INHIBITION OF NEUROINFLAMMATION BY ARTEMISININ AND ITS DERIVATIVES

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A THESIS SUBMITTED TO THE UNIVERSITY OF HUDDERSFIELD IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF HUDDERSFIELD

JULY 2015

(ORIGINAL SUBMISSION: APRIL 2015)

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Abstract

Background: Neuroinflammation in the central nervous system involves the rapid reaction of the CNS through activation of resident immune cells (especially microglia cells) to infection, trauma and stroke, among other stimuli. It is a tightly regulated process but in chronic cases it is detrimental. Uncontrolled neuroinflammation has been shown to manifest itself in various neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS). The Nrf2/HO-1 antioxidant system has also been recently shown to be involved in neuroinflammation. Artemisinin, artemether and artesunate are antimalarial drugs that have been shown to possess anti-inflammatory activity. However, there is no detailed evidence demonstrating that these drugs could inhibit neuroinflammation in lipopolysaccharide (LPS)-activated BV2 microglia cells. Therefore, this study aims to investigate the effects of these compounds in LPS-activated BV2 microglia. This research also attempted to explore the possible involvement of the Nrf2/HO-1 system in the anti-neuroinflammatory action of these compounds.

Methods: Cultured BV2 microglia cells were stimulated with LPS following pre-treatment with artemisinin (5-40 µM), artemether (5-40 µM) or artesunate (0.5-4 µM). Nitrite, used as a measure of nitric oxide production was investigated using the Griess assay. PGE_2 production was investigated with PGE_2 EIA assay, while cytokine production was measured using ELISA. Reporter gene assays in transfected HEK 293 cells were used to investigate effects of the compounds on NF-κB and ARE luciferase activities. Further experiments to elucidate the mechanism of action of artemisinin, artemether and artesunate were carried out by measuring expressions of various target proteins in whole cell and nuclear extracts using western blotting. In addition, NF-κB DNA binding was investigated using the EMSA. Subsequent investigations to understand the role of Nrf2 activity on the inhibitory activity of the compounds on NO, TNFα, IL-6 and PGE_2 production in activated BV2 cells were carried out in Nrf2 silenced cells. To achieve this, BV2 cells were transiently transfected with Nrf2 siRNA followed by pre-treatment with compounds and stimulation with LPS for 24 hours.

Results: Artemisinin, artemether and artesunate inhibited LPS-induced nitric oxide (NO), prostaglandin E_2 (PGE_2) and pro-inflammatory cytokine (TNFα and IL-6) production in LPS-activated BV2 microglia cells. These compounds also down regulated the expression of specific regulatory proteins, inducible NO synthase (iNOS) cyclooxygenase-2 (COX-2) and microsomal prostaglandin E2 synthase-1 (mPGES-1).

Artemisinin, artemether and artesunate exerted anti-neuroinflammatory activity in LPS activated microglia by suppressing LPS-induced NF-κB activity via inhibition of IκB phosphorylation and degradation-mediated nuclear translocation of the p65NF-κB subunit. These compounds also

produced consistent inhibition of DNA binding of NF-κB. All three compounds exhibited a similar profile in interfering with p38MAPK signalling following LPS activation of BV2 microglia by preventing phosphorylation of MKK3/6, p38 and MAPKAPK2. This research showed that artemisinin, artemether and artesunate activated Nrf2 mediated HO-1 antioxidant activity in BV2 cells. These effects appear to be mediated through the activation of the antioxidant response element (ARE). Further association studies revealed that artemisinin; artemether and artesunate caused reduction in TNFα, IL-6 and PGE_2 secretion in LPS-activated BV2 cells through Nrf2-dependent mechanism. However, it appeared that their inhibitory action on NO production was independent of Nrf2 activity.

Conclusion: Taken together, the outcome of this research indicates that artemisinin, artemether and artesunate suppress neuroinflammation in LPS-activated BV2 microglia by interfering with NF-κB and p38MAPK signalling. The compounds also produced consistent activation of the Nrf2/HO-1 antioxidant protective system. This research has also provided the first evidence that the inhibitory actions of these compounds on TNFα, IL-6 and PGE_2 are dependent on their ability to activate the Nrf2 transcription factor.

Keywords

Artemisinin, artemether, artesunate, neuroinflammation, microglia, LPS, Nrf2, HO-1, antioxidant responsive element, sesquiterpene lactones

Publications related to thesis

Okorji UP, Olajide OA (2014) A semi-synthetic derivative of artemisinin, artesunate inhibits prostaglandin E2 production in LPS/IFNγ-activated BV-2 microglia. Bioorganic and Medicinal Chemistry, Jul 11. pii: S0968-0896(14)00510-0. doi: 10.1016/j.bmc.2014.07.007

Conference Posters

Okorji UP, Olajide OA (2014) NF-κB and p38 signalling-dependent inhibition of neuroinflammation by artemether. British Pharmacology Society Conference Proceeding

Okorji UP, Olajide OA (2013) Artesunate inhibits prostaglandin E_2 (PGE_2) production in LPS + IFN-γ activated BV-2 microglia cells. British Pharmacology Society Conference Proceeding

Okorji UP, Olajide OA (2012) Artemisinin inhibits nitrite and prostaglandin production from Lipopolysaccharide (LPS)-stimulated BV-2 microglia. APS PharmSci 2012 Conference Proceeding

Conference Presentation

Okorji, UP, Olajide, OA (2013) The Antimalarial drug artemisinin inhibits neuroinflammation in LPS-activated BV-2 microglia. West African Society for Pharmacology 2013 Conference Proceeding

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Acknowledgements

First, I would like to acknowledge and appreciate my supervisor and mentor, Dr. Olumayokun Olajide, for his guidance, help and advice in both work and general life matters. Thank you for your admirable work ethics and commitment to research. It has indeed rubbed off on me. Thank you for all your help, which has helped me, develop as a great researcher and an individual.

A big thank you to members of Dr. Olajide's group: Ravikanth Velagapudi, Abdelmeneim El-Bakoush, and Anwer Abudheir. Thank you for all your help throughout the course of my work.

To the Biology, Chemistry and Pharmacy Technicians, thank you for all your help throughout my PhD research.

I would like to acknowledge Dr. Nikolaos Georgopoulous, Dr. Patrick McHugh, Dr. Stephano Vanin, Professor Barbara Conway and Professor Rob Brown. Thank you for all your help, advice and encouragements throughout my PhD.

Big thanks to the Huddersfield Association of Women Graduates (AWG). Thank you for acknowledging the contributions of my PhD work and for awarding me the Postgraduate Award 2014. It is indeed a great encouragement.

Thank you to all my amazing friends and colleagues in Huddersfield, who have supported, encouraged and helped me throughout the highs and lows of my PhD journey. Special thanks to my friends Fatima Reine-Traore and Demilade Lawal. Thanks you for all your support and encouragements.

To all, I am very grateful. Thank you.

Dedications

This thesis is dedicated to my amazing family. To my parents, Daniel and Ebele Okorji, my sister, Nkeiruka and brothers, Daniel (Jnr), Chikeluba and Chiagozielam - Thank you so much for your continuous encouragement, love and support. Thank you for the numerous sacrifices you have made to support me to get to where I am today. Above all thank you for being there through the highs and lows.

I also dedicate this work to the amazing women who have been a source of encouragement and inspiration, to getting me to where I am today. To my late aunt Azuka Ojibe, thank you for the inspirational life you lived and the numerous achievements throughout your life. They are a source of encouragement, and constantly challenge me to work towards achieving my dreams. To Professor Ruth Ross, thank you for your exceptional work ethics, commitment to research, attitude to life and your many achievements. It has indeed been a great example. To Ann Parker, thank you for your encouragements, words of advice and positive attitude to life against all odds.

Above this work is dedicated to the father above all.

List of abbreviations

AA	Arachidonic acid
Aβ	Amyloid beta
ACTs	Artemisinin-based combination therapies
AD	Alzheimer's disease
ANOVA	Analysis of variance
ALD	adrenoleukodystrophy
ALS	Amyotrophic lateral sclerosis
AP-1	Activator protein-1
APP	Amyloid precursor protein
ARE	Antioxidant response element
ATP	Adenosine triphosphate
BACE1	Beta-secretase-1
BAFF	B-cell activating factor
BBB	blood brain barrier
BSA	Bovine serum albumin
CIDP	chronic inflammatory demyelinating polyneuropathy
CNC	cap 'n' collar
CNS	Central nervous system
COPD	chronic obstructive pulmonary disease
COX	Cyclooxygenase
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase-2
CpG-ODN	CpG-Oligodeoxynucleotide
CRTH2	Chemoattractant receptor homolo-gous molecule expressed on T-Helper type 2 cell
CYP2A6	Cytochrome P450 2A6
CYP3A4	Cytochrome P450 3A4

CYP3A5	Cytochrome P450 3A5
DHA	Dihydroartemisinin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DP1	Prostaglandin D2 receptor
DTT	Dithiothreitol
ECM	extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
ELK1	ETS domain-containing protein
EMSA	Electrophoretic mobility shift assay
EP (1-4)	Prostaglandin E receptor 1-4
ERK	Extracellular signal-regulated kinase
eNOS	Endothelial nitric oxide synthase
FBS	Foetal bovine serum
FP_A	prostanoid FP_A receptor
FP_B	prostanoid FP_B receptor
GBS	Guillain-Barré syndrome
GST	glutathione S-transferase
GWAS	Genome-wide associated studies
HEK 293	Human Embryonic Kidney 293
HIV	Human immunodeficiency virus
HO-1	heme oxygenase-1
IFN-γ	Interferon gamma
IκB	Inhibitor of Kappa B
IKK	IκB kinase
IL	Interleukin
IL-1	Interleukin-1
IL-1β	Interleukin-1 beta

IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-17	Interleukin-17
IP	prostacyclin receptor
IRAK1	Interleukin-1 receptor associated kinase 1
IRAK4	Interleukin-1 receptor associated kinase 4
iNOS	Inducible nitric oxide synthase
IUPAC	International Union of Pure and Applied Chemistry
JNK	c-Jun N-terminal kinase
kDa	kilo-Dalton
Keap1	Kelch ECH Associating Protein 1
KO	knock-out
LDS	Lithium Dodecyl Sulphate
LPS	Lipopolysaccharide
LT-β	Lymphotoxin beta
LT-βR	Lymphotoxin beta receptor
LTP	Long-term potentiation
M	Molar
MAPEG	Membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK	Mitogen-activated protein kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MAPK3K	Mitogen-activated protein kinase kinase kinase kinase
MCP-1	Monocyte chemo-attractant protein 1
MEM	Minimum essential medium
MES	2-(N-morpholino) ethanesulfonic acid
mg	milligram

MHC	major histocompatibility complex
min	minute
mM	millimolar
MMP9	Matrix metalloproteinase-9
mRNA	messenger RNA
MRP	multi-drug-resistance-associated protein
MS	Multiple Sclerosis
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NEAA	Non-essential amino acid
NED	N-1-naphthylethylenediamine dihydrochloride
NEMO	NF-kappa B essential modulator
NFTs	neurofibrillary tangles
NF-κB	Nuclear factor kappa B
NLRs	nucleotide-binding oligomerization domains (NODs) NOD – like receptor (s)
ng/ml	nanogram per microliter
nm	nanometer
NIK	NF-kappa B inducing kinase
NO	Nitric oxide
NOD	nucleotide-binding oligomerization domain
nNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase
NPC	neural precursor cells
NP-40	nonyl phenoxypolyethoxyl ethanol 40
NQO1	NAD(P)H:quinone oxidoreductase 1
Nrf2	nuclear factor [erythroid-derived 2]-like
NSAID	Nonsteroidal anti-inflammatory drug

OPTI-MEM	Optimised Minimum essential medium
PBS	Phosphate buffered saline
PD	Parkinson's disease
PG	Prostaglandin
PGD_2	Prostaglandin D2
PGE_2	Prostaglandin E2
$PGF_{2\alpha}$	Prostaglandin F2-alpha
PGH_2	Prostaglandin H2
PGI_2	Prostaglandin I2
PLA_2	Phospholipase A2
PMSF	Phenylmethylsulphonyl fluoride
PNI	Peripheral nerve injury
PNS	Periphery nervous system
P/S	Penicillin streptomycin
PTM	Post-translational modification
PVDF	Polyvinylidene fluoride
P2RX7	Purinergic receptor P2X ligand-gated ion channel 7
RAC1	Ras-related C3 botulinum toxin substrate 1
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
rpm	revolutions per minute
RORγt	Retinoic acid-related orphan receptor γt
ROS	Reactive oxygen species
RPMI 1640	Roswell Park memorial Institute 1640 medium
SAP-1	SRF accessory protein 1
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SGL	subgranular layer

SL	Sesquiterpene lactone
STAR1	Striated muscle activator of rho-1
STAT3	Signal transducer and activator of transcription 3
STAT6	Signal transducer and activator of transcription 6
TAK1	TGF-β-activated kinase 1
TBE	Tris borate Ethylene diamine tetra-acetic acid
TBST	Tris buffered saline with Tween 20
TGF-β	transforming growth factor-β
Th17	T helper 17
TLR	Toll like receptor
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor-alpha
TNFR	Tumour necrosis factor receptor
TPα	Thromboxane A2 receptor alpha
TPβ	Thromboxane A2 receptor beta
TRAF	TNF receptor associated factor
TRAF6	TNF receptor associated factor 6
TRAM	Toll-like receptor adaptor molecule 2
TRIF	TIR-domain containing adapter-inducing interferon -β
TxA_2	Thromboxane A2
U/ml	Units per millilitre
V	Volt
VSMC	Vascular Smooth Muscle Cell
WB	Western blot
µg	Microgram
µl	Microliter
µM	Micro molar
%	Percentage

°C	degrees centigrade
&	and
-siNrf2	samples from wild-type cells
+siNrf2	samples from Nrf2 silenced cells.

CHAPTER 1
INTRODUCTION

1. Introduction

In the human body, the immune system plays vital roles in the maintenance of tissue homeostasis, and the response to infection and injury (Glass et al., 2010). Both the innate and adaptive immune systems interact with each other to ensure homeostasis. Inflammation is a host defence response to injury, tissue ischemia, autoimmune responses or infectious agents. According to Ricciotti and FitzGerald (2011), 'inflammation is an intrinsically beneficial event that leads to removal of offending factors and restoration of tissue structure and physiological function'. Hence, it is the body's immune system's response to infection and injury. Inflammation is locally exhibited by the classical features of swelling, redness, heat and pain within tissues outside the brain (Buckley et al., 2013). Inflammation often elicits a generalised sequence of events, which are referred to as the acute phase response. This acute phase response represents another host defence mechanism, which can limit proliferation of invading pathogens. The mechanisms involve the production of acute phase proteins by the liver, activation of sympathetic nervous system, changes in cardiovascular function, altered endocrine status, and behavioural changes leading to energy conservation such as reduced appetite, increased sleep and most common feature of infection, fever which can limit bacterial proliferation (Glass et al., 2010). These mechanisms have been put in place by the body to ensure homeostasis at all times. Thus, the inflammatory response represents a highly regulated biological programme that enables the innate and adaptive immune systems to effectively deal with pathogens. It is also involved in the production of factors that are themselves capable of inducing significant pathology (Glass et al., 2010). Deregulation of the immune system can be detrimental and hence, inflammation has been implicated in the pathogenesis of various diseases – arthritis, cancer, stroke and neurodegenerative and cardiovascular diseases.

The central nervous system (CNS) differs from the other systems and its response to pathogenic challenges is different. Previously, the CNS was regarded as an 'immune privileged' organ which is not susceptible to inflammation or immune activation. It was thought to be largely unaffected by systemic inflammatory and immune responses. However, this hypothesis is now flawed as research findings support the input of the periphery in the CNS especially when the blood brain barrier is affected (Hernández-Romero et al., 2012; Lim et al., 2013).

1.1. Neuroinflammation

Neuroinflammation is the inflammation of a nerve or of the nervous system. It involves the rapid reaction of the CNS through activation of the resident immune cells to infection, trauma, toxins and stroke, among other stimuli (Shastri et al., 2013). It can be initiated by a

variety of cues. The common causes include toxic metabolites (Rao et al., 2012), exosomes, autoimmunity, aging (Pizza et al., 2011), microbes, viruses (Rao et al., 2012), traumatic brain injury (Aungst et al., 2014), air pollution (Levesque et al., 2011), and passive smoke (Moreno-Gonzalez et al., 2013; Shastri et al., 2013). Cellular and molecular immune components such as specialised macrophages (e.g. microglia), cytokines, complement, and pattern-recognition receptors are the contributing players in neuroinflammation (Shastri et al., 2013).

Neuroinflammation is a normal and necessary process. It is tightly controlled but when directed against normal tissue it is damaging (Jha et al., 2012). The harmful effects of neuroinflammation are observed when it manifests itself in diseases. A wide range of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) are associated with chronic neuroinflammation (Cherry et al., 2014; Jha et al., 2012). Neuroinflammation also plays a crucial role in the progression of many neuropsychotic and viral diseases. The detrimental role of neuroinflammation in the pathology of these diseases highlight the importance of understanding the process as well as identifying compounds that could modulate its activity and/ or restore balance in homeostasis especially in disease states.

Inflammation in the brain is characterised by the activation of glial cells. Various types of glial cells are found in different parts of the nervous system. Hence, neuroinflammation can be grouped into peripheral nervous system (PNS) inflammation and central nervous system (CNS) inflammation. Both PNS and CNS neuroinflammation are characterised by infiltration of leukocytes and increased production of inflammatory mediators at these sites (Ji et al., 2014). PNS neuroinflammation involves inflammation of the peripheral nerve and ganglia. It manifests as activation of schwann cells in the nerve and is involved in diseases such as chronic inflammatory demyelinating polyneuropathy (CIDP) (Ydens et al., 2013), peripheral nerve injury (PNI) (Gaudet et al., 2011), chronic pain (Ji et al., 2014), Guillain-Barré syndrome (GBS) (Ydens et al., 2013) and schwannomatosis (Ydens et al., 2013). Satellite glial cells in the ganglia also play a role in neuroinflammation in the PNS.

On the other hand, CNS neuroinflammation involves the brain and spinal cord with the activation of microglia and astrocytes playing an important role. Other glial cells such oligodendrocytes in the spinal cord and brain also play a role in neuroinflammation. CNS neuroinflammation is involved in diseases such as Alzheimer's disease (AD) (Butovsky et al., 2014), Parkinson's disease (PD) (Fan et al., 2014), multiple sclerosis (MS) (Naegele & Martin, 2014; Whalley, 2014) and amyotrophic lateral sclerosis (ALS) (Hooten et al., 2015).

The involvement of various glial cells in neuroinflammation highlights the crucial role played by these cells in the process and the need to understand the signalling pathways involved in their over-activation during chronic neuroinflammation. It also highlights the need to understand the points at which these pathways could be inhibited or attenuated to stop chronic neuroinflammation.

Neuroinflammation can also be classified based on the duration of the inflammation into acute and chronic neuroinflammation. Acute neuroinflammation comprises of the immediate and early response that paves the way for repair of the damaged site in the CNS (Rao et al., 2012). Acute neuroinflammation usually follows injury and is characterised by inflammatory molecules, endothelial cell activation, platelet deposition and tissue oedema. In the acute phase after an injury, neuroinflammation is tightly controlled (Jha et al., 2012).

On the other hand, chronic neuroinflammation involves excessive activation of glial cells, which have harmful effects on other cells including the neurons. This highlights the need to curtail neuroinflammation in chronic neuroinflammatory conditions in the CNS. Brain inflammation has also been reported to be detrimental to neurogenesis, a process by which neurons are generated from neural stem cells and progenitor cells (Whitney et al., 2009). It also explains why neuronal death in diseases like AD is hard to reverse as chronic neuroinflammation is one of the primary underlying factors involved in the progression of the disease. Therefore, it is important to understand the molecular mechanisms involved in chronic neuroinflammation. Moreover, careful selection of targets considered for drug development is also important.

As mentioned earlier, initial response to harmful stimuli in the CNS is achieved by the activation of resident microglial cells. This is followed by the production of inflammatory mediators, which trigger the increased movement of blood-derived immune cells into the injured tissue (Kaminska et al., 2009). Emphasis today has been placed on the microglia, the complement system and inflammatory mediators as playing major roles in chronic neuroinflammation. This highlights the need to understand the role of the microglia and inflammatory mediators in neuroinflammation.

1.1.1. The microglia in neuroinflammation

Microglia cells are small glial cells, which are resident macrophages of the brain and spinal cord (Cherry et al., 2014; Graeber & Streit, 2010). Their morphology differs from ‘conventional’ macrophages by the presence of branch-like processes otherwise known as ramified appearance (Shastri et al., 2013). Microglia cells, which are yolk-sac-derived, remain throughout life, with the population maintained by self-renewal in the healthy CNS with little contribution from the bone-derived macrophages (Salter & Beggs, 2014). Microglia

cells in the adult murine CNS accounts for 5 – 12 % of the total number of glial cells (Gomez-Nicola & Perry, 2015). In humans they account for 0.5 – 16 % of the total nerve cells in the brain (Rojo et al., 2014). Microglial cells acquire their definitive composition after birth, in terms of numbers and phenotype (Gomez-Nicola & Perry, 2015). Microglia plays a beneficial role in scavenging cell debris, tissue healing and repair. But, in chronic state it is damaging to the CNS.

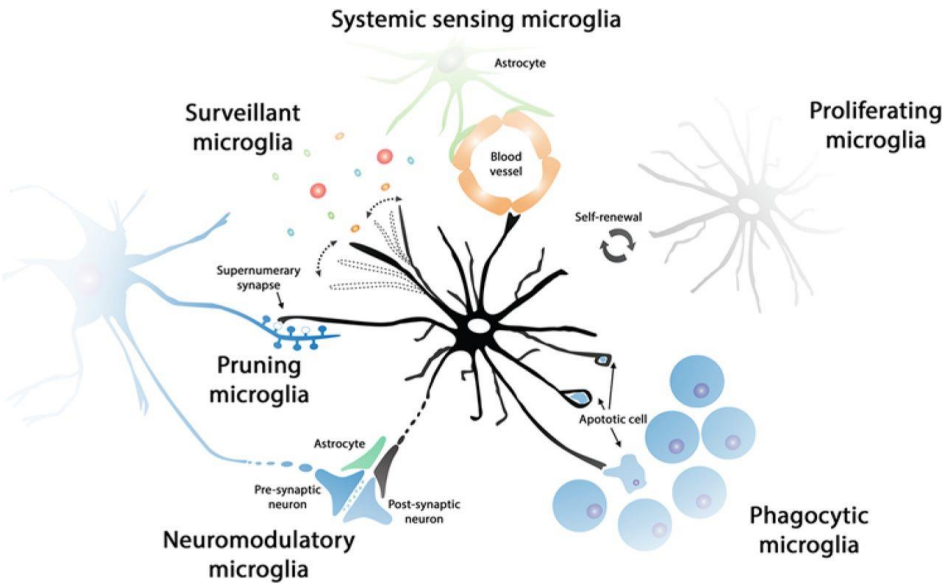

Figure 1: Functional states of microglia in the brain (Gomez-Nicola & Perry, 2015)

The microglia assumes a variety of functional state depending on the conditions it is presented with (Figure 1). Previously, microglia cells in the resting state were thought to be relatively quiescent. Now, microglia cells are known to be constantly active and surveying their environment (Perry & Teeling, 2013).

In the surveillant state, microglia cells constantly and rapidly scan the microenvironment whilst keeping their soma in a fixed position. Aging has been shown to affect the stability of microglia cells (Hefendehl et al., 2014). Aging also leads to disruption in microglia organization, decrease in microglial motility and a remarkable increase in the motility of the soma. This effect of ageing on microglial stability may be the reason why aging is implicated as one of the risk factors of diseases involving neuroinflammation. This may probably also account for the increase in the incidence of neuroinflammatory disorders with increase in age. The maintenance of the surveillant phenotype in microglia cells is achieved by various soluble or membrane-bound factors (Hanisch & Kettenmann, 2007; Kettenmann et al., 2011). There is also a high probability that these various factors that maintain microglia cells in the surveillant phenotype are dysfunctional in neuroinflammatory CNS disorders.

Microglia cells can also exhibit other functional states including pruning and neuromodulation (Figure 1). In pruning, the precise role of microglia cells in the process is known as 'synaptic stripping'. Synaptic stripping is the separation of the presynaptic terminal from an injured postsynaptic neuron (Perry & O'Connor, 2010). Microglial pruning has been described during postnatal development but the mechanism of the involvement of this in early stages of development, during diseases and in pathologies is unclear (Gomez-Nicola & Perry, 2015). Microglia cells play a role in regulating homeostatic synaptic plasticity. In addition, these cells are involved in the regulation of neuronal activity, highlighting their neuromodulatory state. Microglia cells can release neuromodulatory chemicals, which influence neuronal firing and intracellular signalling (Sheridan & Murphy, 2013). This implies that microglia cells not only play a role as the first line of defence in the CNS but also regulate neuronal activity. Therefore, malfunction of the microglia cells during chronic neuroinflammation affect neuronal activity. This further highlights the need to properly inhibit neuroinflammation in CNS disorders and diseases involving chronic neuroinflammation.

Microglia cells can also exist in the phagocytic state (Lyman et al., 2014). The phagocytic activity of microglia cells is one of the features that they share in common with their cellular relative, the macrophages. The phagocytic state of microglia cells helps eliminate bacteria during infection (Gomez-Nicola & Perry, 2015). Microglia cells function as transitory phagocytes responsible for clearing apoptotic neuronal cell bodies during CNS ontogeny. In addition, in the phagocytic state, microglia cells can activate the inflammasome (which activates apoptosis), to ultimately enable tissue regeneration and scarring (Lyman et al., 2014). During this state, microglia cells are very motile and move to the site of injury to clear harmful toxins. This movement is known as microgliosis. Consequently, the phagocytic state of the microglia cells is very important in maintaining homeostasis and ensuring that the CNS is not overcome by the toxic challenge it might be faced with. Microgliosis in neuropathology can be self-limiting by eliminating not only infiltrating, inflammatory cells but also the excess of microglia by apoptosis (Tambuyzer et al., 2009). In a study by Sierra et al (2013), the role of phagocytic microglia cells on the hippocampal neurogenic niche in a healthy brain was investigated. The study shows that phagocytic microglia cells can clear potential toxic proteins such as amyloid beta (Aβ). At the hippocampal subgranular layer (SGL), neurogenic cascade leads to the generation of a population of early neural precursor cells (NPCs), which become finely selected by apoptosis, before completing the maturation to granule cells (Ming & Song, 2011). Sierra et al. (2010) reported that ramified phagocytic microglia cells rapidly and effectively remove dying NPCs in a non-inflammatory fashion. They also showed that when challenged with LPS, NPCs undergo increased apoptosis. It is unclear if this is as a result of the indirect production of pro-inflammatory cytokines or direct

phagoptosis (cell death by phagocytosis). Apart from removal of dead or damaged cells, phagocytic microglia cells can clear axonal and myelin debris (Hosmane et al., 2012) , engulf and prune synapses (Schafer et al., 2012) as well as clear toxic proteins such as Aβ (Sierra et al., 2010). In neurodegenerative diseases, this phagocytic role of microglia cells is not efficient.

Microglial cells produce immunoregulatory molecules including chemokines, cytokines, growth factors, prostanoids and free radicals in their activated state. Nitric oxide and superoxide produced by microglial cells are also modulated by pro- and anti-inflammatory cytokines and/or growth factors. Therefore, the modulation by the pro- and anti-inflammatory cytokines balances each other, leading to a stable state in the healthy brain. However, an imbalance in this system is detrimental and observed in neuroinflammatory related diseases.

Microglia cells also exist in the inflammatory functional state. The inflammatory functional state plays a major role in neurodegenerative diseases and hence considered in scientific literature in mostly diseased (unhealthy) brain. Although this is mostly the case, it may be important to throw some light on the inflammatory profile of the microglia in a healthy brain, under normal physiological conditions. In a study by Hickman et al. (2013) the analysis of the microglial transcriptome shows a profile dominated by ribonucleic acids (RNAs) encoding proteins for sensing endogenous ligands and microbes, jointly referred to as 'microglial sensome'. Microglial cells up-regulate the expression of microbe-recognition genes. These microbe-recognition genes help microglia cells function in the inflammatory functional state. A recent comparison of the transcriptomic profile of the microglia highlights a unique functional signature in microglia cells dominated by the activity of transforming growth factor (TGF)-β (Butovsky et al., 2014). This signature allows specific differentiation of microglia cells when compared with other myeloid cells or resident brain cells. This specific differentiation highlights the particularities of the microglial population. Microglia cells in normal physiological conditions have a down-regulated expression of molecules like CD45, Fc receptors or major histocompatibility complex (MHC) class II when compared to other macrophages (Perry & Teeling, 2013). The MHC class II is one of the markers activated in response to injury or illness, hence, the down-regulation in a healthy brain. However, its expression increases with age.

Upon environmental stimulation or challenges, microglia cells become activated and the morphology changes to an amoeboid appearance where they retract the ramifications (Shastri et al., 2013). These various roles of the microglia highlight its role in maintaining homeostasis. Therefore, an imbalance in the regulatory systems used by microglia cells lead to a disease state. Using a full array of immune receptors such as toll-like receptors (TLRs), nucleotide-binding oligomerization domains (NODs) NOD – like receptors (NLRs) and many

scavenger receptors, microglia cells are able to recognise harmful stimuli and respond to it (Cherry et al., 2014). The inflammatory response of microglia cells could either be acute or chronic.

The acute response is believed to be protective because it avoids further injury and induces tissue repair. Microglia cells achieve this by removal of debris, scavenging neurotoxins as well as secretion of mediators important for neuronal survival (Lima et al., 2012). Lima et al. (2012) also reported that this acute response contributes to wound healing.

Microglia cells also play an important role in chronic inflammation as the intense activation and accumulation of dead cells and activated microglia at the site of injury can induce neuronal damage. This is because dead cells and excessively activated microglia cells release a variety of neurotoxic substances. In neurodegenerative disorders, the common pathophysiological hallmarks are activation of microglia cells and astrogliosis, infiltration of immune system and activation of the adaptive immune system (Kaminska et al., 2009). Hence, the inflammatory functions of microglia cells have a special relevance in understanding the progression of neurodegenerative diseases. A chronic reactive state of microglia cells or an abnormally high proportion of activated microglia cells may become unsafe by increasing the inflammatory burden. At this stage, neuroinflammation has been reported to be self-perpetuating (Lima et al., 2012). Chronic inflammation in the CNS induces neuronal death, and the molecules released by the dead neurons can further activate microglia cells, which enhance cell death. This vicious cycle together with the continuous production of factors that activate microglia contribute to the chronicity of the neuroinflammation process.

1.1.2. Microglia activation states - M1 and M2

Microglial activation through toll-like receptors (TLRs) and NOD – like receptors (NLRs) is considered as the ‘classical’ form of microglial activation. During ‘classical’ activation, innate immune responses including production of pro-inflammatory cytokines like tumour necrosis factor alpha (TNFα), interleukin (IL)-1β and IL-6 and chemokines occurs (Cherry et al., 2014; Rojo et al., 2014). These pro-inflammatory mediators are produced either locally in the CNS or recruited from the peripheral system following the disruption of the blood brain barrier (BBB). The classical activation leads to adaptive immune response by expressing major histocompatibility class II molecules and interaction with T cells. The classical activation is generally typified by the production of pro-inflammatory cytokines and reactive oxygen species (ROS) (Cherry et al., 2014). The cytokine production is essential for the polarization of the microglia into the classical state, otherwise known as the M1 phenotype (Figure 2). The M1 profile is a rapid response of microglia characterised by hypertrophic bodies, with

fewer, thicker, and shorter processes than those of surveillance microglia (Rojo et al., 2014). The M1 microglia is generally considered as potent effector cells that kill and engulf microorganisms as well as tumour or otherwise damaged cells. The M1 response is reinforced rapidly by coactive factors including ROS and reactive nitrogen species (RNS) released by the microglia and surrounding cells. Therefore, a potential distinction and an important component of M1 microglia is their ability to produce ROS and RNS. A key microglia enzyme, which is very much associated with this process, is iNOS, which utilises arginine to produce nitric oxide (NO) (Cherry et al., 2014; Rojo et al., 2014).

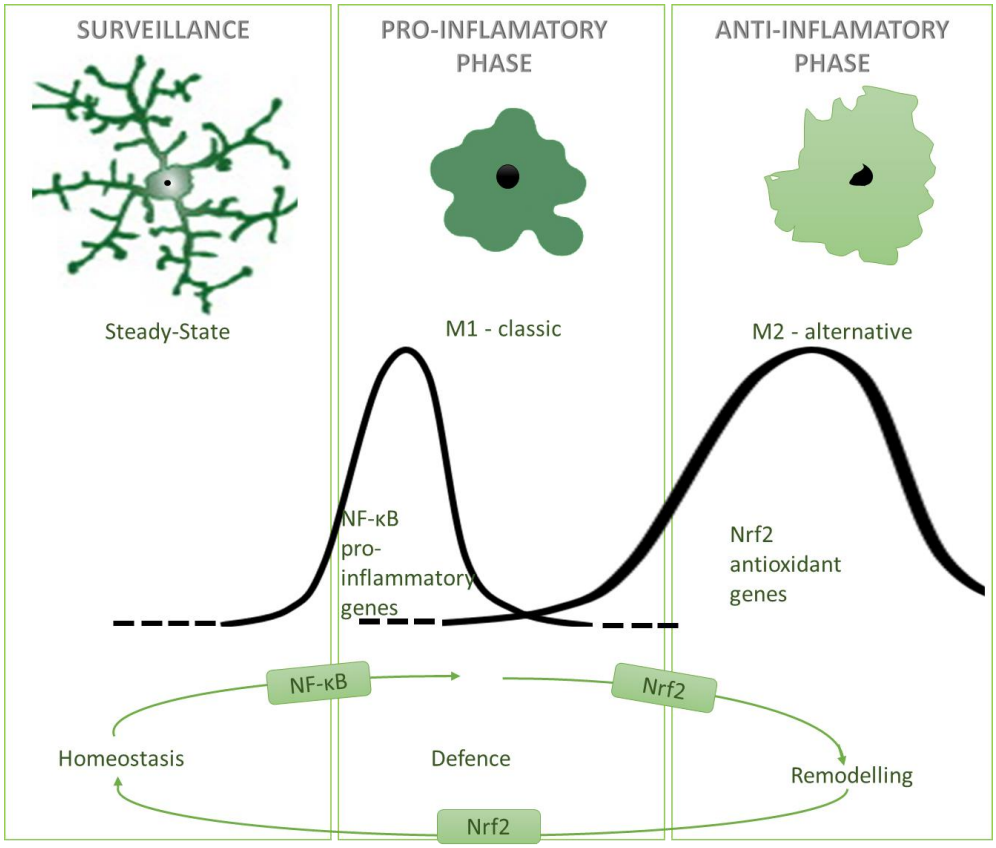

Figure 2: M1 and M2 dynamics in microglial activation (adapted from Rojo et al., 2014**)**

The effective regulation of the cytokines, ROS and RNS produced during the classical activation is important as over production of these mediators can be detrimental to the CNS. Optimal regulation of M1 also helps in smooth progression into M2 phenotype, which helps in recovery after a challenge (Figure 2). Although the M1 phenotype seems to have straightforward identification markers based on characteristics, classifying these cells in vivo has proven to be more challenging. The NF-κB transcription factor is central to the acquisition of the pro-inflammatory M1 polarization (Rojo et al., 2014).

The pro-inflammatory polarization of the microglia (M1) is often followed by a long-lasting repair stage (Hu et al., 2015; Wang et al., 2014). The transition from the M1 phenotype to

the M2 phenotype is very vital in homeostasis. This infers that in the pathology of most CNS neuroinflammatory diseases, the challenge is that the microglia cells do not transit to the M2 phenotype, which initiates the long lasting repair. This long lasting repair stage is the second state of activation of the microglia known as the alternate activation or M2 phase/phenotype (Figure 2). In the M2 phenotype, the microglia display hypertrophic cell bodies with thick and ramified processes and high phagocytic capacity (Rojo et al., 2014). During the M2 phase, the microglia takes on an anti-inflammatory phenotype which is involved in wound healing and debris clearance (Cherry et al., 2014); Hu et al., 2015). The M2 phenotype is activated by the anti-inflammatory cytokines such as IL-4, IL-3 and IL-10, immunoglobulin complexes/ TLR, TGF-β and glucocorticoids (Rojo et al., 2014). The division of the M2 is based on observations that stimulation with various cytokines yield different sets of receptor profiles, cytokines production, chemokine secretion and function (Cherry et al., 2014). Even though the profiles of the cells classified in the M2 phenotype are diverse, the one feature that places them all in the M2 classification is their ability to express mediators or receptors that have the capacity to down regulate repair, or protect the body from inflammation. The M2 response is crucial for restoration of normal tissue homeostasis, because it leads to the switching off of the proinflammatory response, scavenging of debris and restructuring of the damaged extracellular matrix (ECM). The signalling cascades involved in the acquisition of the M2 phenotype are less well understood, yet cytokines such as interleukin -4 (IL-4) which signals through signal transducer and activator of transcription 3 (STAT3) and signal transducer and activator of transcription 6 (STAT6) appear to be important. However, the induction of anti-oxidant and cytoprotective genes is a signature of M2 polarization. New findings indicate that the nuclear factor [erythroid-derived 2]-like (Nrf2) signalling favours an M2 phenotype (Rojo et al., 2014).

1.1.3. Role of Nitric oxide (NO) in neuroinflammation

Nitric oxide is a key messenger involved in physiological functions including regulation of inflammatory and immune responses (Förstermann & Sessa, 2012; Virarkar et al., 2012). It is a bioactive radical, which plays a crucial role as both neurotransmitter and neuromodulator in the CNS (Banuls et al., 2014). NO is involved in a vast range of functions including neurotransmission, regulation of food intake, control of the sleep-wake cycle, modulation of hormone release, thermal regulation, neuroprotection and neurotoxicity (Cespuglio et al., 2012; Godinez-Rubi et al., 2013; Morley et al., 2011; Sagi et al., 2014). At low concentrations, NO has physiological roles in the function of neuronal and vascular cells. On the other hand, at higher concentrations, it is implicated in the pathogenesis of various neurological diseases including stroke, neurodegenerative diseases, and demyelination and neuroinflammatory diseases (Drechsel et al., 2012; Ghasemi & Fatemi, 2014). This suggests

the importance of this inflammatory mediator and the need to inhibit its overproduction in chronic CNS neuroinflammatory diseases.

NO is enzymatically produced from an amino acid, L-arginine, by the activity of NO synthase (NOS) enzyme. Three NOS subtype exists: neuronal (n) NOS, endothelial (e) NOS and inducible (i) NOS (Förstermann & Sessa, 2012; Joshua & Thomas, 2014). The third subtype iNOS (or NOS2) indicates that the expression of the enzyme is induced by inflammatory stimuli. It is expressed in various cell types especially the glial cells, astrocytes and microglia. These glial cells do not express iNOS constitutively but express the enzyme in pathological conditions such as ischemia, trauma, and neurotoxic and inflammatory damage. Several line of evidence have demonstrated that glial NO is involved in the pathophysiology of various neurological diseases including demyelination (e.g. multiple sclerosis [MS], experimental allergic encephalopathy, and X-linked adrenoleukodystrophy [ALD]), neuronal death during ischemia (e.g. stroke) and trauma, and neurological diseases (e.g. Alzheimer's disease [AD], Parkinson's disease [PD], Huntington's disease [HD], Human immunodeficiency virus (HIV)-associated dementia and ALS) (Virarkar et al., 2012). In addition, the formation of nitotyrosine (a marker of nitrosative stress) has been documented in patients with PD and AD (Butterfield et al., 2011; Chinta & Andersen, 2011; Tao et al., 2012). These observations provide evidences for the role of NO in the pathogenesis of these diseases. Consequently, an understanding of the intracellular signalling pathways involved in glial iNOS-derived NO production can provide novel therapeutic approaches for targeting these pathways.

1.1.4. Cytokines in neuroinflammation

Cytokines are a class of small proteins that act as signalling molecules at picomolar or nanomolar concentrations to regulate inflammation and modulate cellular activities including growth, survival and cell differentiation (Ramesh et al., 2013). Cytokines are a large and diverse group of pro- or anti-inflammatory factors that are grouped into families based upon their structural homology or that of their receptors. Anti-inflammatory cytokines including interleukin 10 (IL-10), interleukin 4 (IL-4) and transforming growth factor beta (TGF-β) are important in neuronal and glial survival during neuroinflammation. On the other hand, pro-inflammatory cytokines generated during neuroinflammation including tumour necrosis factor alpha (TNFα), interleukin 6 (IL-6), interferon gamma (IFN-γ) and interleukin-1 (IL-1) are involved in blood brain barrier (BBB) breakdown, tissue destruction, leukocyte emigration into the CNS, cytotoxic factors production, free radical formation, neuronal and glial dysfunction, apoptosis and neurodegeneration (Ramesh et al., 2013). The involvement of pro-inflammatory cytokines in all these detrimental situations strongly highlights the need to inhibit pro-inflammatory cytokines involved in chronic neuroinflammation.

1.1.4.1. Interleukin-6 (IL-6)

IL-6 is a pleiotropic inflammatory cytokine involved in the regulation of immune response, haematopoiesis, cell regeneration and inflammation (Brunssen et al., 2013; Gruol, 2014). Under physiological conditions, IL-6 and IL-6 receptor are expressed in several regions of the brain including the hippocampus, striatum, hypothalamus, neocortex and brainstem. IL-6 has both pro- or anti-inflammatory actions, depending on the signalling pathway activated (Scheller et al., 2011). The dual function of IL-6 as a pro- or anti-inflammatory cytokine is a well-balanced system. However, during chronic neuroinflammation, this balance is tilted towards pro-inflammatory function. Hence, the pro-inflammatory function is considered in neuroinflammatory conditions.

In a study by Baune et al. (2009), the possible relationship between MRI changes in the aging brain and peripheral cytokine levels was reported. It has also been reported that one of the main protein kinases, Cdk5/p35 complex, which is involved in tau hyper-phosphorylation in neurodegenerative disorders, appears to be up regulated by the effects of IL-6 as well as IL-1 (Banzhaf-Strathmann et al., 2014).

IL-6 production has been reported in activated glia cells and its role in neuroinflammation and neurodegeneration is also associated with cognitive impairment (Rojo et al., 2008). These authors also suggest that the mechanisms for impairing cognitive functions used by IL-6 may result from its own activity or related to the production of other cytokines. This is because in LPS-induced neurodegeneration, messenger RNA (mRNA) levels of IL-1β and TNFα in the hippocampus are significantly higher in IL-6 wild type animals and very low in IL-6 knock-out (KO) mice.

During neuroinflammation, IL-6 is up regulated following an infection or injury in the CNS or in a number of CNS diseases. Various studies have shown that IL-6 was expressed, produced and/or up regulated in various diseases including viral meningitis, experimental cerebral malaria, and traumatic brain injury (Erta et al., 2012). Therefore, proper regulation of IL-6 is important in neuroinflammatory disorders.

1.1.4.2. Tumour Necrosis Factor alpha (TNFα)

TNFα is a potent multifunctional cytokine, which exerts inflammatory effects in the CNS during chronic inflammation. Upon binding to its associated receptor, TNFα is considered to be a master regulator of cellular cascades that control a number of diverse processes (Frankola et al., 2011) like physiological roles in synaptic transmission and plasticity. It plays a central role in initiating and regulation the cytokine cascade during an inflammatory response (Rubio-Perez & Morilla-Ruiz, 2012). The levels of TNFα in the healthy brain are low but high in inflammatory states. In the CNS, although astrocytes and neurons are able to

produce TNFα, microglia cells are the major source of this cytokine during neuroinflammation (Hanisch, 2002; Welser-Alves & Milner, 2013).

Excessive TNFα levels have an inhibitory effect on glutamate transporters, resulting in increased glutamate concentration in the CNS parenchyma (Olmos & Llado, 2014). Therefore, even slight increases in TNFα induced Ca^{2+} permeable-AMPA and/or NMDA receptor trafficking becomes toxic for neurons (Olmos & Llado, 2014). Toxicity in neurons leads to neuronal death, suggesting the need for homeostasis in TNFα production. Microglial activation and up regulation of TNFα expression is a common feature in chronic neuroinflammation. Hence, TNFα may represent a viable target for pharmacological intervention.

During neuroinflammation, different stimuli trigger TNFα production via different signalling pathways including p38MAPK (Mir et al., 2008) and NF-κB (Lawrence, 2009), thus, making it difficult to determine which of the signalling pathway(s) is implicated in the induction of TNFα expression (Olmos and Llado, 2014). TNFα can also activate microglia cells by itself. Therefore, the TNFα generated from microglia cells in turn activates the microglia. TNFα knock out (KO) in mice has been shown to inhibit microglial activation (Zhao et al., 2007). This suggests that TNFα is an important cytokine in neuroinflammation. Hence, modulation of TNFα production is important.

TNFα has been strongly implicated in neurodegenerative diseases. For instance, microglia derived TNFα has been shown to be the major cytokine responsible in promoting neuronal cell cycle events in the pathogenesis of Alzheimer's disease (Bhaskar et al., 2013). Therefore, attenuation of TNFα expression in these diseases is vital to restoring homeostasis.

1.1.5. Prostaglandin E_2 in neuroinflammation

Prostaglandin E_2 (PGE_2) is a lipid autacoid derived from arachidonic acid. PGE_2 is formed when arachidonic acid (AA), a 20-carbon unsaturated fatty acid is released from the plasma membrane by phospholipases and metabolised by the sequential actions of PGG/H synthase or by cyclooxygenase (COX) and their respective synthases (Ricciotti & FitzGerald, 2011) (Figure 3).

PGE_2 is produced by the action of cyclooxygenases, which mediate the first step in its synthesis from arachidonic acid. PGE_2 plays a role in both physiology and pathology. PGE_2 production in uninflamed tissues is generally low but increases immediately during inflammation (Riccotti & FitzGerald, 2011). The constitutively active COX-1 is responsible for the majority of the PGE_2 produced under homeostatic conditions. However, COX-2 is responsible for the massive release of PGE_2 during inflammation (acute or chronic) (Fiebich

et al., 2014). Due to the crucial role of COX-2 in neuroinflammation, COX-2 derived PGE_2 production is also considered to be crucial in neuroinflammation. In addition, PGE_2 produced in excess during neuroinflammation act on prostaglandin E_2 receptor (EP receptors), which is expressed in the microglia. Activation of microglial EP2 receptors is known to enhance neurotoxic activities (Schlachetzki et al., 2010). Therefore, inhibition of PGE_2 in neuroinflammatory conditions is important in restoring homeostasis.

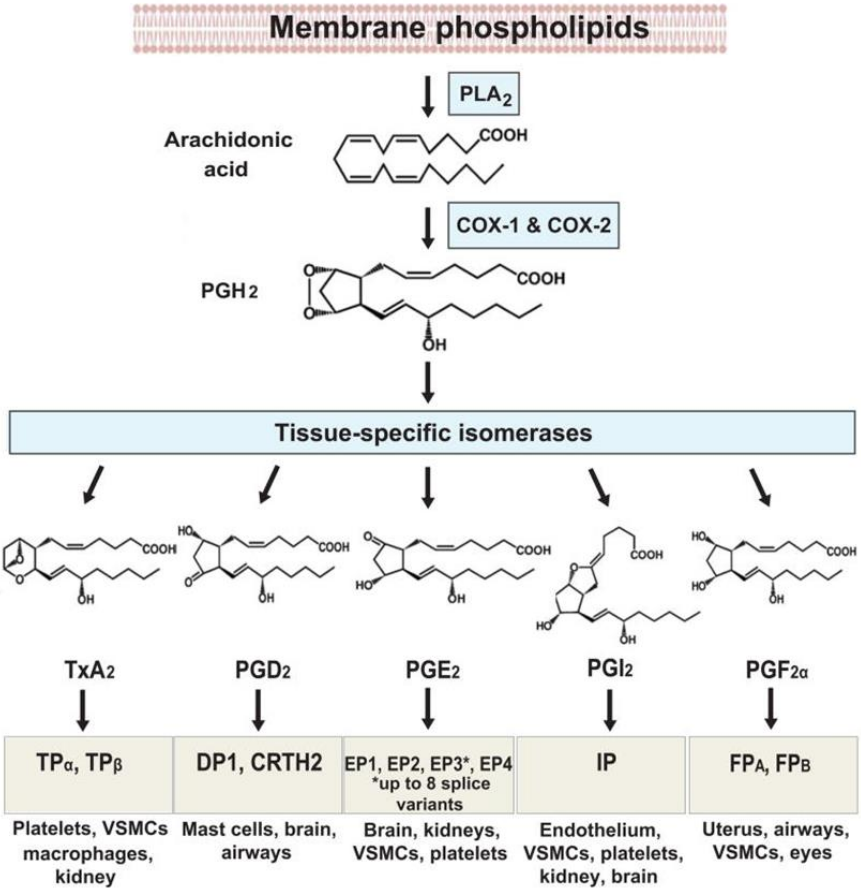

Figure 3: Prostaglandin production - the biosynthetic pathway. (Ricciotti & FitzGerald, 2011)

1.1.5.2. Cyclooxygenases

Cyclooxygenases have two isoforms: COX-1 and COX-2 which share 60% homology in their amino acid sequence and have comparable kinetics (Aid & Bosetti, 2011). They also show individual differences; COX-1 is expressed constitutively in most cells and is involved in housekeeping functions. Inflammatory stimuli, hormones and growth factors induce COX-2. COX-2 is the important source of prostanoids formation in inflammation and in proliferative diseases.

In the CNS, both cyclooxygenases are constitutively expressed (Aid & Bosetti, 2011). COX-2 is detected in the perinuclear, dendritic and axonal domains of neurons, particularly in

cortex, hippocampus, amygdale and dorsal horn of the spinal cord of both rodent and human CNS. It is highly inducible by inflammatory stimuli (Moilanen, 2014) and has been traditionally considered as the most appropriate target for anti-inflammatory drugs. COX-1 isoform is predominantly expressed by microglia in rodent and human brains and its brain expression increase with aging (Choi et al., 2013). In a study using COX-1 and COX-2 selective inhibitors, the comparable role of each COX isoform in neuroinflammatory response induced by β-amyloid peptide (Aβ) was investigated. Results showed that treatment with either COX-1 or COX-2 selective inhibitor or their combination equally decreased the level of TNFα, PGE_2 and cleaved caspase-3 and attenuated astrogliosis and neuronal cell loss (Dargahi et al., 2011). Dargahi et al (2011) also showed that treatment with COX-1 selective inhibitor or the combined COX inhibitors prevented the induction of COX-2. These findings suggested that the activity of both isoforms might be detrimental in neuroinflammatory conditions associated with Aβ. However, there are no indications that COX-1 expression levels in microglia are changed under neuropathological or neuroinflammatory conditions (Hoozemans et al, 2008). Therefore, COX-2 activity is the main source of PGE_2 increment and the main cyclooxygenase involved in neuroinflammation.

COX-2 has been well established to be involved in neuroinflammation. Inhibition or genetic deletion of COX-2 has been shown to exacerbate neuroinflammatory response to an endotoxin challenge (Aid et al., 2010). Using LPS-induced neuroinflammation, COX-2 deletion has been shown to increase neuronal damage, glial activation and expression of brain cytokines and ROS-expressing enzymes – a major source of superoxide during neuroinflammation (Aid et al., 2010). Also, COX-2 isoform plays an important role in synaptic plasticity, memory consolidation and cortical development in neurons and mediates neuroinflammation in microglia (Fiebich et al., 2014). Therefore, proper regulation of COX-2 is vital to homeostasis; highlighting the need to inhibit excessive production of COX-2 in neuroinflammatory disorders.

1.1.5.3. Microsomal prostaglandin E_2 synthase-1 (mPGES-1)

The cyclooxygenases have been better understood upon the discovery of prostaglandin E_2 synthase (PGES), which belongs to a superfamily known as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) (Jakobosson et al., 1999; Samuelsson et al., 2007). PGESs regulate the final step in the synthesis of PGE_2. mPGES-1 is responsible for the conversion of prostaglandin (PG) H_2, produced by the COX enzymes into PGE_2, a powerful mediator of inflammation (Leclerc et al., 2013). To date, there are three known PGES: cytosolic (c) PGES, microsomal (m) PGES-1 and mPGES-2. mPGES-1 is induced by pro-inflammatory stimuli (such as LPS, IL-1β and TNFα), down regulated by anti-

inflammatory glucocorticoids and functionally coupled with COX-2 in marked preference to COX-1 (de Oliveira et al., 2008). In addition, microglia-specific expression of mPGES-1 contributes to LPS-induced PGE_2 production (Ikeda-Matsuo et al., 2005). Enhanced mPGES-1 expression has been demonstrated in several pathologies including rheumatoid arthritis (Westman et al., 2004), osteoarthritis (Li et al., 2005) and Alzheimer's disease (Chaudhry et al., 2008). In addition, in a study using mPGES-1 knockout mice treated with IL-1β, it was shown that activation of mPGES-1 is involved in inflammation (Siljehav et al., 2012). These suggest that mPGES-1 is important in neuroinflammation and its inhibition is vital in modulating this process.

1.2. Nuclear factor kappa B (NF-κB) signalling pathway

NF-κB, a transcription factor, is a complex dynamic protein interaction network of several interacting components that regulate each other. Most of the components of the NF-κB signalling are regulated by each other or by upstream, downstream or neighbouring signalling molecules at the level of protein synthesis. NF-κB is sometimes called a sequence-sequence DNA-binding factor with specificity on binding to specific sequence, thereby controlling the flow of genetic information from DNA to RNA. It is a heterodimeric protein comprising of different combinations of members of the Rel family of transcription factors. There are five members of the transcription factor NF-κB family: RelA (p65), RelB and c-Rel, and the precursor proteins NF-κB1 (p105) and NF-κB2 (p100) which are processed into p50 and p52 respectively (Oeckinghaus et al., 2011).

At resting conditions, NF-κB dimers are bound to inhibitory I-kappa B (IκB) proteins that sequester NF-κB complexes in the cytoplasm. Degradation of IκB by an inflammatory stimulus (e.g. LPS) is initiated through the phosphorylation by IκB kinase (IKK) complex, which consists of catalytically active kinases, IKKα and IKKβ, and the regulatory subunit IKKγ (NEMO). Phosphorylated IκB is targeted for ubiquitination and proteasomal degradation, releasing bound NF-κB dimers to translocate into the nucleus where transcriptional activity of nuclear NF-κB is regulated by post-translational modifications (PTMs). Two main activating pathways of NF-κB have been reported (Oeckinghaus et al., 2011); the classical (canonical) and alternate (non-canonical) pathways. These pathways are shown in Figure 4 below. Upon activation, NF-κB dimers translocate from the cytoplasm to the nucleus. In the nucleus, they bind to the DNA and regulate transcription of pro-inflammatory mediators.

NF-κB transcription factors are critical regulators of immunity, stress responses, apoptosis and differentiation (Rojo et al., 2014). NF-κB dependent transcription is not only tightly

controlled by positive and negative regulatory mechanisms, but also closely coordinated with other signalling pathways, making it a complex pathway. This crosstalk is crucial to shaping the diverse biological functions of NF-κB into cell type- and context specific responses.

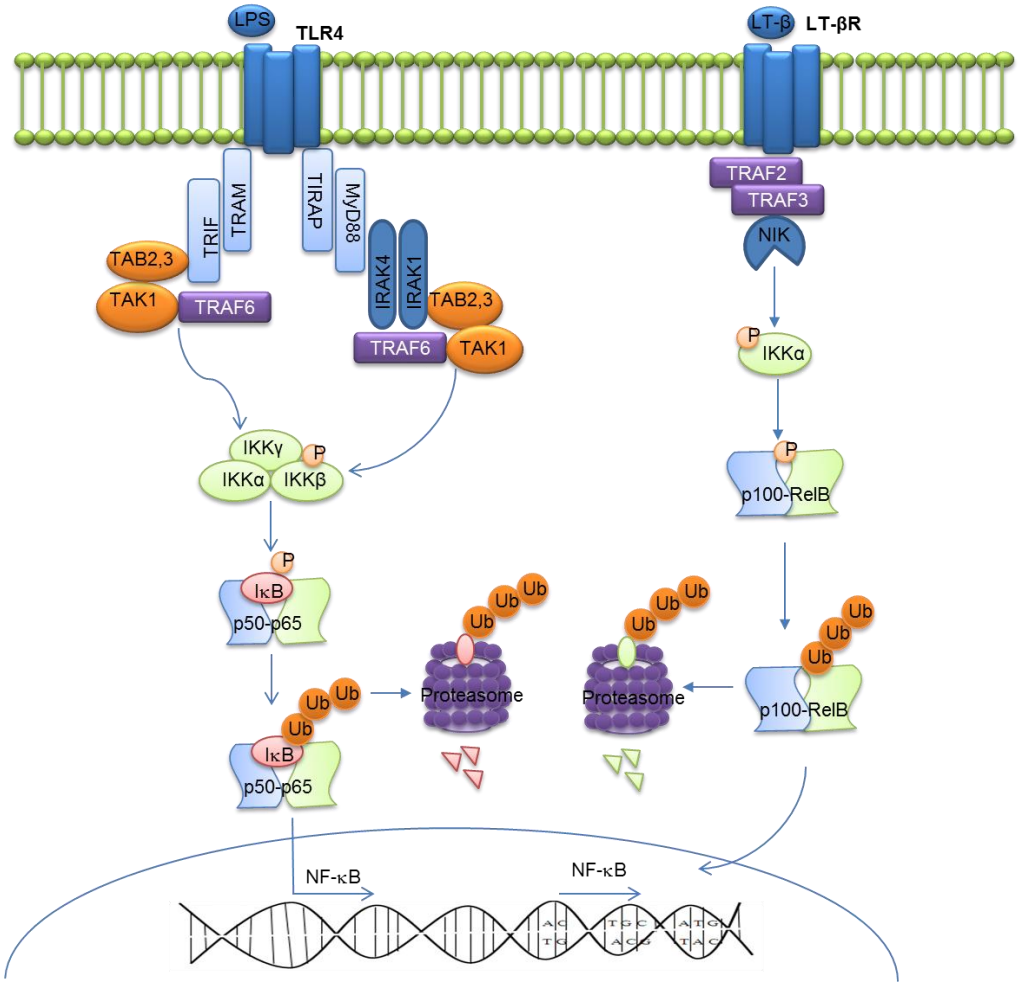

Figure 4: The canonical and non-canonical pathways of NF-κB activation.
Upon activation by a stimulus, NF-κB dimer is released, translocate to the nucleus and cause transcription of NF-κB genes (adapted from Oeckinghaus et al., 2014; Gerondakis et al., 2014).

In the CNS, NF-κB has been reported to have diverse functions. Activated NF-κB can be transported in a retrograde fashion from activated synapses to the nucleus to translate short-term processes to long-term changes (Kaltschmidt & Kaltschmidt, 2009). In glia cells, NF-κB is inducible and regulates the process of inflammation that exacerbates diseases involving neuroinflammation. The microglia express TLR4 receptors, which upon activation by LPS initiates the signalling cascade leading to the translocation of p65NF-κB subunit from the cytoplasm to the nucleus where it induces various NF-κB regulated genes. Activation of NF-κB in the periphery has been found to be regulated in a similar way in the CNS supporting that NF-κB plays a role in immune and inflammatory responses in the brain as in the periphery.

It has also been reported that NF-κB levels increase as a consequence of brain injury. For instance, an increase in NF-κB activity has been reported in ischemia (Harari & Liao, 2010). In addition, the potential role of NF-κB in neurodegenerative diseases in which inflammation in the CNS is likely to be important has also been reported especially in diseases like multiple sclerosis (MS) and Alzheimer's disease (AD). Moreover, certain viral infections in the CNS have been suggested to involve NF-κB as a regulator of their transcription or in viral clearance. This was first shown for HIV of which the replication is implicitly dependent on NF-κB (Hong & Banks, 2014).

NF-κB is an important signal in neurodegenerative diseases. For example, it has been reported that the neurotoxic peptide amyloid beta (Aβ), which is deposited in plaques of AD patients, can activate NF-κB in glia and neuronal cells (Kaltschmidt et al., 1997). Also, NF-κB controls the expression of APP and BACE1, which enhances the formation of Aβ (Buggia-Prevot et al., 2008; Chami et al., 2012). Previous studies found that the activation of NF-κB contributes to the increase in β-secretase in neuronal cells. In AD brains, increased levels of BACE1 and p65 NF-κB has been reported (Chen et al., 2012). Chen et al. (2012) also reported that p65 NF-κB expression leads to an increase in BACE1 transcription and knockout of p65 NF-κB decreases gene expression of BACE1 in cells. These suggest that NF-κB is an important transcription factor in the progression of CNS neurodegenerative diseases.

1.3. p38 Mitogen-activated protein kinase (p38 MAPK) signalling

The Mitogen-activated protein kinase-signalling pathway is used by eukaryotes to transduce external signals to intracellular responses (Coulthard et al., 2009). The responses are modulated by the MAP kinases, which include c-Jun N-terminal kinases (JNKs), extracellular signal- regulated kinases (ERK 1/2) and p38 isoforms. Activation of MAPKs occurs via a three-tiered phosphorylation cascade. Stimuli such as inflammatory cytokine or osmotic shock initiate GTPase-dependent activation of several upstream kinases, the mitogen-activated protein kinase kinase kinases (MAPKKKs).

The p38MAPK is a 38kDa polypeptide, which exists in 4 isoforms (α, β, γ, δ), which are encoded by separate genes, expressed in different tissues and cell types, and are often functionally distinct. The p38MAPKs are described as the stress-activated protein kinases as they are primarily activated through inflammatory insults, extracellular stresses and cytokines (Corrêa & Eales, 2012; Krementsov et al., 2013). p38MAPK regulates inflammation and cell death but has also been shown to play a non-conventional role in cell survival (Krementsov et al., 2013). Activated p38MAPK up-regulates cytokine production by

direct phosphorylation of transcription factors and downstream kinase stabilization and translation of mRNAs encoding for pro-inflammatory cytokines (Krementsov et al., 2013; Sabio & Dais, 2014). These observations have identified p38α and p38β as principal mediators of inflammatory response. Hence, great effort has gone into identification of p38α alone or both p38α and β antagonist for inflammatory disorders over the years. Although the p38α and p38β MAPK are both widely expressed, the p38α is the best-characterized isoform and the most abundant isoform (Krementsov et al., 2013). The p38MAPK is activated downstream from the activation of TLR activation and promote the production of multiple pro-inflammatory and T cell polarizing cytokines.

The p38 MAPK pathway has been reported to play a central role in the expression and activity of pro-inflammatory cytokines. Upon activation by a stimulus (e.g. LPS), MAPKKKs phosphorylate and activate the dual specific MAPK kinases (MKKs such as MKK3, MKK4, and MKK6), which in turn phosphorylate p38 MAPK (Figure 5).

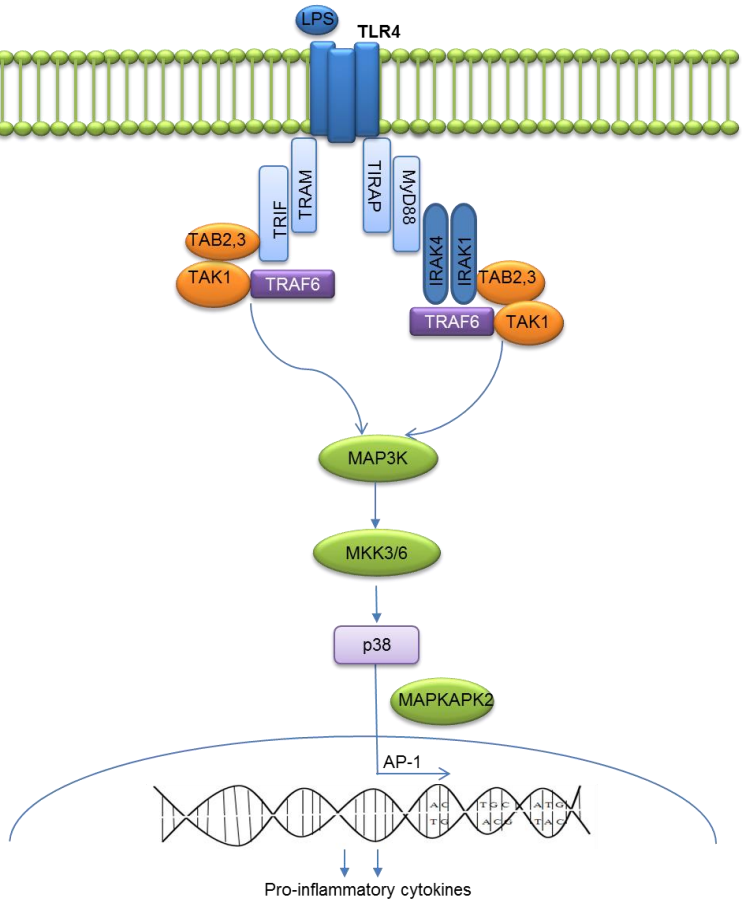

Figure 5: p38MAPK signalling.
Activation of TLR4 by a stimulus (e.g. LPS) activates p38MAPK through activation of MAP kinases, which activate p38 and regulates the production of pro-inflammatory cytokines such as TNFα and IL-6 (adapted from Kimura et al., 2013).

This pathway phosphorylates and enhances the activity of many transcription factors, such as NF-κB, ETS domain-containing protein (ELK-1), heat shock transcription factor-1 and SRF accessory protein 1 (SAP-1) (Myers & Shubayev, 2011). In microglia-driven inflammation, p38 MAPK cascades have been shown to contribute to transcriptional and post-transcriptional regulation of iNOS and TNFα gene expression in LPS-activated glial cells (Bhat et al., 1998). Previous studies have shown the importance of this signalling pathway in the CNS. For example, a study demonstrated that the deficiency of microglial p38α MAPK rescues neurons and reduces synaptic protein loss by suppressing LPS-induced TNFα production (Xing et al., 2011). In a recent study using cortical neurons from wild-type or p38β knockout mice and wild-type neurons treated with two highly selective inhibitors of p38α MAPK, results show that neuronal p38β is not required for neurotoxicity induced by multiple toxic insults (Xing et al., 2014). This finding suggests the critical importance of microglial p38α. It has also been reported that pharmacological inhibition of p38α MAPK in an AD mouse model decreases brain pro-inflammatory cytokine production and attenuates synaptic protein loss (Munoz & Ammit, 2010). These research findings demonstrate that microglial p38α MAPK-mediated TNFα and interleukin overproduction is crucial to inflammation-induced neurotoxicity. Therefore, inhibition of p38α MAPK signalling is crucial in modulating neuroinflammation. p38MAPK signalling shares a common signalling node with NF-κB signalling, TNF receptor associated factor (TRAF). There are seven members of the TRAF family (TRAF1-7) defined by the presence of domains, which mediates binding to receptor and signalling mediators. TRAF6 represents a central point of divergence for activation of NF-κB and p38MAPK signalling pathways (Figure 6) through toll-like receptor (TLR) 4 signalling.

In TLR4 signalling, TRAF6 induces activation of TAK1, a mitogen activated protein kinase kinase kinase kinase (MAPK3K), to trigger activation of both AP-1 (p38MAPK) and NF-κB. This activation shows that NF-κB and p38MAPK represent common outcomes of TRAF-dependent signalling but the underlying regulatory mechanisms differ (Oeckinghaus et al., 2011).

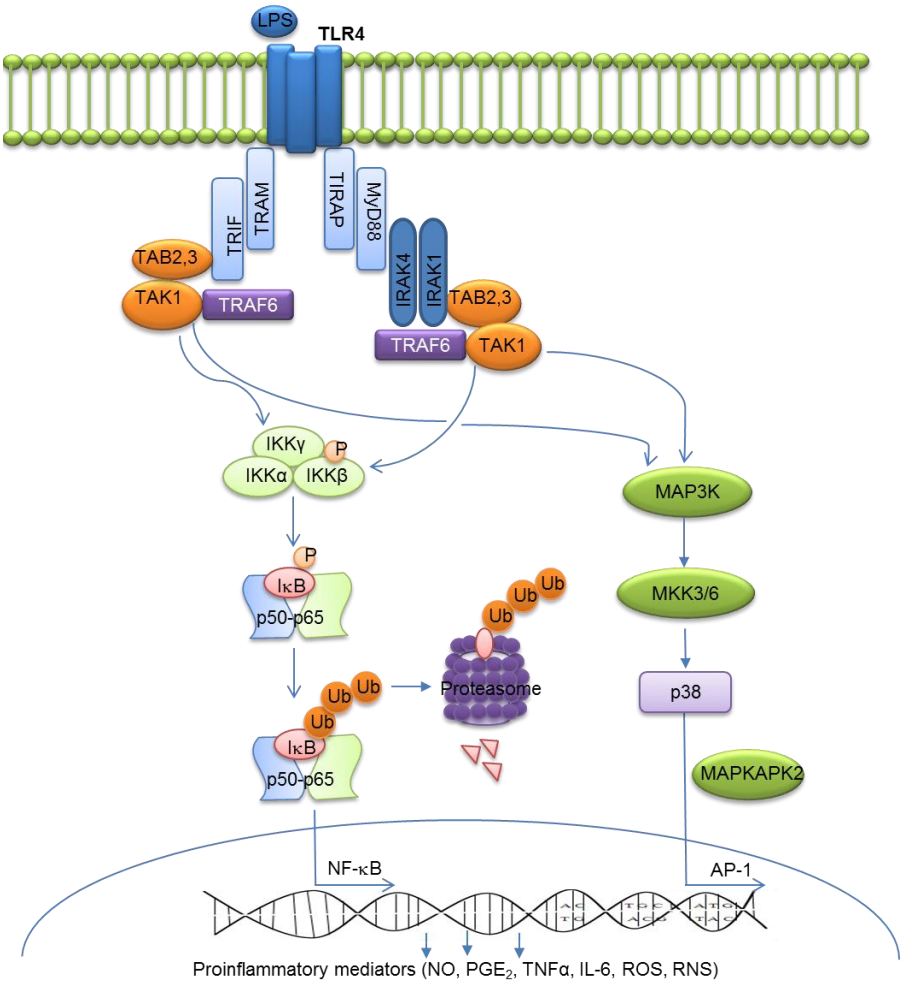

Figure 6: Convergence point for p38MAPK and NF-κB through TRAF6-dependent signalling. In Toll-like receptor (TLR) signalling, upon activation of TLR4 by LPS, TRAF6 induces activation of TAK1, a MAPK3K, to trigger activation of both AP-1 (p38MAPK) and NF-κB. This subsequently leads to the production of proinflammatory mediators (adapted from Kimura et al., 2013).

1.4. Nuclear factor [erythroid-derived 2]-like 2 (Nrf2) antioxidant signalling pathway

After the M1 (pro-inflammatory) phase in microglial activation, the microglia transits into the M2 phase which is responsible for remodelling and restoration of homeostasis. The nuclear factor [erythroid-derived 2]-like 2 (Nrf2) signalling pathway regulates the M2 phase.

Nrf2 transcription factor is widely expressed in human and mouse tissues (Hayes & Dinkova-Kostova, 2014). Nrf2 controls the expression of many Phase I and Phase II drug metabolising enzymes (Hayes & Dinkova-Kostova, 2014). Nrf2 detoxifies ROS mainly through the transactivation of a series of genes known as antioxidant response elements (AREs) (Xiang et al., 2014). These genes are responsible for the expression of antioxidants and phase 2 detoxifying enzymes such as NAD (P) H: quinone oxidoreductase 1 (NQO1), glutathione S-transferase (GST), heme oxygenase-1 (HO-1) and glutathione peroxidase, which maintain cell homeostasis (Hayes & Dinkova-Kostova, 2014; Niture et al., 2014).

Nrf2 belongs to the cap 'n' collar (CNC) family of basic region leucine zipper transcription factors. Nrf2 comprises of seven highly conserved domains called Nrf2-ECH homology domains 1-7 (Neh1 – 7). Kelch ECH Associated Protein 1 (Keap1) binds to Nrf2 in the cytoplasm under normal conditions. Keap1 is a cytoskeleton protein predominantly present in cytosol, and it acts as a stress sensor and negative repressor of Nrf2 (Xiang et al., 2014). It is also capable of binding actin filaments (Bellezza et al., 2010). Nrf2 in its bound form to Keap1 is targeted towards ubiquitination and subsequent proteasomal degradation in a Keap1-dependent fashion. The interaction between Nrf2 and Keap1 occurs via a 'two-site tethering' mechanism, otherwise known as the 'hinge and latch' mechanism (Rojo et al., 2014). At oxidative conditions, Nrf2 is released from Keap1 repression, translocate to the nucleus, forms a heterodimer with small Maf proteins, recognizes and binds to ARE promoter site in nucleus encoding detoxifying and antioxidant enzymes such as HO-1, GSH, SOD and NADPH: quinone oxidoreductase. Protein phosphorylation, as the major post-translational mechanism in signalling processes, has a central role in regulating the Nrf2 liberation process, stability, and nuclear translocation. Phosphorylation stabilises Keap1, de-phosphorylation promotes rapid degradation of Keap1 and hence stabilization of Nrf2 (Bellezza et al., 2010). Under unstressed condition, Nrf2 is constantly degraded via Keap1-mediated ubiquitination that is counter balanced by constitutive Nrf2 translocation.

Keap1-mediated ubiquitination is impeded under oxidative conditions, while Nrf2 nuclear translocation is elevated. This results in the expansion of the pool of free Nrf2 proteins. The nuclear influx of Nrf2 is still determined by the intensity of oxidative stress and Keap1 still modulates the redox-sensitivity of Nrf2 by controlling the availability of free Nrf2 proteins (Xiang et al., 2014). HO-1 is implicated mainly in immunomodulation. Induction of HO-1 protects cells from oxidative stress-related cytotoxicity and apoptotic cell death.

1.4.1. Interaction(s) between Nrf2 and NF-κB

Induction of Nrf2 by compounds of different chemical classes is directly correlated to the inhibition of COX-2 and iNOS expression (Baird & Dinkova-Kostova, 2011; Liu et al., 2008). However, the anti-inflammatory effects of these molecules are only partially Nrf2-dependent and the exact relationship between Nrf2-induction and anti-inflammatory properties remains to be clarified. Nrf2-deficient mice, subjected to a moderately severe head injury, show greater cerebral NF-κB activation compared with wild-type animals (Jin et al., 2008). Induction of Nrf2 over expression has been shown to suppress NF-κB-DNA binding activity (Song et al., 2009). In addition, NF-κB p65 subunit represses the Nrf2-ARE pathway at transcriptional level (Bellezza et al., 2010). This suggests an interaction between the Nrf2 and NF-κB signalling pathways.

Studies on hippocampus of LPS-treated mice have shown that deletion of Nrf2 gene results to an increase in inflammatory markers (iNOS, IL-6 and TNFα) in microglial cells (Innamorato et al., 2008). In addition, Nrf2 activation results in a decrease in LPS-induced activation of p38 and inflammatory markers (TNFα, IL-1β, IL-6, PGE_2 and NO) in microglial cells (Koh et al., 2009).

Therefore, agents that activate Nrf2 and inhibit NF-κB may be beneficial in the treatment of chronic neuroinflammatory diseases.

1.5. Neuroinflammation in diseases

1.5.1. Alzheimer's disease (AD)

Alzheimer's disease, which is the most common form of dementia, is one of the most common age-related neurodegenerative diseases (Alzheimer's-Association, 2014). The symptoms of AD are characterised by memory loss, progressive impairment of cognition and various behavioural and neuropsychiatric disturbances (Nordberg, 2014). The pathological hallmarks of AD in the brain include extracellular amyloid plaque comprising aggregated, cleaved products of amyloid precursor protein (APP) and intracellular neurofibrillary tangles (NFTs) generated by hyper phosphorylated forms of microtubule-binding protein tau (Holtzman et al., 2011).

The observation that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) protects the brain from the onset or progression of neurodegenerative diseases especially AD has been a great link in pointing neuroinflammation to AD (Hoozeman et al., 2011). The evidence of an inflammatory response in AD includes changes in microglia morphology – from ramified (resting) to amoeboid (active) and astrogliosis (manifested by an increase in the number, size and motility of astrocytes surrounding the senile plaques (Glass et al., 2010). They also reported that the microglia surrounding the plaques stained positive for activation markers and pro-inflammatory mediators, including MHC class III, COX-2, monocyte chemo-attractant protein 1 (MCP-1), and pro-inflammatory cytokines TNFα, IL-1β and IL-6. A recent study on several innate genes in genome-wide association studies (GWAS) of AD demonstrated a causal relationship between inflammation and the disease (Escott-Price et al., 2014).

Aβ aggregates as well as products derived from dead cells can trigger microglia and astrocytes through TLR and RAG-dependent pathways leading to local inflammation (Liu et al., 2012) (Figure 7). The local inflammation may further amplify neuronal death in the CNS. The activation of signal-dependent transcription factors such as NF-κB (Srinivasan & Lahiri, 2015) and AP-1 by various trigger (including possibly Aβ) could result in the production of a

vast array of amplifiers including the pro-inflammatory cytokines (Glass et al., 2010). The pro-inflammatory cytokines TNFα and IL-6 produced from the activation of the microglia may act directly on neurons to induce apoptosis. It may also act on astrocytes activating NF-κB and p38MAPK signalling. This further activation can generate proinflammatory cytokines, which may also act directly on neurons to induce apoptosis. On the other hand, the factors released by the astrocytes can also activate the microglia there by causing a vicious cycle that eventually leads to neuronal death. These evidences highlight the huge role played by TNFα and IL-6 in neuroinflammation in AD.

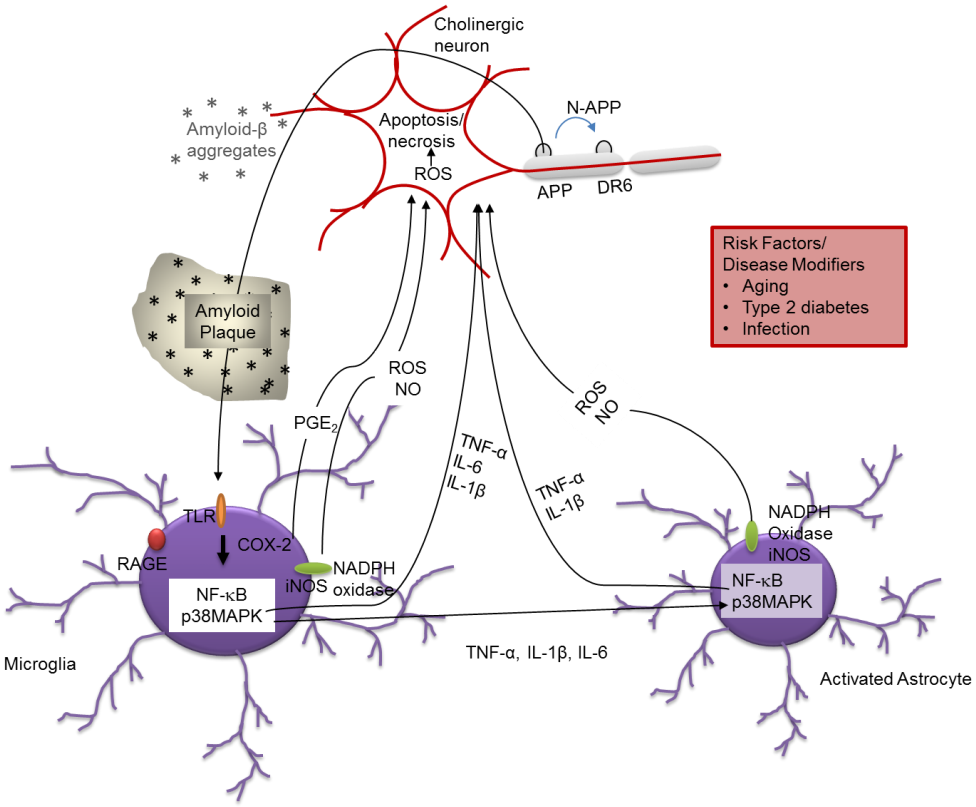

Figure 7: Inflammatory components in Alzheimer's disease (adapted from Glass et al., 2010).

1.5.2. Parkinson's disease (PD)

Parkinson's disease is the second most common neurodegenerative disease after AD (Gandhi & Wood, 2010). It is also the most common movement disorder. The prominent clinical features are motor symptoms (such as bradykinesia, tremor, rigidity and postural instability) and non-motor-related symptoms (such as olfactory deficits, autonomic dysfunction, depression, cognitive deficits, and sleep disorders) (Shulman et al., 2010). Similar to AD, PD is a proteinopathy; characterized by the accumulation and aggregation of misfolded α-synuclein (Breydo et al., 2012). The neuropathological hallmarks are intracellular inclusions containing α-synuclein called Lewy bodies and Lewy neuritis and the

loss of dopaminergic neurons in the substantia nigra of the midbrain and in other brain regions as well (Breydo et al., 2012; Glass et al., 2010). Microglial activation and an increase in astroglia and lymphocyte infiltration also occur in the neuropathological alteration in PD outside the loss of dopaminergic neurons. Microglial activation leads to the production of various pro-inflammatory mediators.

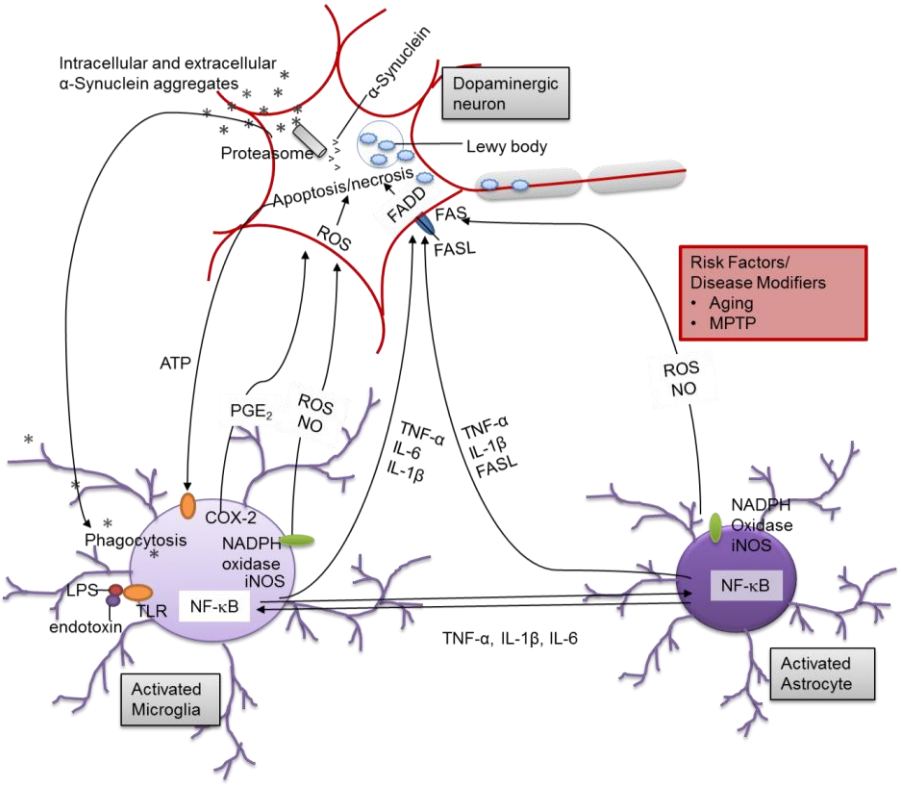

Figure 8: Inflammatory components in Parkinson's disease (adapted from Glass et al., 2010).

These inflammatory mediators such as ROS, NO, TNFα and IL-1β derived from the microglia modulate the progression of neuronal cell death in PD (Hald & Lotharius; 2005, Tansey & Goldberg, 2010) (Figure 8). The inflammatory mediators generated from microglial activation also activate the astrocytes. The combination of the various factors that are produced in both the activated microglia and astrocyte in turn may cause neurotoxicity (Tansey & Goldberg, 2010).

In addition, PD-associated activated microglial cells release NO produced by iNOS as well as ROS. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the major source of ROS production in activated microglia in PD (Hunot et al., 1996) (Figure 8). Microglial cells phagocytise the extracellular α-synuclein, resulting in the activation of NADPH oxidase and ROS production (Zhang et al., 2005). The nitration of α-synuclein induced by oxidative stress is also a potent inducer of microglial activation. These different activators of the microglia in PD facilitate neuroinflammation subsequently leading to

neuronal death. Recently, it has been reported that microglial activation influences neuronal function in PD dementia (Fan et al., 2014). This observation further confirms the influence of neuroinflammation on surrounding neurons in PD.

1.5.3. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) (also known as Lou Gehrig's disease) is a progressive fatal neurodegenerative disease that affects motor neurons in the brainstem, spinal cord, and motor cortex (Hooten et al., 2015). The clinical features of ALS involve degeneration of motor neurons producing fasciculation, muscle wasting and weakness, increased spasticity and hyper-reflexia (Ajround-Driss & Siddique, 2015; Maniecka & Polymenidou, 20015). ALS is universally fatal, with a median age of onset of 55years and a survival of 2-5 years after the onset of symptoms (Glass et al., 2010).

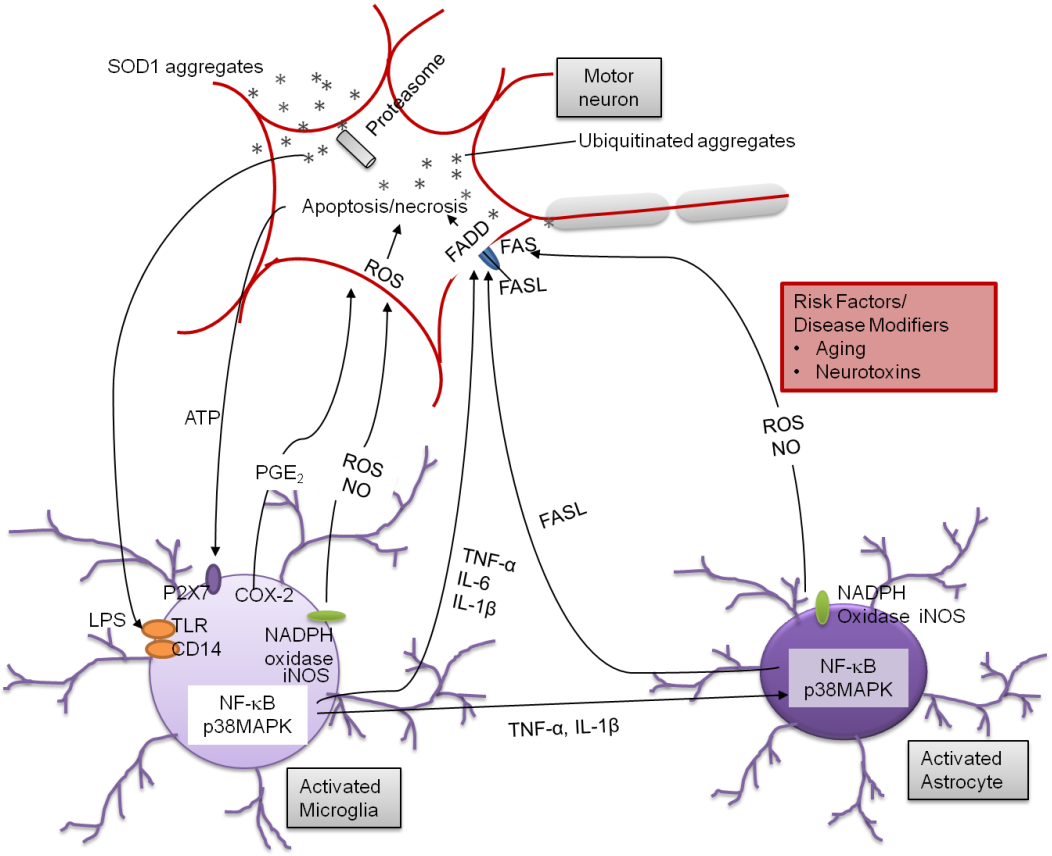

Figure 9: Inflammatory components in Amyotrophic Lateral Sclerosis (adapted from Glass et al., 2010).

Majority of ALS cases are sporadic with only 10% of the cases familial. The major determinants of motor neuron death in ALS are yet to be established. However, the motor neuron death is ALS could be as a consequence of multiple factors. Although the main cells affected are motor neurons, there is increasing evidence that neighbouring glia cells are

involved during the pathogenesis of the disease (Clement et al., 2003; Yamanaka et al., 2008).

There is a possibility that toxic aggregates in ALS can induce inflammatory responses in microglia via TLR and CD14. Therefore, neuroinflammation in this case might be involved in the disease progression. Activated microglia cells can activate astrocytes by producing cytokines, NO and ROS. Activated microglia and astrocytes amplify the damage in the motor neurons by further activation of the NF-κB and p38MAPK signalling pathways in the microglia and astrocytes. The dying motor neurons release ATP, which can activate microglia though, purinergic receptor P2X ligand-gated ion channel 7 (P2RX7) expressed by the microglia (DiVirgilio et al., 2009) (Figure 9). This further activates the microglia resulting in a vicious cycle thereby increasing the chronicity of neuroinflammation.

1.5.4. Multiple sclerosis

Multiple Sclerosis (MS) is a heterogeneous and complex autoimmune disease that is characterized by inflammation, demyelination and axon degeneration in the CNS (Naegele & Martin, 2014). Recently, neuroinflammation has been implicated in the demyelination of the neurons (Ellwardt & Zipp, 2014). The cause is from a primary defect in the immune system that targets components of the myelin sheath, thereby resulting in secondary effects on the neurons. MS predominantly affects young adults and 2-3 times more in females than in males (Glass et al., 2010).

Both the innate and acquired immune systems play a part in the pathology of MS. Dysfunction of the BBB, migration of immune cells into the CNS and attack of antigens on neurons and oligodendrocytes account for the major inflammatory events in MS (Ellwardt & Zipp, 2014). Immune cells outside the CNS such as dendritic cells have been reported to play a role in the pathogenesis of MS (Naegele & Martin, 2014). Also, CNS inflammatory cells, microglia and astrocytes, play a role in progression of MS (Miljkovic et al., 2011). Bacterial infection, viruses or other environmental stimuli activate the microglia in MS (Kakalacheva & Lunemann, 2011). Upon activation, the microglia cells produce inflammatory mediators through the NF-κB (Srinivasan & Lahiri, 2015) and p38MAPK signalling pathways. Naïve T cells recognise the myelin-derived antigen-presented by antigen-presenting cells. In the presence of inflammatory mediators like IL6 and TGF-β, the naïve T cells are induced to express retinoic acid-related orphan receptor γt (RORγt) and differentiate to T helper 17 (Th17) cells (Korn et al., 2009). Activated microglia and astrocytes also secrete inflammatory cytokines (Interleukin 13 (IL-13)), which induces the differentiated Th17 cells to produce proinflammatory cytokines (including TNFα). This results in the damage of the myelin sheaths, which protects the nerve axons. This leads to demyelination of the neurons. In

addition, the astrocytes when activated secrete B-cell activating factor (BAFF), a survival factor for auto reactive B cells (Dalakas, 2008), which in turn differentiate into plasma cells and produce anti-myelin antibodies. ROS and NO are also generated by the microglia and the astrocyte. These inflammatory mediators also contribute to the damage caused on the neurons and in the destruction of the myelin sheaths on the neurons (Ellwardt & Zipp, 2014).

Microglial activation with LPS has been reported to cause an increased production of ATP which signals to astrocytes to produce increased excitatory postsynaptic current in hippocampal neurons (Pascual et al., 2012). Therefore, the activation of microglia cells subsequently influences surrounding cells, including astrocytes and neurons.

1.6. Artemisinin and its derivatives

Artemisinin (Figure 10) is a natural product isolated from the Chinese herb *Artemisia annua* ('qinghao' or sweet wormwood) identified in 1972 during the Vietnam War by Tu Youyou a Chinese scientist from the Chinese Academy of Traditional Chinese Medicine (Miller & Su, 2011; Neill, 2011). Artemisinin has also been isolated from other *Artemisia* species such as *A. scoparia* (Singh & Sarin, 2010). It is a sesquiterpene lactone (SL) with well-known antimalarial activity. Sesquiterpene lactones have been isolated from numerous genera of the asteraceae family and are described as the active constituents of a variety of medicinal plants, which are used in traditional medicine for the treatment of inflammatory diseases (Hall et al., 1979; Hall et al., 1980). Artemisinin was first reported in 1979 but biological chemists were puzzled by the apparent stability of the hitherto unknown 15-carbon (sesquiterpene) peroxide structure (White, 2008). Hence, a full chemical synthesis was conducted subsequently and four years later reported.

Artemisinin, which has been used in traditional Chinese medicine for over 2 millennia, still remains one of the 'gems' of traditional Chinese medicine (Efferth et al., 2008). It is still one of the most promising natural products investigated in the past two decades. Over the years, a couple of semi-synthetic derivatives of artemisinin have been made including artemether and artesunate (Figure 10). Most of the derivatives of artemisinin have been reported to be useful in the treatment of malaria. Today, artesunate, artemether and arteether are the more widely used derivatives of artemisinin (Ho et al., 2014).

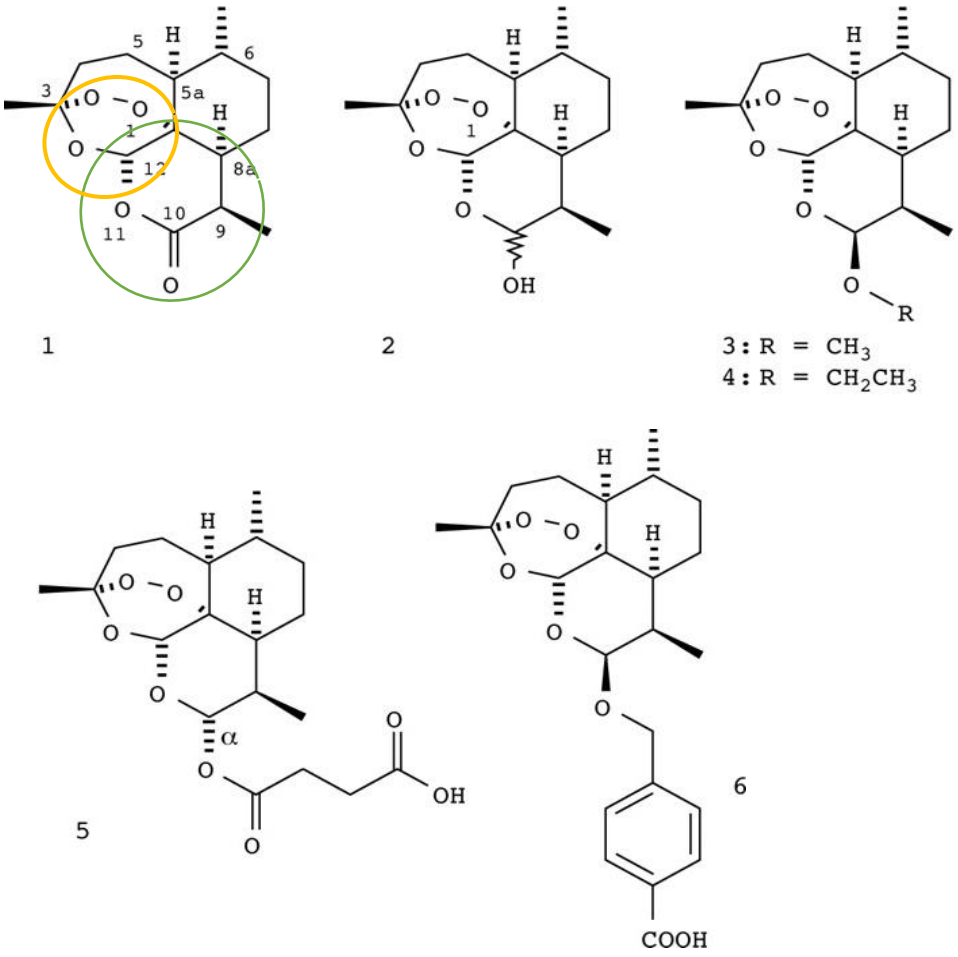

Figure 10: Artemisinin (1) and derivatives dihydroartemisinin (2), artemether (3), arteether (4), artesunate (5) and artelinate (6).
Circled in green is the lactone ring while the ring circled in yellow is the trioxane ring (Woodrow et al., 2005).

1.6.1. Structure and chemistry of artemisinin and its derivatives- artemether and artesunate.

The IUPAC name, molecular formula, molar mass and other properties of artemisinin and its derivatives artemether and artesunate are shown in table 1 below. Artemisinin has a tetracyclic structure with a trioxane ring and a lactone ring. The trioxane ring contains a peroxide bridge, the active moiety of the molecule. Reduction of artemisinin derivatives to dihydroartemisinin give rise to a chiral centre, that may ultimately lead to the formation of 'prodrugs' which could be oil soluble or water soluble (O'Neill & Posner, 2004). The most accessible site in artemisinin is the lactone group.

Table 1: Details of some properties of artemisinin and its derivatives artemether and artesunate. (* Log D value of artemisinin was not reported in literature).

Compound	IUPAC name	Molecular formula	Molar Mass	Log P	Log D
Artemisinin	(3R, 5aS, 6R, 8aS, 9R, 12S, 12aR)-octahydro-3, 6, 9 – trimethyl-3, 12-epoxy-12H-pyrano [4, 3-j]-1, 2-benzodioxepin-1093H)-one	$C_{12}H_{22}O_5$	282.332g/mol	2.94 (Abraham and Acree, 2013)	*
Artemether	(1R,4S,5R,8S,9R,10S,12R,13R)-10-methoxy-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclo[10.3.1.0^(8,13)] hexadecane	$C_{16}H_{26}O_5$	298.3746g/mol	3.53 (Avery et al., 1995)	3.1 (Schlagenhauf-Lawlor, 2007)
Artesunate	(3R,5aS,6R,8aS,9R,10S,12R,12aR)-Decahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyranol [4,3-j]-1,2-benzodioxepin-10-ol, hydrogen succinate	$C_{19}H_{28}O_8$	384g/mol	2.77 (Avery et al., 1995)	-9.3 x 10-2 (Schlagenhauf-Lawlor, 2007)

Reduction of artemisinin by sodium borohydride produced dihydroartemisinin in which the lactone group is converted to a lactal group (D'Acquarica et al., 2010). Ether substitution gives artemether, which is oil soluble. The water-soluble derivative artesunate is the succinate ester of dihydroartemisinin. Currently, artemisinin and its derivatives artesunate, a water-soluble derivative, and artemether, an oil soluble derivative, are the most important drugs for the treatment of malaria and are used in artemisinin-based combination therapies (ACTs) recommended by the World Health Organization (Breman et al., 2007). The strength of artemisinin and its derivatives in antimalarial therapy lies in their unique chemical structures, which differ from the standard quinolone (Ho et al., 2014).

Artemisinin and its derivatives kill the asexual stages of parasite development in the blood and also affect the sexual stages of *Plasmodium (P) falciparum* (gametocytes), which transmit the infection to others. Artemisinin derivatives are also used in cerebral malaria, one of the most severe complications of *P. falciparum* infections (Pasvol, 2005). A clinical trial done in China of which some of the patients had cerebral malaria showed that artemisinin and its derivatives were effective in treating malaria including cerebral malaria (Li et al, 1994). In another study by Hien et al (1992), artemisinin suppositories, intravenous artesunate and intravenous quinine were compared in the treatment of cerebral malaria. Findings from the study suggested that artemisinin suppositories are as effective as

artesunate and quinine given intravenously. These studies suggest that artemisinin and its derivatives might be able to cross the blood-brain barrier. However, there is a possibility that mostly dihydroartemisinin penetrate into the CNS.

Cerebral malaria is mostly characterized by hyper-parasitaemia and excessive production of type 1 pro-inflammatory cytokines followed by up-regulation of endothelial cell adhesion molecule expression which contributes to the sequestration of parasitized erythrocytes in the brain microvasculature (Mimche et al., 2011; Schofield & Grau, 2005). Research finding suggests that in cerebral malaria, trypsin-resistant membrane components and soluble factors of *Plasmodium falciparum*-infected red blood cells contribute to the impedance of the blood brain barrier (BBB) in a multistep and multifactorial process (Tripathi et al., 2007). This suggests that the integrity of the BBB is compromised for artemisinin and its derivatives to permeate into the CNS to exert action. In another study by Coghi et al (2009), the interaction of artemisinin and its derivatives with oxyhemoglobin Hb-FeII, Hb-FeII, carboxyHb-FeII, heme-FeII and carboxyheme FeII have been suggested to play an important role in the mode of action in cerebral malaria. In a recent study, artemisinin derivatives have been reported to inhibit metalloproteinase-9 (MMP9) proteins, which play a role in the pathogenesis of cerebral malaria by destroying endothelial tight junctions and increasing BBB permeability (Magenta et al., 2014). It has also been reported that p-glycoprotein which plays a role in BBB permeability, is inhibited by artemisinin derivatives (Davis et al., 2003; Steglich et al., 2012). There has been no report on the effect of the parent compounds on the inhibition of MMP9 and p glycoprotein. However, reports done by Coghi et al (2009) suggest artemisinin does permeate the BBB. These highlight the need for further investigation of the permeability of these drugs and / or their metabolite DHA into the CNS.

1.6.2. Artemisinin Metabolism

Artemisinin derivatives are metabolised primarily to a principal metabolite dihydroartemisinin (DHA) and then to inactive metabolites through hepatic cytochrome P-450 and other enzyme systems (Golenser et al., 2006; O'Neil & Posner, 2004). Conversion to DHA differs between derivatives at varying rates (Woodrow et al., 2005). However, artemisinin itself is not metabolised to DHA (Golenser et al., 2006). Artesunate is hydrolysed to DHA rapidly by CYP2A6 while artemether is slowly demethylated to DHA by CYP3A4 and CYP3A5 and arteether is converted to DHA slowly by CYP3A4 (Golenser et al., 2006). DHA is further converted to inactive metabolites through glucuronidation, which is catalysed by UDP-glucuronosyltransferase (Woodrow et al., 2005). Other metabolites including deoxyartemisinin, deoxydihydroartemisinin and 9, 10-dihydrodeoxyartemisinin, which lack the endoperoxide bridge, are inactive and are excreted when metabolised. A diagram on the metabolism of artemisinin is shown in Figure 11.

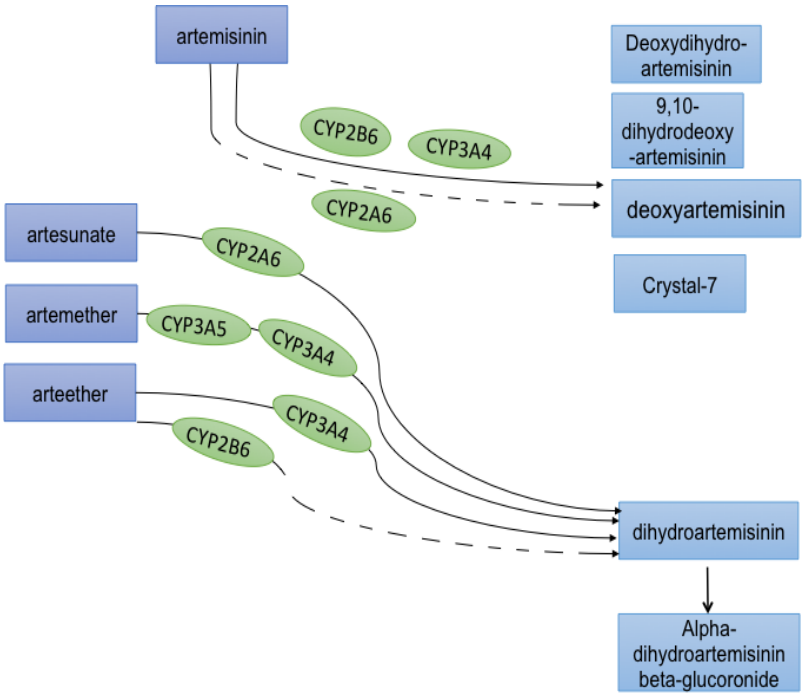

Figure 11: Metabolism of artemisinin and its derivatives (adapted from Woodrow et al., 2005; Golenser et al., 2006).

The metabolism artemisinin derivatives to DHA may affect the bioavailability and activity of the drugs. For example, artesunate is rapidly metabolised while artemether is converted to DHA slowly. Some pharmacokinetics data of the drugs are shown in table 2 below.

Table 2: Some pharmacokinetics parameters of artemisinin and derivatives (Balint, 2001).

Drug	Absorption	Distribution volume (L/kg)	Oral plasma clearance (L/hr)	Elimination half-life (hr)	Bioavailability (%)	Peak plasma concentration (hr)	First-pass effect	Usual oral (dose/kg)
Artemisinin	Rapid and incomplete	-	400	2-5	8-10	< 2	Extensive	20mg
Artemether	Rapid and incomplete	0.67		3-11	54	3	Extensive	4mg
Artesunate	Rapid and incomplete	0.14	2.33	<1	82		Extensive	4mg
Dihydroarte misinin		0.90	1.10	3.1	85	0.65		

Artemisinin has a low bioavailability and high protein binding of 80% (Balint, 2001). This might affect the activity of the drug. It is important to note that artesunate has a high bioavailability of 82% while dihydroartemisinin has a bioavailability of 85%. On the other hand, artemether has a 54% bioavailability and longer elimination half-life. These pharmacokinetics data give an insight what happens to the drug upon absorption. It is important to note that all the drugs are rapidly absorbed.

1.6.3. Anti-inflammatory actions of artemisinin, artemether and artesunate

Previous researches investigated the effect of artemisinin in various models of inflammation. Artemisinin and its derivatives have been shown to be effective in autoimmune diseases, allergic inflammation and septic inflammation (Ho et al., 2014). Artemisinin and its derivatives have also been proven very efficient in the treatment of cerebral malaria as they can inhibit TNF and NO production (Shakir et al., 2011). Among the family of artemisinin derivatives, artesunate is the most studied analogue of artemisinin. This is due to the addition of a hemi-succinate group, which confers substantial water-solubility and high oral bioavailability, which results to a more favourable pharmacological profile (Ho et al., 2014). However, there has not been a comprehensive study on the effect of the artemisinin or its derivatives artemether and artesunate in LPS-activated BV2 microglia cells, a model of neuroinflammation.

In macrophages, artemisinin and its derivatives artesunate and artemether diminish the secretion of macrophage-derived proinflammatory cytokines particularly TNF. Artemisinin and its derivatives have been found to interrupt signalling pathways especially the NF-κB system involved in the expression of genes related to cytokine production, inflammation, adhesion molecules and apoptosis *in vivo* (Pahl, 1999). In another study on the evaluation of the role of the NF-κB system in hemozoin- and 15-HETE-mediated activation of matrix metalloproteinase-9 (MMP-9), artemisinin was reported to abolish NF-κB-mediated and MMP-9 related enhanced release of TNF and IL-1β in human adherent monocytes (Prato et al., 2010). In an *in vivo* study using mouse models, artesunate has been shown to produce inhibition of TNFα (Li et al., 2010). In addition, artemisinin and its derivatives have been shown to inhibit NO production. In a study in RAW 264.7 cells, artesunate significantly inhibited NO production and iNOS mRNA expression (Konkimalla et al., 2008). Also artemisinin has been reported to supress iNOS mRNA expression and NO production in LPS-activated macrophages (Kang et al., 2010). Kang et al (2010) observed that the suppression of NO/iNOS was due to inhibition of IFN-β production and blockage of striated muscle activator of rho (STAR)-1 signalling rather than NF-κB degradation. Artemisinin has been reported to inhibit TNFα and IL-6 production in a dose- and time-dependent manner. In animal models of sepsis induced by CpG-Oligodeoxynucleotide (CpG-ODN), LPS, heat-killed *E. coli* 35218 or live *E. coli* in RAW 2647 cells, artemisinin produced potent anti-inflammatory effects with inhibition of NF-κB activation as a possible mechanism (Wang et al., 2006).

The water-soluble derivative artesunate has been reported to possess immunomodulatory properties as well as anti-inflammatory properties in experimental colitis. Most recently, it

was reported to activate Nrf2 pathway and suppress NO/iNOS production in activated BV2 microglia (Lee et al., 2012). Artemether, the oil-soluble derivative, which is also known as dihydroartemisinin methyl ether, has been shown to have anti-inflammatory effect in mouse model of colitis (Wu, 2011).

As mentioned in section 1.6.2, artemisinin derivatives are metabolised to the active compound DHA. Reports have also shown that DHA possess anti-inflammatory actions. In a study by Yu et al (2012), it was reported that artemisinin and DHA inhibited proinflammatory cytokines TNFα and IL-6 as well as iNOS mediated NO production in RAW264.7 cells following stimulation with LPS. DHA has also been reported to inhibit inflammation in RAW264.7 cells following stimulation with phorbol ester (Kim et al., 2013).

There has also been suggestion that artemisinin and its derivatives might be neurotoxic. This raises a question on the suitability of these compounds. For instance, in a study by Classen et al (1999) neuronal and secondary axonal damage was observed in dogs. It is important to note that the neurologic signs were observed at high doses only. Therefore, lower concentrations are potentially safe. In a review by Woodrow et al (2005) it has been noted that despite pre-clinical evidence of toxicity in brainstem in animals, no major toxicity to humans have been documented. This highlights a discrepancy between animal and human toxicity. This also suggests that artemisinin and its derivatives are safe. However, there is also the need for all drugs to be monitored for toxicity. In addition, reported neurotoxicity might be due to the formation of free radicals, which is a proposed mechanism for artemisinin and its derivatives (Li & Zhou, 2010). Studies by Schmuck et al. (2002) suggested that artemisinin might have induced neurotoxicity by inhibition of mitochondrial function and induction of oxidative stress. These reports suggest the need to monitor the concentrations used to ensure toxicity does not account for the anti-inflammatory actions observed. However, it is also important to note that the possible concentrations to be used for these compounds in neuroinflammatory conditions should be different from that used in malaria or cerebral malaria as these are two different indications. Hence, in this study possible signs of toxicity would be monitored using the MTT assay.

1.7. *In vitro* models of Neuroinflammation

Various approaches have been developed over the years to investigate neuroinflammation *in vitro*. These models have been developed to achieve the aims of the national centre for the replacement, refinement and reduction of animals in research.

A very common approach is the use of lipopolysaccharide (LPS) to activate toll-like receptor in microglia cells (Lull & Block, 2010; Olajide et al., 2013). It involves the stimulation of mono-layer culture of glial cells with LPS. This proven model is cost effective and easy to

run. It is very effective in the measurement of various parameters involved in neuroinflammation. However, a major limitation for this model is the inability to investigate the role relationship between the microglia and neurons. Another model which addresses this limitation involves the use of LPS to activate microglia cells is microglia-neuron co-cultures (Gresa-Arribas et al., 2012). An advantage of the microglia-neuron co-culture is the provision of an avenue to measure neuroprotective effects of anti-inflammatory compounds *in vitro*. Therefore, the microglia-neuron co-culture is a useful model for both the study of neuroinflammation and neuroprotection *in vitro*. Although this approach is solely *in vitro*, it is an expensive model. The use of organotypic membrane culture of postnatal rat hippocampal slices is another *in vitro* model to investigate neuroinflammation (Huuskonen et al., 2005). This approach does reduce the number of animals used however it still involves some animals. In addition, this approach is expensive. Another approach is a model that involves the induction of interleukin-6 in murine astrocytes with Theiler's murine encephalomyelitis virus (Rubio et al., 2011). An advantage of this model is that it blocks out oestrogen, which plays a role in neuroinflammation. However, it is limited because it has used astrocytes instead of microglia, which is widely accepted, as the main glia cell involved in neuroinflammation. These various models have their advantages and disadvantages and can be used for the study of neuroinflammation depending on the experimental design.

In this present study, in other to understand the effect of artemisinin and its derivatives in neuroinflammation, LPS-activated BV2 microglia, an *in vitro* model was used because it is cost effective. In addition, it provides an adequate model to study molecular mechanism of action of the compounds.

1.8. Gap in knowledge

Several studies have shown that artemisinin, artemether and artesunate possess anti-inflammatory activity. However, there is no detailed evidence that the drugs could inhibit neuroinflammation in LPS-activated microglia cells. Also, the exact mechanism(s) of anti-neuroinflammatory action of these drugs are not fully understood.

1.9 Aims and objectives

1.9.1 General aim

The study aims to investigate the effects of artemisinin, artemether and artesunate in LPS-activated microglia.

1.9.2 Specific Objectives

The specific objectives of the research were:

1. To investigate the effects of artemisinin, artemether and artesunate on neuroinflammation in LPS-activated BV2 microglia.
2. To determine whether these compounds produce inhibition of neuroinflammation through interference with NF-κB and/or p38 MAPK signalling, and
3. To explore the role of Nrf2/HO1 antioxidant protective mechanism in the anti-neuroinflammatory effects of the compounds.

1.10 Structure of thesis

In this thesis the various objectives that have been identified in section 1.8.2 will be investigated for the different compounds artemisinin, artemether and artesunate.

Chapter 2 will focus on the general methods that were employed for investigating the three compounds. Studies on artemisinin, artemether and artesunate will be reported in chapters 3, 4, and 5 respectively and results discussed briefly at the end of each chapter. Chapter 6 will discuss results obtained from the research, their implications to knowledge and drug discovery.

CHAPTER 2

GENERAL METHODOLOGY

2. General Methodology

2.1. Cell culture

2.1.1. BV2 cell culture

BV2 mouse microglia cells ICLC ATL03001 (Interlab Cell Line Collection, Banca Bilogica e Cell Factory, Italy) were maintained in RPMI 1640 medium with 10 % heat-inactivated foetal bovine serum (FBS) (Sigma-Aldrich, Dorset, UK), 2 mM l-glutamine (Sigma-Aldrich, Dorset, UK), 100 U/ml penicillin and 100 mg/ml streptomycin (P/S) (Sigma-Aldrich, Dorset, UK) in a 5% CO_2 incubator. BV2 cells are semi-adherent cells. They were sub-cultured 1:10 when they reached confluence. To achieve this, suspended cells were collected into a 50 ml tube (Sarstedt, Dorset, UK). Adherent cells were harvested from the flask by incubating cells at 37°C with 2 ml of 0.25 % trypsin/EDTA solution (Sigma-Aldrich, Dorset, UK) for 1 minute after washing with phosphate buffered saline (PBS) (Sigma-Aldrich, Dorset, UK). After 1 minute incubation, trypsin/EDTA was neutralised by adding 8ml of complete RPMI 1640 medium containing 10 % heat-inactivated foetal bovine serum (FBS) (Sigma-Aldrich, Dorset, UK), 2 mM l-glutamine (Sigma-Aldrich, Dorset, UK), 100 U/ml penicillin and 100 mg/ml streptomycin (P/S) (Sigma-Aldrich, Dorset, UK). Harvested adherent cells were collected into 50ml tube containing suspended cells. Cells were centrifuged and culture media supernatants aspirated out. Cells were re-suspended in 10 ml of RPMI 1640 medium containing 10 % heat-inactivated foetal bovine serum (FBS) (Sigma-Aldrich, Dorset, UK), 2 mM l-glutamine (Sigma-Aldrich, Dorset, UK), 100 U/ml penicillin and 100 mg/ml streptomycin (P/S) (Sigma-Aldrich, Dorset, UK). Subsequently, BV2 cells were sub-cultured into a new flask by adding 1 ml of cell suspension to 9ml od RPMI 1640 medium containing 10 % heat-inactivated foetal bovine serum (FBS) (Sigma-Aldrich, Dorset, UK), 2 mM l-glutamine (Sigma-Aldrich, Dorset, UK), 100 U/ml penicillin and 100 mg/ml streptomycin (P/S) (Sigma-Aldrich, Dorset, UK) and grown at 37°C in a 5 % CO_2 incubator. After 48 hours, media was replaced. BV2 cells were used from passage 4 to passage 18.

For experimental purposes, cells were counted using a haemocytometer following harvesting and seeded out in 96-well (seeding density: 0.0125 x 10^6 cells per well), 24-well (seeding density: 0.05 x 10^6 cells per well), 12-well (seeding density: 0.1 x 10^6 cells per well) or 6-well (seeding density: 0.3 x 10^6 cells per well) tissue culture plates as required, at 2 x 10^5 cells/ml and incubated for 48 hours at 37°C. Tissue culture plates used are transparent, adherent surface, non-polygenic, non-cytotoxic polystyrene flat well plates from Sarstedt (Leicester, UK). At 70% confluence, media was replaced with serum free RPMI 1640 and incubated at 37°C for 3 hours prior to experiments.

Other consumables like sterological pipettes (5ml, 10ml and 25ml), pipette tips (10µl, 200µl, and 1ml), microcentrifuge tubes (or eppendorf tubes; 1ml) and cell scrappers were obtained from Sarstedt (Leicester, UK).

2.1.2. Human Embryonic Kidney (HEK) 293 cell culture

HEK 293 cells (European Collection of Cell Cultures, Cat no: 85120602, Salisbury, UK) were maintained in MEM medium with 10 % foetal bovine serum (FBS) (Sigma-Aldrich, Dorset, UK), 2 mM l-glutamine (Sigma-Aldrich, Dorset, UK), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich, Dorset, UK) in a 5 % CO_2 incubator. HEK 293 cells are fully adherent cells. Cells were sub-cultured 1:8 when they reached confluence. To achieve this, MEM media was aspirated out of the flask containing cells and washed with PBS (Sigma-Aldrich, Dorset, UK). Subsequently, 2 ml of 0.25 % trypsin/EDTA solution (Sigma-Aldrich, Dorset, UK) was added to adherent cells and incubated at 37°C for 1 minute. Thereafter, 8ml of MEM medium with 10 % foetal bovine serum (FBS) (Sigma-Aldrich, Dorset, UK), 2 mM l-glutamine (Sigma-Aldrich, Dorset, UK), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich, Dorset, UK) was added. Cell suspension was collected into a 50ml tube (Sarstedt, Leicester, UK), centrifuged and re-suspended in 10ml of MEM medium with 10 % foetal bovine serum (FBS) (Sigma-Aldrich, Dorset, UK), 2 mM l-glutamine (Sigma-Aldrich, Dorset, UK), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich, Dorset, UK). Media was replaced every 48 hours until cells became confluence. HEK293 cells were used from passage 4 to passage 15.

For transfection experiments, HEK 293 cells were counted using a haemocytometer following harvesting and seeded out in 96- well (seeding density: 0.0125 x 10^6 cell per well) solid white tissue culture adherent, flat well plates (Sarstedt, Leicester, UK) at 4 x 10^5 cells/ml.

2.2.1. Determination of LPS and drug concentration(s) used

In other to determine the concentration of LPS used for the study, BV2 cells were stimulated with various concentrations of LPS (0.05 – 1 µg/ml) for 24 hours. Subsequently, a nitrite assay was done to determine the concentration used. Result obtained (Figure 12) showed that at 1µg/ml, nitrite production was highest and significant. Hence, this concentration was used for the study.

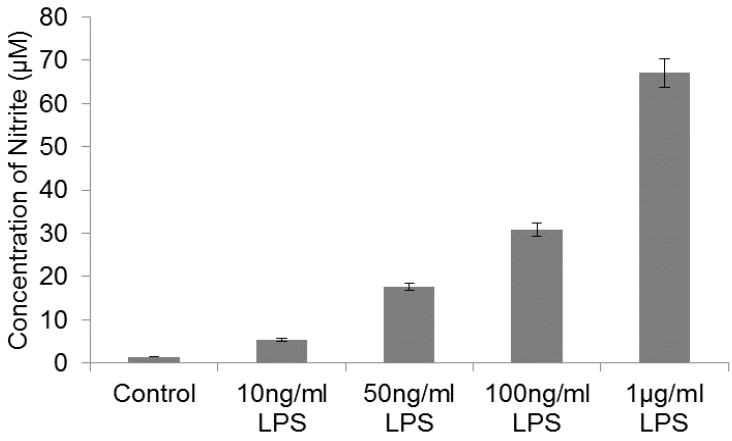

Figure 12: Effect of various concentrations of LPS (0.05-1 μg/ml) on nitrite production following 24 hour stimulation.

In other to determine the concentration of artemisinin, artemether and artesunate used in the study, an MTT assay was carried out for various concentrations. The results obtained for artemisinin (5-40 µM), artemether (5-40 µM) and artesunate (0.5-4 µM) are reported in sections 3.4, 4.4 and 5.4 respectively. These concentrations were found to be non-toxic to the cells and hence, they were further investigated. Results from the nitrite assay on artemisinin (section 3.1.1), artemether (section 4.1.1) and artesunate (section 5.1.1) also served as a preliminary result to suggest that the compounds might possess anti-inflammatory actions.

2.2.2. Drugs and treatment

Artemisinin (Sigma-Aldrich, Dorset, UK), artemether (Sigma-Aldrich, Dorset, UK) and artesunate (Sigma-Aldrich, Dorset, UK) were dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Dorset, UK) to a concentration of 0.1 M. Aliquots were stored at -80°C and diluted in DMSO (Sigma-Aldrich, Dorset, UK) before use. BV2 cells were pre-treated with either artemisinin (5-40 µM), artemether (5-40 µM) or artesunate (0.5-4 µM) for 30 minutes and stimulated with Lipopolysaccharide (LPS) (1 µg/ml) (*Salmonella enterica* serotype typhimurium SL1181, Sigma-Aldrich, Dorset, UK). Control cells were not treated with compounds or stimulated with LPS. LPS treated BV2 cells were only stimulated with LPS. The treatment plan is shown in table 3.

In experiments to test effects of compounds on HO-1 activation, BV2 cells were treated with only artemisinin (5-40 µM), artemether (5-40 µM) or artesunate (0.5-4 µM). In experiments to investigate Nrf2 activation, cells were treated with only artemisinin (40 µM), artemether (40 µM) or artesunate (4 µM). For HO-1 and Nrf2 experiments there were no LPS controls but there were untreated cells, which represent the negative control.

Table 3: Treatment plan for study

Treatment Plan		
Negative control	LPS/ TNFα Control	Drug treatment
↓	↓	↓
Untreated Cells	Cells treated with inflammatory stimulant (LPS or TNFα)	Cells pre-treated with artemisinin (5-40μM), artemether (5-40μM), or artesunate (0.5-4μM) followed by stimulation with stimulant (LPS or TNFα) or treated with compounds alone.

In NF-κB luciferase activity experiments using HEK 293 cells, cells were pre-treated with artemisinin (5-40 μM), artemether (5-40 μM) or artesunate (0.5-4 μM) for 30 minutes and stimulated 1 ng/ml TNFα (eBiosciences, Hatfield, UK). The negative control group were treated with no compounds or TNFα. The TNFα control cells were stimulated with TNFα (1 ng/ml).

For experiments on ARE luciferase activity experiments, HEK 293 cells were treated with only artemisinin (5-40 μM), artemether (5-40 μM) or artesunate (0.5-4 μM). The negative control cells represent the untreated cells. There was no TNFα control group in ARE luciferase activity experiments.

2.3. Measurement of cell viability - MTT Assay

The colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay is a simple method of assessing the ability of live but not dead cells to reduce a water soluble tetrazolium-based compound (MTT) to a purple formazan water-insoluble product (Figure 13). This reduction is carried out in cells by mitochondrial reductase. The amount of purple formazan formed is an indicative of the viability of the cells.

MTT assay was performed to determine the viability of BV2 microglia stimulated with or without LPS (1 μg/ml) in the presence of test compounds for 24 hours. MTT powder (Sigma-Aldrich, Dorset, UK) was dissolved in double deionised water. Two hundred microliters of MTT solution (5 mg/ml) diluted in culture media was added to each well of BV2 microglia in a 96-well plate. The plate was then incubated at 37°C in a CO_2-incubator for 4 hours. Thereafter, 200 μl of medium was removed from every well without disturbing the cell clusters and 150 μl of DMSO solution added to each well to dissolve the formazan crystals. Shaking the plate for a few seconds before the absorbance was read at 540 nm with a plate reader (Tecan F50, Reading, UK) facilitated thorough mixing. Experiments were performed at least three times and in triplicates.

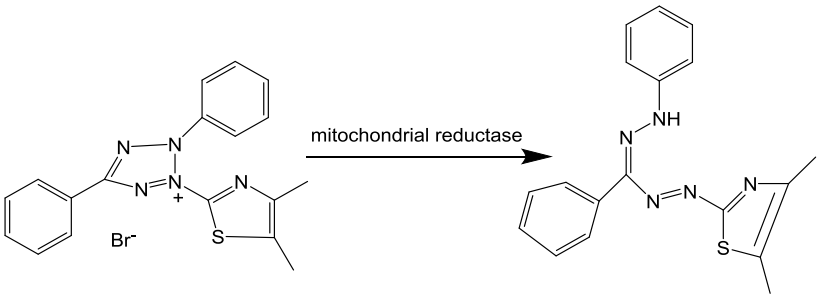

Figure 13: The chemistry of MTT assay.
Live but not dead cells reduce a water-soluble tetrazolium-based compound (MTT) to a purple formazan water-insoluble product (van Meerloo et al., 2011).

2.4. Determination of nitrite production (Griess assay)

Nitric oxide is a physiological messenger molecule in many biological systems. One of the methods of investigating nitric oxide is to measure nitrite (NO_2^-), one of the two primary products of NO breakdown. Nitrite is stable and non-volatile and hence measured in the assay. The Griess reaction is based on the chemical reaction, which uses sulphanilamide and N-1-naphthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions (Figure 14).

Accumulation of nitrite, an indicator of NO levels (and indirectly of inducible nitric oxide synthase (iNOS) activity) in culture supernatant was measured using the Griess reagent. In experiments to determine effects of compounds on nitrite production, cells were seeded out and stimulated in phenol red-free, serum-free RPMI media for 24 hours. Levels of nitrite in culture media were measured using commercially available Griess assay kit (Promega, Southampton, UK).

Briefly, 50 µl of supernatants were incubated with 50 µl of sulphanilamide solution for 10 minutes in the dark at room temperature. Thereafter, 50 µl of napthyethylene-diamine (NED) solution was added and incubated for a further 5-10 minutes at room temperature in the dark. Absorbance was measured within 30 minutes at 540 nm in a microplate reader (Infinite F50, Tecan, Reading, UK). Actual nitrite concentrations in the various treatments were obtained from the nitrite standard reference curve. Experiments were performed at least three times and in triplicates.

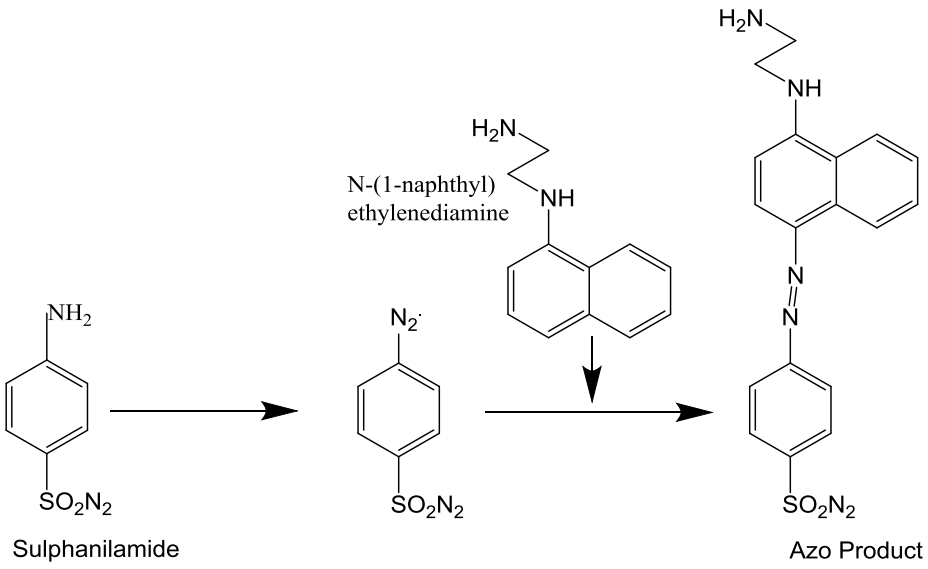

Figure 14: Principle of the Griess assay.
Reaction of sulphanilamide with nitrite and N- (1-naphthyl) ethylenediamine (NED) to produce an azo product measured in Griess assay (Griess, 1879).

2.5. Determination of prostaglandin E_2 (PGE_2) production using enzyme immunoassay (EIA)

Prostaglandins are synthesised from arachidonic acid by COX-1 or COX-2. This is processed further to PGE_2 or other prostanoids by cytosolic or microsomal prostaglandin synthases. PGE_2 is produced by a wide variety of tissues and in several pathological conditions, including inflammation. In LPS-activated BV2 microglia cells, PGE_2 is produced. Consequently, the effect of artemisinin (5-40 µM), artemether (5-40 µM) and artesunate (0.5-4 µM) on PGE_2 production was investigated.

BV2 cells were treated with artemisinin (5-40 µM), artemether (5-40 µM) or artesunate (0.5-4 µM) for 30 minutes and stimulated with LPS (1 µg/ml) for 24 hours. Thereafter, cells supernatants were collected. Levels of PGE_2 in culture supernatants were measured using commercially available enzyme immunoassay kit (Arbor Assays, Ann Arbor, Michigan, USA). 100 µl of samples were incubated in a clear plastic microtiter 96 well plate pre-coated with goat anti-mouse IgG together with 25 µl of PGE_2 conjugate (a prostaglandin E_2-peroxidase conjugate in stabilising solution) and 25 µl of mouse monoclonal antibody (specific for PGE_2) for 2 hours at room temperature with shaking at 250 rpm. Then, wells were washed 4 times with a wash buffer. In the next step, 100 µl of 3,3', 5,5'-Tetramethylbenzidine (TMB) solution was added into the wells of the clear plastic microtiter 96 well plate followed by a further incubation for 30 minutes at room temperature without shaking. At the end of the incubation period, the reaction was stopped using a 1 M solution of hydrochloric acid. Absorbance was measured at 450 nm in a microplate reader (Infinite F50, Tecan, Reading, UK) within

30 minutes of stopping the reaction. Actual PGE_2 concentration was obtained from the PGE_2 standard reference curve, ranging from 31.25-1000 pg/ml. Experiments were performed at least three times and in triplicates.

2.6. ELISA for the determination of TNFα, and IL-6

Pro-inflammatory cytokines have been shown to play an important role in the progression of neuroinflammation. Therefore, the effect of artemisinin (5-40 µM), artemether (5-40 µM) or artesunate (0.5-4 µM) was investigated in LPS activated BV2 microglia.

BV2 cells were seeded in 6 well plates (2×10^5 cells/ml) and cultured for 48 hour. Thereafter, cells were treated with artemisinin (5-40 µM), artemether (5-40 µM) or artesunate (0.5-4 µM) for 30 minutes and stimulated with LPS (1 µg/ml) for 24 hours. Supernatants were collected, centrifuged at 1200 rpm for 5 minutes and stored away in -80°C until when required for experiments. Determinations of TNFα and IL-6 concentration were measured using a commercially available mouse ELISA kits (R&D systems, Abingdon, UK).

Prior to use for experiments, samples were allowed to thaw completely and come to room temperature. Microplates coated with antibody specific for TNFα or IL-6 was washed four times with 1x wash buffer. Next wells were blocked using assay diluent at room temperature for 1 hour. Subsequently, wells were washed four times using 1x wash buffer. Diluted samples and standards were added to the wells and incubated for 2 hours at room temperature with shaking at 250 rpm. Following incubation, wells were washed four times and 100 µl detection antibody added in each well and incubated for a further 1 hour with shaking at 250 rpm at room temperature. Next, 100 µl of avidin-hrp solution was added in each well and incubated for 30 minutes following washing. At the end of the incubation period, wells were washed five times, 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate solution (R & D systems, Abingdon, UK) was added to each well and incubated in the dark for 30 minutes. Finally, 100 µl stop solution (2 N sulphuric acid, R&D systems, Abingdon, UK) was added to each well and absorbance read at 450 nm using a microplate reader (Infinite F50, Tecan, Reading, UK). Concentrations of TNFα or IL-6 production were read off a TNFα or IL-6 standard curve. Experiments were performed at least three times and in triplicates.

2.7. Preparation of whole cell extracts

Whole cell (cytoplasmic) extraction was performed on cells lysed in a radioimmunoprecipitation assay (RIPA) buffer containing 2 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, Dorset, UK).

At the end of cell stimulation, suspended BV2 cells were collected, centrifuged, and washed in 1x PBS while adherent cells were washed with 1x PBS (Sigma-Aldrich, Dorset, UK). RIPA lysis buffer containing 2mM PMSF was added to adherent and suspended cells and left on ice for 10 minutes to allow swelling. Subsequently suspended cells were vortex-mixed vigorously while adherent cells were scrapped and collected into the eppendorf tubes containing lysed suspended cells. Lysates were centrifuged at 13000 rpm for 10 minutes and whole cell extracts (supernatants) collected and stored away at -80°C until when required.

2.8. Preparation of nuclear cell extracts

Nuclear extraction was carried out using a fractionation procedure. First the whole cell extract was collected followed by further extraction for the nuclear extract using Epi Extraction kit (Abcam, Cambridge, UK). At the end of the experiment, cells were washed with 1x PBS and re-suspended in whole cell extraction buffer (ENE1 [1x]) containing DTT and protease inhibitor. Cells were left on ice for 10 minutes to allow swelling. Thereafter, they were vortex-mixed vigorously and centrifuged at 13000 rpm for 1 minute at 4°C. The supernatant (whole cell extract) was collected in a fresh ice-cold eppendorf tubes and frozen away in -80°C. The pellet left in the tube was re-suspended in nuclear extraction buffer (ENE2) containing DTT and protease inhibitor and left on ice for 15 minutes with vigorous vortex mixing every 3 minutes at 2000 rpm. Thereafter, samples were vortex mixed again before centrifugation at 4°C for 10 minutes at 13500 rpm. At the end of the centrifugation, the nuclear extract was collected and stored away at -80°C.

2.9. Protein determination

Whole cell extracts and nuclear extracts were diluted 1:15 and 1:20 respectively in deionised water. Next, 5 µl of diluted protein samples and pre-diluted BSA standards (Thermoscientific, Loughborough, UK) were added to a 96 well plate in duplicates. 250 µl of Coomasie reagent (Thermoscientific, Loughborough, UK) was added to each well and mixed for 30 seconds with a plate shaker. Samples were subsequently incubated at room temperature for 5 minutes. Absorbance was read using a plate reader (Infinite, Tecan, Reading UK) at 595 nm. The protein concentration in each sample was measured from the standard curve, ranging from 125-2000 µg/ml.

2.10. Western blot

Western blot is used to separate and identify proteins. A mixture of proteins is separated based on their molecular weights using gel electrophoresis. Separated proteins are transferred to a membrane producing a band for each protein. The membrane is incubated

with label antibodies specific to the protein of interest. This procedure has been used to identify the effect of artemisinin, artemether and artesunate on protein expression.

Protein samples were prepared by adding 4× LDS buffer (Life Technologies, Paisley, UK) and 10× sample reducing agent (Life Technologies, Paisley, UK) at a volume that results in 1×final dilution in the sample. The mixture was then heated at 70°C for 10 minutes. Samples were vortex mixed before and after heating. Next, samples were centrifuged briefly and loaded into the wells of a polyacrylamide gel (4-12 % Bis-Tris gel, Life Technologies, Paisley, UK). Subsequently after loading the wells, antioxidant (Life Technologies, Paisley, UK) was added in the 1x SDS MES running buffer (Life Technologies, Paisley, UK).

Protein samples were separated by SDS-PAGE for 35 minutes at 200 V, blotted onto polyvinylidene difluoride (pvdf) membrane (Millipore, Hertfordshire, UK) for 2 hours at 25 V. Thereafter, membrane was incubated in readymade blocking buffer (Licor, Cambridge, UK) or 5 % non-fat milk in 1x tris-buffered saline and tween 20 (TBST) for 1 hour at room temperature. Thereafter, membrane was washed three times using 1x TBS-T at 2000 rpm for 10 minutes/ wash.

After washing, membrane was incubated with the required primary antibody at 4°C with shaking overnight. Table 4 above shows the list of antibodies used for the experiments and the dilution factor used.

After overnight incubation, membranes were washed extensively (three times for 10 minutes each using 1x TBS-T) and incubated in secondary antibody Alexa Fluor 680 goat anti-rabbit (1:10000) (Invitrogen, Paisley, UK) at room temperature for 1 hour in the dark. Subsequently, membrane was washed three times for 5 minutes each using 1x TBST. Protein detection was done using a LICOR Odyssey Imager. Each experiment was performed in triplicate.

Table 4: List of the antibodies used in western blot analysis with the details of the source and dilution factor

Antibody	Details	Type	Source	Dilution for WB	Molecular Weight
MAPKAPK2	p-Thr334	Rabbit polyclonal	Assay bio Tech	1:1000	45kDa
p-MKK3(S189) MKK6(S207)	22A8	Rabbit monoclonal	Cell Signalling	1:1000	40kDa(P-MKK3) 41kDa(P-MKK6)
NOS2 (iNOS)	sc-650	Rabbit polyclonal	Santa Cruz	1:1000	130kDa
p-IκBαSer32/36	sc-101713	Rabbit polyclonal	Santa Cruz	1:250	41kDac
p-p38α Thr180/Tyr182-R	sc-17852-R	Rabbit polyclonal	Santa Cruz	1:1000	38kDa
p-NFκB p65 (Ser 536)	sc-101752	Rabbit polyclonal	Santa Cruz	1:250	65kDa
COX-2 (M-19)	sc-1747-R	Rabbit polyclonal	Santa Cruz	1:1000	70 – 72kDa
IκBα (Total)		Rabbit polyclonal	Santa Cruz	1:500	40kDa
Heme Oxygenase 1 (H-105)	sc-10789	Rabbit polyclonal	Santa Cruz	1:1000	32kDa
Nrf2 (c-20)	sc-722	Rabbit polyclonal	Santa Cruz	1:500	Nrf2/poly-ubiquitinated Nrf2:57/100kDa
mPGES1	AS03 031	Rabbit polyclonal	Agrisera	1:10000	17kDa
Anti-Actin (20-33)	A5060	Rabbit polyclonal	Sigma	1:1000	42kDa
p38α (C-20,Total)	sc-535	Rabbit polyclonal	Santa Cruz	1:1000	38kDa
p-IKKα/β (Ser180/Ser181)-R	sc-23470-R	Rabbit polyclonal	Santa Cruz	1:500	85kDa
IKKα/β (H-470) (Total)	sc-7607	Rabbit polyclonal	Santa Cruz	1:1000	85kDa
Alexa Fluor 680	A21076	Goat anti-rabbit	Invitrogen	1:10000	N/A (Secondary antibody)

2.10.1. Stripping and re-probing

The PVDF membranes were stored in an incubation box containing TBS-T (1x) buffer at 4°C, until re-probing was required. Antibodies were stripped from the PVDF membrane by incubating it in restore fluorescent stripping buffer (1x, Thermo Fisher Scientific, Hempstead, UK) for 5 minutes at room temperature with shaking (20 rpm). Stripping buffer was then discarded and membrane washed three times for 10°minutes each with TBS-T to get rid of

stripping buffer. Subsequently, membranes were blocked with blocking buffer, washed and re-exposed to primary antibody.

2.11. Transient transfection and NF-κB reporter gene assay

Cell transfection is a procedure that involves introducing foreign nucleic acids into cells to produce genetically modified cells (Kim & Eberwine, 2010). This is an important analytical procedure as it can be used to investigate gene function and regulation, and protein function. Reporter assays are versatile and sensitive methods of assaying targets in high throughput drug screening (Liu et al., 2009). Promoters are used to drive reporter gene expression and can be activated by a broad range of selective activation. HEK-293 cells have been transfected with NF-κB construct and the reporter gene assay has been used to investigate the effect of artemisinin, artemether and artesunate on NF-κB activity.

HEK 293 cells were grown in MEM media supplemented with foetal bovine serum (FBS), P/S, L-glutamine and sodium pyruvate. At confluence, the cells were sub-cultured (at a ratio of 1:3) 24 hours before transfection. Thereafter, cells were harvested and re-suspended at 4 x 10^5 cells/ml in Opti-MEM containing 5 % FBS and 1 % NEAA. Cells were seeded out in a solid, white 96-well plates and incubated with 50 µl Cignal® NF-κB reporter (0.5µg) (SA Biosciences, Manchester, UK), using TransIT®-LT1 transfection reagent (diluted 1:3 in Opti-MEM serum free media) (Mirus, Cambridge, UK) and incubated for a further 16 hours at 37°C in 5 % CO_2 incubator.

Following HEK 293 cell transfection with NF-κB vector, media was changed to OPTI-MEM (0.5 % FBS, 1 % NEAA, 100 U/ml Penicillin and 100µg/ml Streptomycin) and incubated for another 8 hours. Thereafter, transfected cells were treated with artemisinin (5-40 µM), artemether (5-40 µM) or artesunate (0.5-4 µM) and incubated for 30 minutes at 37°C followed by TNFα (1 ng/ml) for 6 hours. At the end of the stimulation, NF-κB-mediated gene expression was measured by using One-Glo luciferase Assay kit (Promega, Southampton, UK). 100 µl of luciferase buffer containing luciferase substrate was added to each well at the end of the 6 hours incubation and luminescence read with FLUOstar OPTIMA reader (BMG LABTECH, Bucks, UK). Experiments were performed at least three times and in triplicates.

2.12. Transient transfection and ARE reporter gene assay

Nrf2 responds to oxidative stress by binding to ARE in the promoter of genes coding for antioxidant enzymes like NADPH and HO-1. To understand the effect of artemisinin, artemether and artesunate on ARE, cell transfection was used to introduce ARE construct to HEK 293 cells. Subsequently, the effect of artemisinin, artemether and artesunate on ARE activity was measured in a reporter gene assay.

HEK 293 cells were grown in MEM supplemented with foetal bovine serum (FBS), penicillin/streptomycin, and L-glutamine and sodium pyruvate. At confluence, cells were harvested and re-suspended at 1.5 x 10^5 cells/ml in OPTI-MEM (Life Technologies, Paisley, UK) supplemented with 10 % FBS and sodium pyruvate. Subsequently, cells were seeded out and incubated in solid white 96-well at 37°C for 24 hours. Transfection cocktail was made by diluting ARE vector (pGL4.37 [luc2P/ARE/Hygro]; Promega, Southampton, UK) at a concentration of 1 ng DNA/µl in transfection reagent (FUGENE 6) (Promega, Southampton, UK) diluted at a ratio of 3:1 in OPTI-MEM. Transfection cocktail was incubated at room temperature for 20 minutes. Transfection cocktail (8 µl) was added in each well and incubated for 18 hours at 37°C.

Following transfection of HEK 293 cells with ARE vector, culture media was changed to 75 µl of OPTI-MEM (supplemented with 0.5 % FBS) per well and incubated for 6 hours at 37°C. Subsequently, cells were treated with artemisinin (5-40 µM), artemether (5-40 µM) or artesunate (0.5-4 µM) and incubated at 37°C for 18 hours. Following incubation, plates were allowed to cool at room temperature for 15 minutes. Thereafter, 80 µl of luciferase buffer containing luminescence substrate was added to each well and luminescence read with FLUOstar OPTIM reader (BMG LABTECH, Bucks, UK). Experiments were performed at least three times in triplicates.

2.13. Electrophoretic mobility shift assay (EMSA)

EMSA is a simple, rapid and sensitive means of detecting sequence-specific DNA-binding of proteins. Hence it is used to study protein: DNA complexes and interaction. EMSA was used to investigate the effect of artemisinin; artemether and artesunate on the ability of NF-κB to bind in a sequence-specific manner to a labelled oligonucleotide probe and retard its migration through a non-denaturing polyacrylamide gel.

IRDye 700-labeled double-stranded DNA oligonucleotide (Licor, Cambridge, UK) was used for the experiments. DNA oligonucleotides were incubated with nuclear extract samples and binding buffer for 30 minutes at room temperature in the dark in a reaction cocktail containing 25 mM DTT/2.5 % Tween 20, 1 µg/µl poly (di.dc), 1 % NP-40 and 100 mM $MgCl_2$.

Thereafter, native tris-borate-EDTA (TBE) gel (Life technologies, Paisley, UK) was pre-run for 30 minutes. Subsequently, separation of protein-DNA complexes was performed at 10 V/cm at 4°C for 1 hour in the dark. At the end of the electrophoresis, migration was visualised using LICOR Odyssey Imager (Cambridge, UK).

2.14. Silencing experiments

Eukaryotic cells have many sophisticated ways of controlling gene expression. In the complex environment of the cell, these processes need to be precisely targeted. A group of mechanisms that use small RNA molecules to direct gene silencing is known as RNA interference (RNAi). RNAi is achieved with the use of small interfering RNA (siRNA), micro RNA or small hairpin RNA (shRNA).

It has been shown that the induction of Nrf2 by compounds of different chemical classes is directly correlated to the inhibition of pro-inflammatory responses (Lui et al, 2008; Baird & Dinkova-kostoova, 2011). Hence, to understand if the inhibitory effect of artemisinin, artemether and artesunate on NO, TNFα, IL-6 and PGE_2 is dependent on Nrf2, Nrf2 gene was knocked out in BV2 cells. Subsequently, the cells were stimulated with LPS following pre-treatment with artemisinin, artemether or artesunate. The effects of these compounds on NO, TNFα, IL-6 and PGE_2 were investigated in wild-type cells and compared to that of siNrf2 cells.

Small interfering RNA (siRNA) targeted at Nrf2 (Santa Cruz biotechnology, Texas, USA) was used to knockdown Nrf2. BV2 cells were seeded in six-well culture plate, and incubated at 37°C in a 5 % CO_2 incubator until 70-80 % confluent. Then, 2 µl Nrf2 siRNA duplex were diluted into 100 µl of siRNA transfection medium (Santa Cruz biotechnology, Texas, USA). In a separate tube, 2 µl of transfection reagent (Santa Cruz biotechnology, Texas, USA) was diluted into 100 µl of siRNA transfection medium. The dilutions were mixed gently together and incubated for 30 minutes at room temperature.

Next, cells were incubated in Nrf2 siRNA transfection cocktail for 6 hours at 37°C. Following 6 hours transfection, media change was done on all cells (wild-type and siNrf2) to complete supplemented media and incubated for a further 18 hours. Thereafter, cells were pre-treated with artemisinin (40 µM), artemether (40 µM) or artesunate (4 µM) for 30 minutes and stimulated with LPS (1 µg/ml) for 24 hours. Cell supernatants were collected and stored in -80°C until analysis.

For result analysis, samples from Nrf2 silenced cells were denoted as '+siNrf2' while samples from wild-type cells were denoted as '-siNrf2'.

2.15. Statistical analyses

Values of all experiments are represented as a mean ± SEM of at least 3 experiments. For each experiment, percentage relative to untreated control, LPS control or TNFα control have been done before exporting values obtained to GraphPad. Statistical analysis was

performed using One-way ANOVA followed by a post-hoc Student Newman-Keuls test, using the GraphPad Prism statistics software.

CHAPTER 3

STUDIES ON ARTEMISININ

3. Studies of Artemisinin

3.1. Background

Artemisinin has been shown to have promising anti-inflammatory property in various experimental conditions. Previous studies have shown that artemisinin interrupts NF-κB signalling pathway *in vivo* (Pahl, 1999). It has also been demonstrated that artemisinin is capable of inhibiting neuroinflammation in a model of AD in APPswe/PS1dE9 double transgenic mice (Shi et al., 2013). However, the effects of artemisinin in LPS-activated BV2 microglia are not fully understood. To explore these effects, BV2 microglia cells were pre-treated with artemisinin and challenged with LPS. Subsequently, effects of artemisinin on secreted pro-inflammatory proteins (TNFα, IL-6, PGE_2 and NO) were investigated. In order to establish the molecular mechanisms of action of the compound, possible interference with NF-κB and p38 MAPK signalling were also investigated.

3.1.1. Artemisinin inhibits iNOS-mediated nitrite production from LPS-activated BV2 microglia

One of the prominent features of chronic neuroinflammation is the excessive production of nitric oxide. To assess whether artemisinin could inhibit iNOS-mediated NO production, BV2 cells were stimulated with LPS (1 µg/ml) in the absence or presence of artemisinin (5-40 µM) for 24 hours. Subsequently, cell supernatants and whole cell extracts were collected. Griess assay was used to assess the effect of artemisinin on NO production in supernatants while immunoblotting was used to investigate the effect of artemisinin on iNOS protein expression.

As shown in Figure 15 a, treatment with LPS (1 µg/ml) resulted in a marked increase in NO production in BV2 cells when compared to the untreated control ($p<0.001$). However, pre-treatment with artemisinin produced a concentration-dependent suppression of nitrite produced by the cells ($p< 0.001$).

To further investigate the results obtained in experiments on NO production, the effect of artemisinin (5-40 µM) on iNOS protein expression was investigated using western blot. Results show that LPS treatment caused a marked increase ($p<0.001$) in iNOS protein expression when compared to untreated control cells (Figure 15 b).

However, pre-treatment with artemisinin (20 and 40 µM) significantly inhibited expression of iNOS protein expression in LPS-activated BV2 cells. At concentrations of 20 µM and 40 µM, pre-treatment with artemisinin resulted in 65 % and 50 % iNOS protein expression respectively, when compared with LPS-treated cells. These observations suggest that higher concentrations of artemisinin reduced NO production in LPS activated BV2 cells through inhibition of iNOS protein expression.

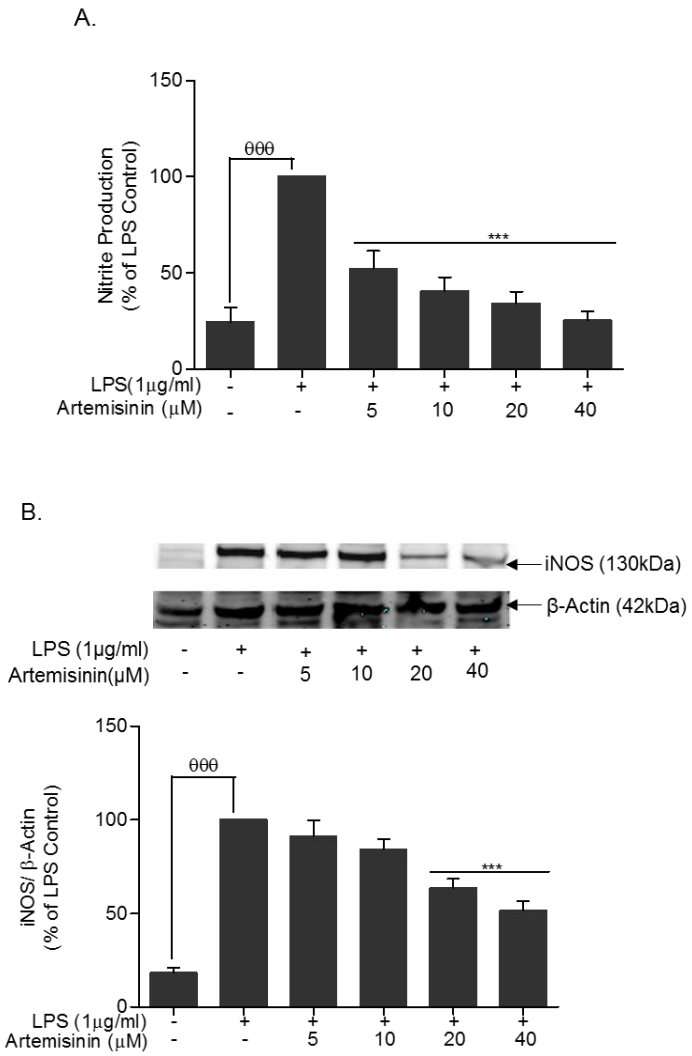

Figure 15: Artemisinin suppressed iNOS-mediated nitrite production in LPS-activated BV2 microglial cells.
(A) Artemisinin (5-40 µM) inhibited nitrite production in LPS activated BV2 microglia cells. (B) Artemisinin (20 and 40 µM) inhibited iNOS protein expression in LPS-activated BV2 microglia cells. 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. *p<0.05, ***p<0.001 in comparison with LPS control. $^{\theta}$p<0.05, $^{\theta\theta\theta}$p< 0.01 in comparison with negative control (untreated cells).

3.1.2. Artemisinin suppresses PGE_2 production in LPS stimulated BV2 microglia by inhibiting COX-2 and mPGES-1 proteins.

To assess whether artemisinin inhibits the production of PGE_2, BV2 cells were stimulated with LPS (1 µg/ml) in the presence or absence of artemisinin (5-40 µM) for 24 hours. BV2 cells treated with LPS (1 µg/ml) showed a marked increase in PGE_2 production (Figure 16 a).

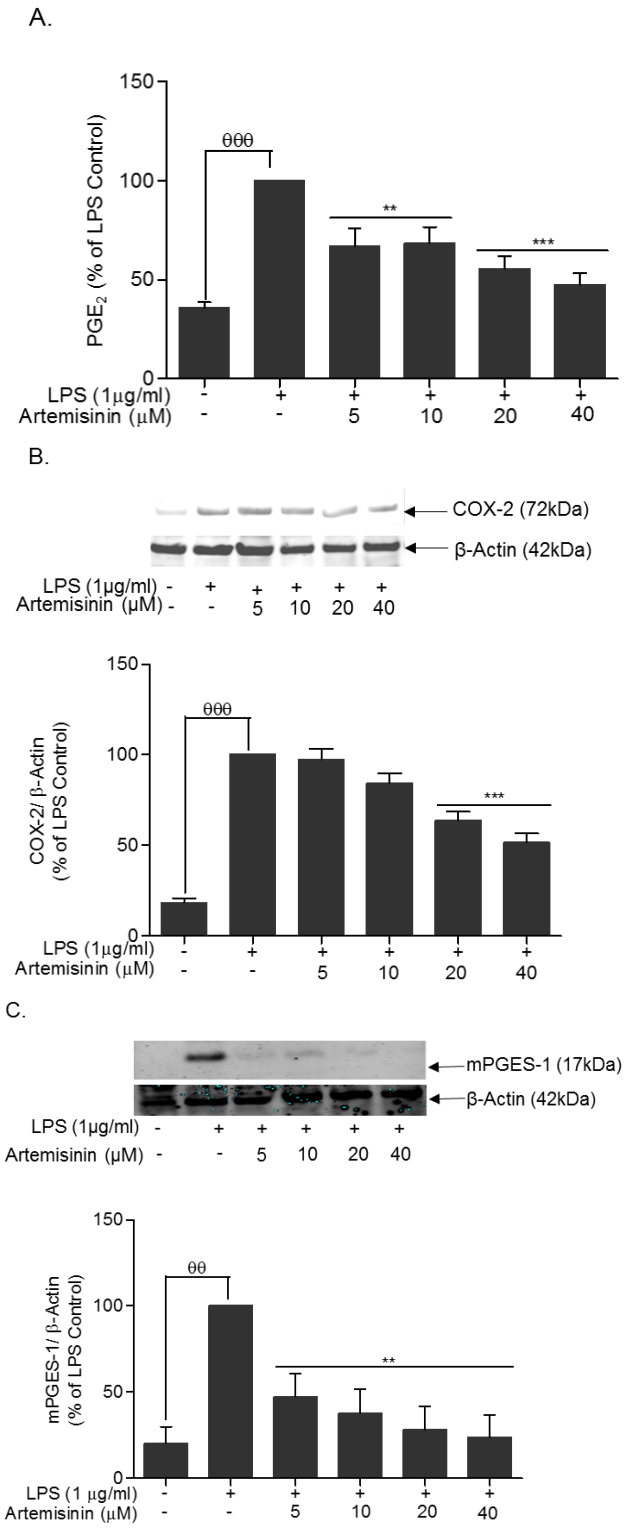

Figure 16: Artemisinin suppressed COX-2- and mPGES-1-mediated PGE_2 production in LPS-activated BV2 microglial cells.

(A) Artemisinin (5-40 µM) inhibited PGE_2 production in LPS activated BV2 microglia cells. (B) Artemisinin (20 and 40 µM) suppressed COX-2 protein expression in LPS-activated BV2 microglial cells. (C) Artemisinin (5-40 µM) suppressed mPGES-1 protein in LPS- activated BV2 activated microglial 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. *p<0.05, ***p<0.001 in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p< 0.001$ in comparison with negative control (untreated cells).

Pre-treatment with artemisinin (5-40 µM) significantly ($p<0.05$) suppressed PGE_2 production in LPS-activated BV2 microglia cells at all concentrations investigated. At 20 and 40 µM of the compound, PGE_2 production was 55.7 % and 47.6 %, respectively when compared to the LPS control ($p<0.001$).

Thereafter, the effect of artemisinin on COX-2 protein expression was investigated. Figure 16 b shows that BV2 microglia cells stimulated with LPS produced a significant expression of COX-2 protein expression when compared to the untreated control ($p<0.001$). However, pre-treatment with artemisinin (20-40 µM) resulted in a significant ($p<0.05$) reduction in COX-2 protein expression. At 20 µM, pre-treatment with artemisinin resulted in 79.7 % COX-2 protein compared to LPS control. At 40 µM, COX-2 protein expression was 64.4 % when compared to the LPS control. These findings suggest that at 20 and 40 µM concentrations, inhibition of PGE_2 production in LPS activated BV2 microglia cells by artemisinin were mediated through inhibition of COX-2 protein expression.

mPGES-1 is an inducible terminal enzyme for PGE_2 biosynthesis (Kudo & Murakami, 2005). It is also coupled to COX-2 has been reported to be involved in neuroinflammation (de Oliveira et al., 2008). Consequently, the effect of artemisinin on mPGES-1 protein expression was also studied. Treatment of BV2 microglia cells with LPS (1 µg/ml) resulted in a marked increase in mPGES-1 protein expression (Figure 16c).

However, it was observed that pre-treatment with artemisinin (5-40 µM) significantly reduced mPGES-1 protein expression (Figure 16 c). These results appear to suggest that the effect of artemisinin on PGE_2 production was mediated through inhibition of mPGES-1 protein at much lower concentrations than its inhibition on COX-2.

3.1.3. Artemisinin suppresses TNFα and IL-6 production in LPS activated BV2 microglia cells.

Pro-inflammatory cytokines (TNFα and IL-6) have been reported to play a vital role in chronic neuroinflammation (Brunssen et al., 2013; Olmos & Llado, 2014). Therefore the effect of artemisinin on these cytokines was investigated in LPS-activated BV2 microglia cells. BV2 cells were stimulated with LPS (1 µg/ml) in the absence or presence of artemisinin (5-40 µM) for 24 hours. Subsequently, supernatants were collected.

Result showed that LPS (1 µg/ml) stimulation resulted in a significant increase in TNFα (Figure 17 a). However, in samples collected from cells pre-treated with artemisinin (10-40 µM) a concentration-dependent and significant ($p<0.05$) inhibitory effect of TNFα production was observed.

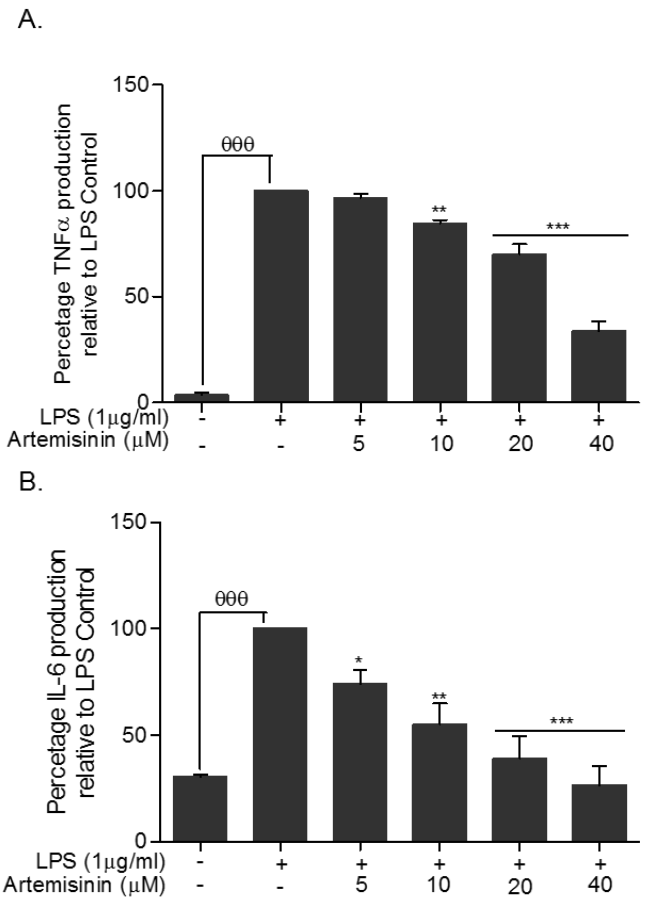

Figure 17: Artemisinin suppressed production of pro-inflammatory cytokines TNFα and IL-6 in LPS activated BV2 microglial cells.
(A) Artemisinin (10-40 µM) suppressed TNFα production in LPS activated BV2 microglia cells. (B) Artemisinin (5-40 µM) inhibited IL-6 production in LPS activated BV2 microglia 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. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p< 0.001$ in comparison with negative control (untreated cells).

The effect of artemisinin on IL-6 production was also investigated. Figure 17 b shows that artemisinin (5-40 µM) significantly inhibited the production of IL-6 in a concentration-dependent manner. At 20 µM and 40 µM of artemisinin, 37 % and 25 % IL-6 was produced respectively, when compared with LPS control.

3.1.4. Artemisinin inhibits neuroinflammation in LPS-activated BV2 microglia by targeting IκB/NF-κB signalling.

NF-κB plays an important central role in the regulation of pro-inflammatory genes like iNOS which controls nitrite production, and COX-2 which mediates PGE_2 production in stimulated microglia. Consequently, its effect on NF-κB signalling was investigated. To understand whether artemisinin shows a general effect on NF-κB mediated gene transcription, a

luciferase reporter gene assay was conducted. HEK 293 cells were transiently transfected with an NF-κB reporter construct, and then stimulated with TNFα (1 ng/ml) in the absence or presence of artemisinin (5-40 µM). The experiment revealed that artemisinin (10-40 µM) produced significant ($p<0.01$) inhibition of NF-κB-regulated luciferase reporter gene expression in a dose-dependent manner following stimulation with TNFα (1 ng/ml) (Figure 18).

NF-κB exists in an inactive form in the cytoplasm where it is bound to IκB complex. Upon phosphorylation by IKK, active NF-κB (p65) translocate to the nucleus while IκB gets degraded. Next, the effect of artemisinin on phosphorylation and degradation of IκB-α was investigated. BV2 microglia cells were stimulated with LPS (1 µg/ml) for 1 hour in the presence or absence of artemisinin (5-40 µM). Whole cell extracts were obtained and analysed for phospho-IκBα and total IκB-α proteins.

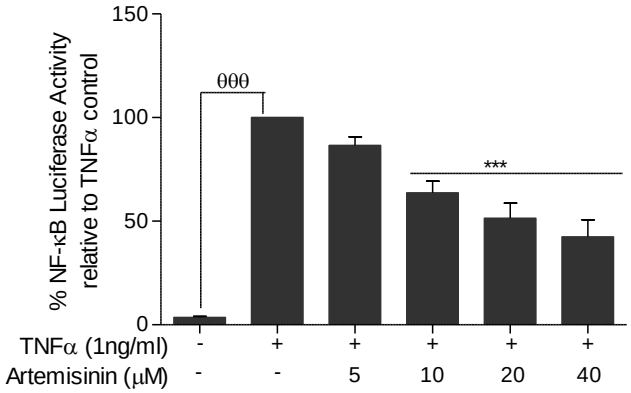

Figure 18: Artemisinin (5-40 µM) suppressed NF-κB activity in TNFα stimulated HEK293 cells transiently transfected with NF-κB luciferase plasmid.
HEK293 cells were stimulated with TNFα (1 ng/ml) in the absence or presence of artemisinin (5-40 µM) for 6 hours. Luminescence measurement was used as a measure of NF-κB activity. 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. *$p<0.05$, ***$p<0.001$ in comparison with TNFα control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p< 0.001$ in comparison with negative control (untreated cells).

Figure 19 a shows that upon stimulation of BV2 microglia cells with LPS, there was a marked increase ($p<0.001$) in phospho-IκB-α protein. However, pre-treatment with artemisinin (5-40 µM) decreased phospho-IκB-α protein expression significantly ($p<0.05$). Treatment with LPS alone resulted in an increase in IκBα degradation. However, pre-treatment of BV2 cells with artemisinin (20-40 µM) inhibited degradation of IκBα by increasing total IκBα protein expression (Figure 19 a).

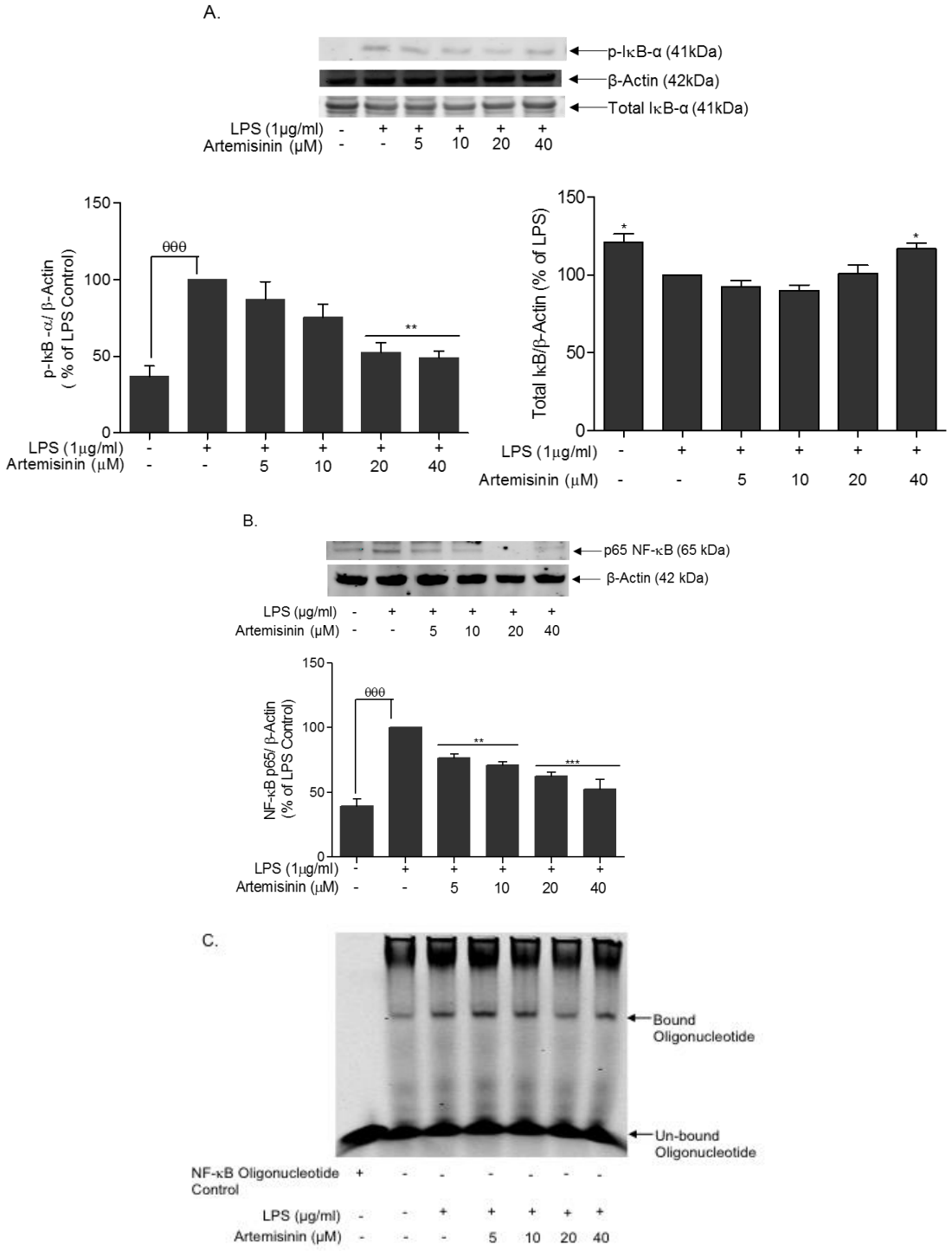

Figure 19: Artemisinin inhibited NF-κB signalling pathway in LPS-activated BV2 microglia cells. (A) Artemisinin inhibits IκB-α phosphorylation and degradation in LPS-activated BV2 microglia cells. Whole cell lysates from 1 hour stimulation were analysed for p-IκB-α and total IκB-α protein expressions using western blot. (B) Artemisinin inhibited p65 nuclear translocation in LPS-activated BV2 microglial cells. Nuclear extracts from 1 hour stimulation were analysed for p65 protein expression using western blotting. (C). Artemisinin inhibited NF-κB DNA binding in LPS-activated BV2 microglia cells. Nuclear extracts from 1 hour stimulation were investigated for NF-κB binding using EMSA. 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. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p< 0.001$ in comparison with negative control (untreated cells).

In the nucleus, p65 induces the transcription of various genes regulated by NF-κB. Transcription of these genes result in the production of pro-inflammatory mediators including PGE_2/COX-2 and NO/iNOS, as well as pro-inflammatory cytokines. The effect of artemisinin (5-40 µM) on nuclear phosphorylated p65 subunit was therefore investigated. At concentrations of 20 µM and 40 µM, artemisinin significantly ($p<0.001$) inhibited phosphorylated p65 protein expression in LPS activated BV2 microglia cells (Figure 19 b).

When NF-κB translocate to the nucleus, it binds to the NF-κB promoter region where it activates the transcription of pro-inflammatory genes. The effect of artemisinin on NF-κB binding to DNA was investigated using EMSA. Results obtained (Figure 19 c) show that artemisinin inhibited DNA binding of NF-κB. This result suggests that not only does artemisinin inhibit NF-κB translocation to the nucleus but it also inhibits DNA binding.

In NF-κB signalling, activation of NF-κB occurs due to IκB kinase (IKK)-mediated phosphorylation and proteasomal degradation of IκB. This process allows active NF-κB subunits to translocate to the nucleus and induce target gene expression. To further elucidate the molecular mechanisms involved in the anti-neuroinflammatory activity of artemisinin, its effect on phospho-IKK protein was investigated. Results showed that artemisinin did not have significant inhibitory effect on phospho-IKK protein expression in LPS-activated BV2 cells (Figure 20).

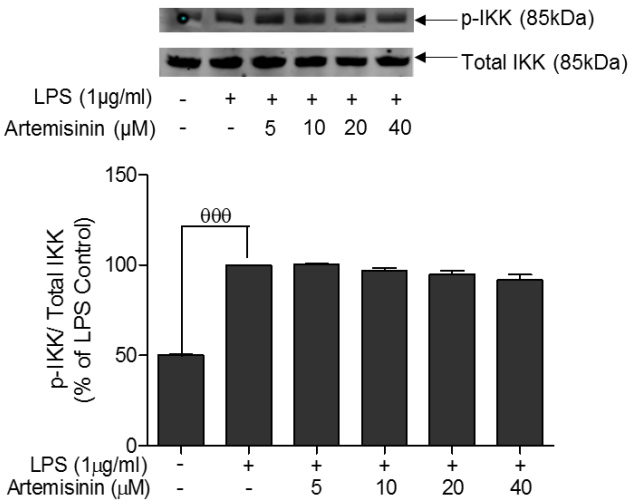

Figure 20: Artemisinin does not inhibit IKK activity in LPS-activated BV2 microglial cells. BV2 cells were stimulated with LPS (1 µg/ml) in the presence or absence of artemisinin (5-40 µM) for 10 minutes. Whole cell extracts were analysed for phospho-IKK and total IKK protein expression using western blot. 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. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p< 0.001$ in comparison with negative control (untreated cells).

It therefore appears artemisinin interferes with NF-κB signalling by directly targeting IκB phosphorylation and degradation.

3.1.5. Artemisinin interferes with p38MAPK signalling pathway through inhibition of MKK3/6

The p38MAPK signalling pathway has been reported to play a crucial role in the expression and activity of pro-inflammatory cytokines by contributing to their transcriptional and post-transcriptional regulation (Bhat et al., 1998). In addition, in primary rat and human microglia, LPS stimulated p38MAPK signalling has been reported to mediate the production of pro-inflammatory mediators including NO and TNFα (Lee et al., 2000; Munoz & Ammit, 2010). Based on these evidences that strongly implicate p38MAPK in neuroinflammation, the effect of artemisinin on its signalling was investigated.

First, the effect of the compound on the phosphorylation of p38-MAPK was investigated. BV2 microglia cells were pre-treated with artemisinin (5-40 µM) for 30 minutes followed by stimulation with LPS (1 µg/ml) for 1 hour. Subsequently, whole cell extracts were collected and the effect of artemisinin (5-40 µM) on the phosphorylation of p38 was investigated. Figure 21 shows that on LPS stimulation, a significant increase in the phosphorylation of p38 protein was observed.

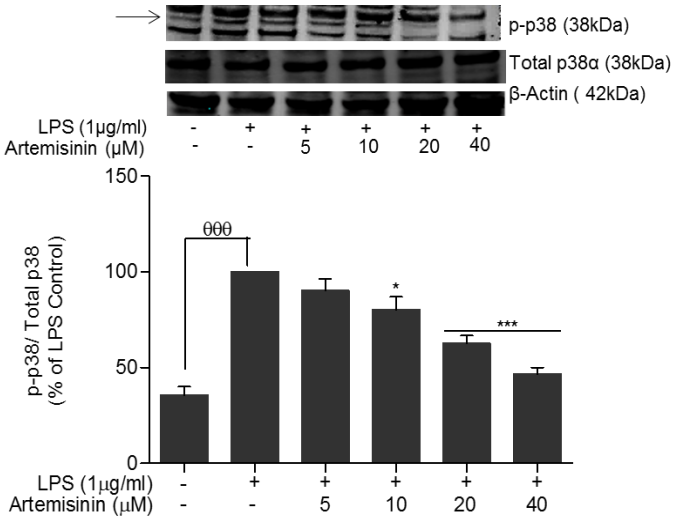

Figure 21: Artemisinin inhibited phosphorylation of p38 in LPS-activated BV2 microglial cells. Whole cell extracts from 1 hour stimulation were analysed for p-p38 and total p38 protein using western blot. All values are expressed as mean ± SEM for at least 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with Negative control (untreated cells).

However, pre-treatment with artemisinin (10-40 µM) resulted in a concentration-dependent decrease in phospho-p38 protein expression. At 20 µM and 40 µM artemisinin p-p38 protein

expression was 63 % ($p<0.001$) and 48 % ($p<0.001$) respectively when compared to the LPS control.

LPS stimulation of the microglia is known to phosphorylate p38MAPK by MKK3/6. Therefore, to further understand the effect of artemisinin on p38MAPK signalling, the effect of the compound on phospho-MKK3/6 was investigated. BV2 microglia cells were pre-treated with artemisinin and subsequently stimulated with LPS (1 µg/ml) for 1 hour. Phosphorylated MKK3/6 in BV2 microglia cells stimulated with LPS was significantly increased when compared to the control (Figure 22 a).

However, pre-treatment with artemisinin (10 and 20 µM) resulted in a decrease in phosphorylated MKK3/6 protein expression. Once activated, p38MAPK further activates transcription factors by acting on its substrate MAPKAPK2 (Bachstetter & Eldik, 2010). In addition, MAPKAPK2 has been linked to microglial cell activation (Culbert et al., 2006). Stimulation of BV2 microglia with LPS caused a marked rise in phospho-MAPKAPK2 expression (Figure 22 b). Pre-treatment with artemisinin (20 and 40 µM) resulted in a decrease in phopho-MAPKAPK2 protein. These results show that artemisinin the effects of artemisinin on p38MAPK signalling was more evident at 10 and 40 µM.

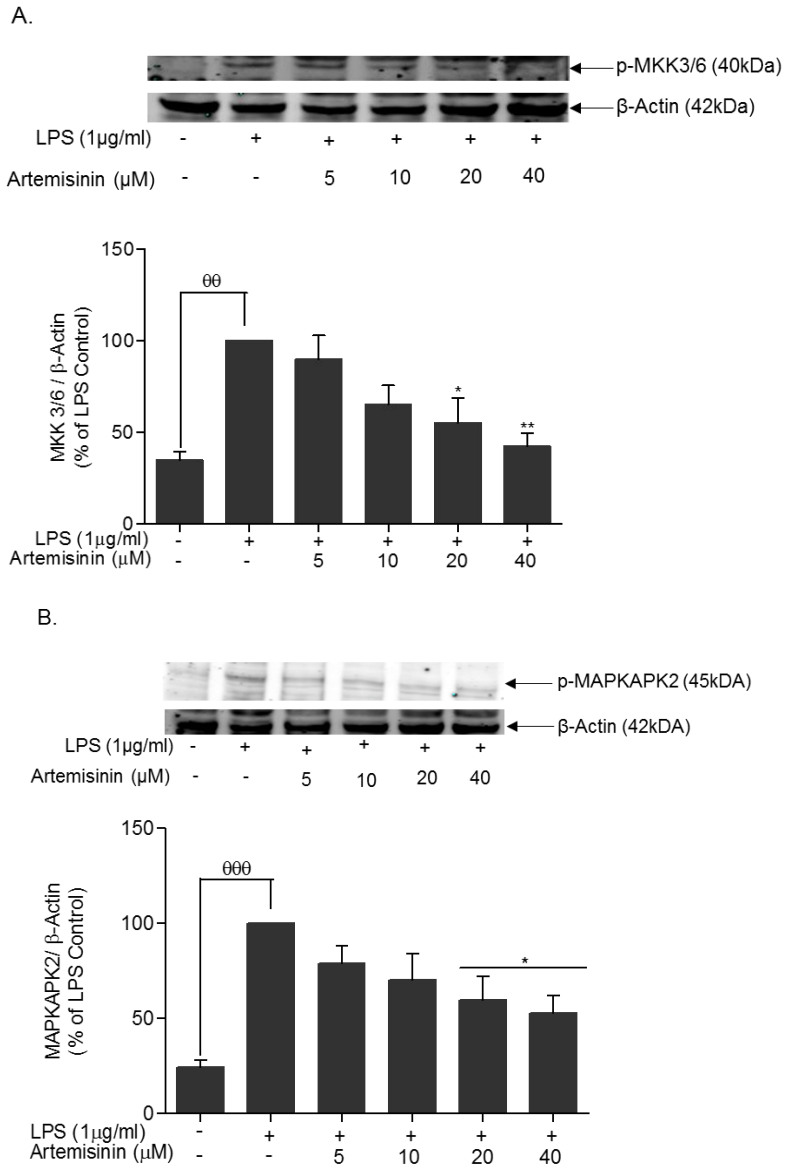

Figure 22: Artemisinin inhibited phosphorylation of MKK3/6 and MAPKAPK2 in LPS activated BV2 microglial cells.
(A.) Artemisinin (20-40 µM) inhibited MKK3/6 phosphorylation in LPS activated BV2 microglia cells. (B.) Artemisinin (20-40 µM) inhibited phosphorylation of MAPKAPK2 in LPS activated BV2 microglia 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. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p< 0.001$ in comparison with negative control (untreated cells).

3.2. Artemisinin activates the Nrf2/HO-1 antioxidant protective system.

Nrf2 is a transcription factor that is widely expressed in human and mouse tissues (Hayes & Dinkova-Kostova, 2014). Nrf2 activates a battery of antioxidant and cytoprotective genes that have in common a cis-acting enhancer sequence termed antioxidant response element (ARE) that include heme oxygenase-1 (HO-1) (Innamorato et al., 2008). Nrf2/HO-1 axis has been reported to be relevant in down-regulation of neuroinflammation by restoring

homeostasis (Innamorato et al., 2008; Rojo et al, 2014). Therefore, the effect of artemisinin on Nrf2/HO-1 antioxidant protective system was investigated.

To elucidate the effect of artemisinin on HO-1, BV2 cells were treated with artemisinin (5-40 µM) for 24 hours, and HO-1 protein expression investigated. Results showed that artemisinin (5-40 µM) significantly ($p<0.05$) increased the expression of HO-1 (Figure 23). At 40 µM, artemisinin produced 3-fold increase in HO-1 expression, suggesting marked activation of the protein.

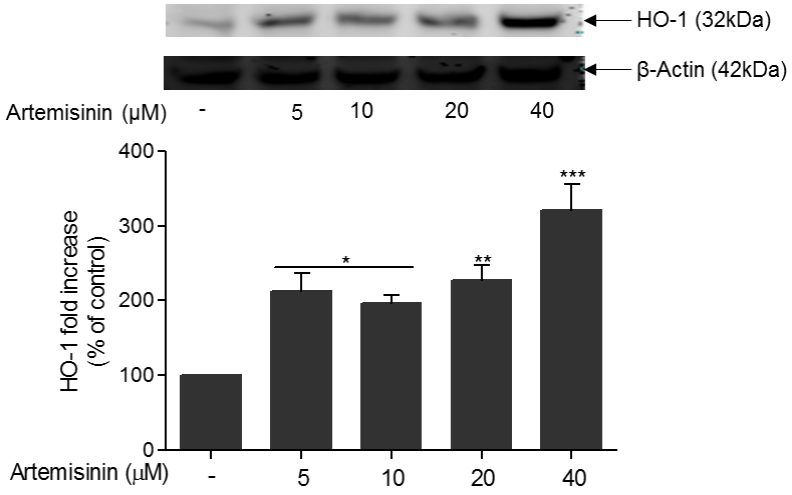

Figure 23: Artemisinin activated HO-1 protein in BV2 microglial cells.
BV2 cells were treated with artemisinin (5-40 µM) for 24 hours. Whole cell extracts were collected and analysed for HO-1 protein expression using western blot. All values are expressed as mean ± SEM for at least 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with control.

To further understand the effect of artemisinin on Nrf2-ARE protective mechanisms, the effect of artemisinin on ARE activity was investigated. HEK293 cells were transfected with ARE luciferase reporter gene. Treatment with artemisinin (5-40 µM) resulted in a concentration-dependent increase in ARE luciferase activity (Figure 24). At 20 µM and 40 µM, artemisinin treatment resulted in 510 % and 638 % ARE luciferase activity respectively relative to control. Up regulation of Nrf2 transcription factor has been reported to enhance HO-1 activity (Innamorato et al., 2008). To further understand the mechanism involved in the pharmacological up regulation of HO-1 protein by artemisinin, its effect on Nrf2 was investigated.

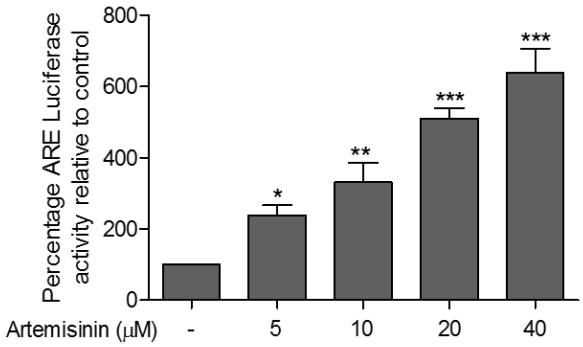

Figure 24: Artemisinin activated ARE luciferase activity in HEK293 cells transfected with ARE construct.
HEK293 cells were transfected with ARE-reporter construct for 18 hours. Thereafter, cells were treated with artemisinin (5-40 μM) for 8 hours and luciferase activity measured. All values are expressed 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<0.001 in comparison with control.

In the experiment, the highest concentration of artemisinin (40 μM) was investigated because at this concentration, the percentage HO-1 produced was significant (p<0.001) compared with the untreated control. In addition, at this concentration, ARE luciferase activity was significant (p<0.001) compared to the control. BV2 cells were treated with artemisinin (40 μM) for 15, 30, 60, 120 and 180 minutes. Interestingly, artemisinin produced significant (p<0.001) activation of nuclear Nrf2 after 15 and 120 minutes of treatment (Figure 25).

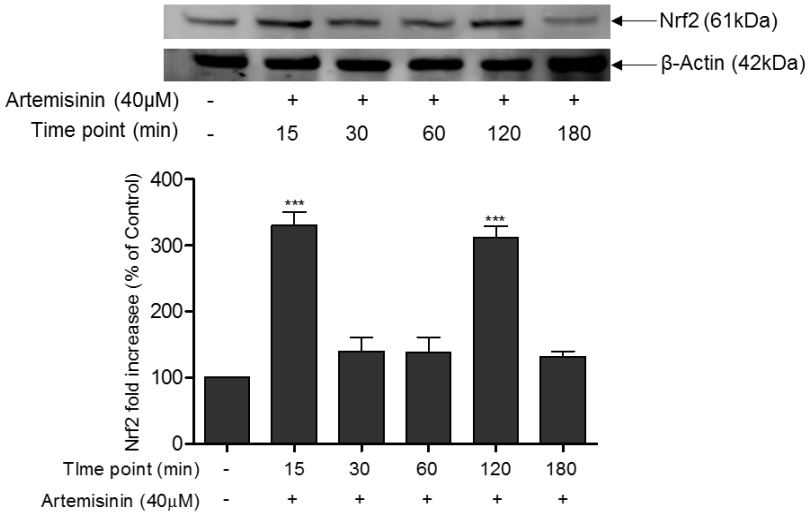

Figure 25: Artemisinin (40 μM) activated Nrf2 protein in BV2 microglial cells.
BV2 microglia cells were treated with artemisinin (40 μM) for 0 - 180 minutes. Thereafter, nuclear extracts were collected and analysed for Nrf2 protein expression using western blot. All values are expressed as mean ± SEM 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<0.001 in comparison with control.

3.3.1. Inhibition of nitric oxide production by artemisinin is independent of Nrf2

Nrf2-knockout BV2 cells were pre-treated with artemisinin (40 µM) prior to LPS stimulation for 24 hours, followed by determination of nitrite. Results show that Nrf2 gene silencing enhanced nitrite production with LPS stimulation (Figure 26). However, pre-treatment with artemisinin did not reverse the inhibitory effect of artemisinin on nitrite production in Nrf2 knockout cells. This observation suggests that the effect of artemisinin on nitrite production in LPS-activated BV2 microglia cells was independent of Nrf2 activity.

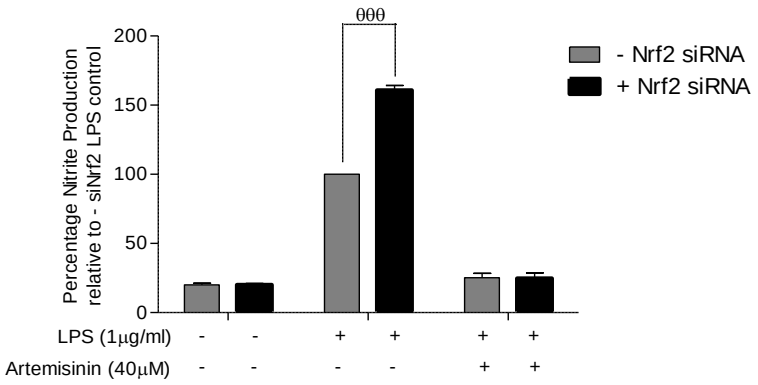

Figure 26: Inhibition of NO production in BV2 microglia by artemisinin is independent of Nrf2 activity. BV2 microglia cells were transiently transfected with Nrf2 siRNA, treated with artemisinin (40 µM) for 30 minutes and stimulated with LPS (1 µg/ml) for 24 hours. Cell supernatants were collected and analysed for NO production using Griess Assay. 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. $^{\theta}p<0.05$, $^{\theta\theta\theta}p< 0.001$ in comparison with - Nrf2 siRNA LPS control.

3.3.2. Inhibition of TNFα and IL-6 production in LPS-stimulated BV2 cells by artemisinin is dependent on Nrf2

Next, the effect of artemisinin (40 µM) on TNFα production in Nrf2 silenced cells was investigated. Figure 27 a shows that Nrf2 silencing enhanced TNFα production following LPS stimulation. However, pre-treatment with artemisinin (40 µM) reversed the inhibitory action of artemisinin following LPS stimulation ($p<0.001$). The outcome of this experiment suggests that the inhibitory action of artemisinin on TNFα production in LPS-activated BV2 microglia is dependent on Nrf2 activity.

Similarly, Nrf2 silencing significantly enhanced LPS induced IL-6 production in BV2 microglia (Figure 27 b). Pre-treatment with artemisinin (40 µM) significantly ($p<0.001$) reversed the inhibitory effect of artemisinin on IL-6 production in Nrf2 knockout cells.

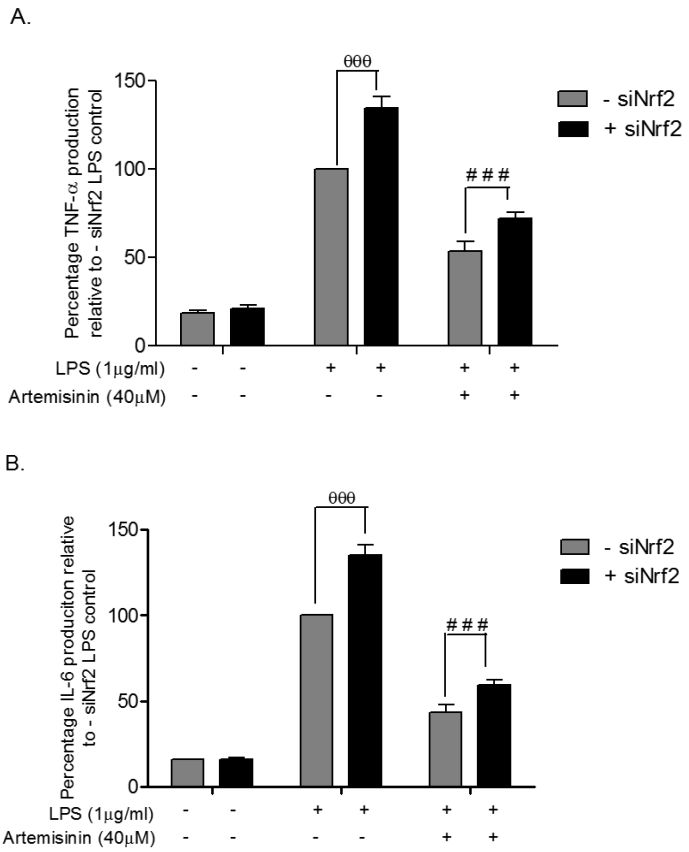

Figure 27: Inhibition of pro-inflammatory cytokines TNF and IL-6 by artemisinin in LPS activated BV2 cells is dependent on Nrf2 activity.
BV2 microglia cells were transfected with Nrf2 siRNA, treated with artemisinin (40 µM) for 30 minutes and stimulated with LPS (1 µg/ml) for 24 hours. Cell supernatants were analysed using an ELISA. (A) Inhibition of TNFα production in LPS-activated BV2 cells by artemisinin is dependent on Nrf2 activity. (B) Inhibition of IL-6 production in LPS-activated BV2 cells by artemisinin is dependent on Nrf2. 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. $^{\theta}p<0.05$, $^{\theta\theta\theta}p< 0.001$ in comparison with -siNrf2 LPS control. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ in comparison with -siNrf2 40 µM artemisinin treatment.

3.3.3. Inhibition of PGE_2 production in LPS-stimulated BV2 cells by artemisinin is dependent on Nrf2.

Nrf2-silenced BV2 cells were pre-treated with artemisinin (40 µM) for 30 minutes prior to LPS stimulation for 24 hours. Thereafter, PGE_2 concentration was determined. Figure 28 show that Nrf2 knockout significantly ($p<0.001$) enhanced PGE_2 production following LPS stimulation. Pre-treatment of Nrf2-silenced BV2 cells with artemisinin (40 µM) significantly ($p<0.001$) reversed the inhibitory effect of artemisinin on PGE_2 production. This result suggests that the effect of artemisinin on PGE_2 production in LPS activated BV2 cells is dependent of Nrf2.

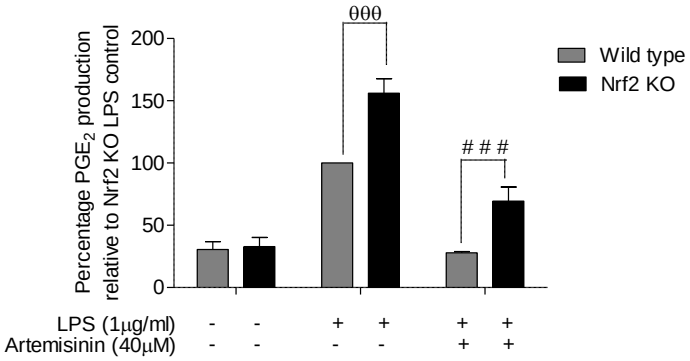

Figure 28: Inhibition of PGE_2 production in LPS-activated BV2 microglia cells by artemisinin is dependent on Nrf2 activity.
BV2 microglia cells were transfected with Nrf2 siRNA, pre-treated with artemisinin (40 µM) for 30 minutes and stimulated with LPS (1 µg/ml) for 24 hours. Cell supernatants were collected and analysed for PGE_2 production using PGE_2 EIA assay. 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. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with -siNrf2 LPS control. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ in comparison with -siNrf2 40 µM artemisinin treatment.

3.4. Treatment with artemisinin did not affect cell viability

Results of viability experiments show that artemisinin (5-40 µM) did not affect cell viability of BV2 microglia cells (Figure 29).

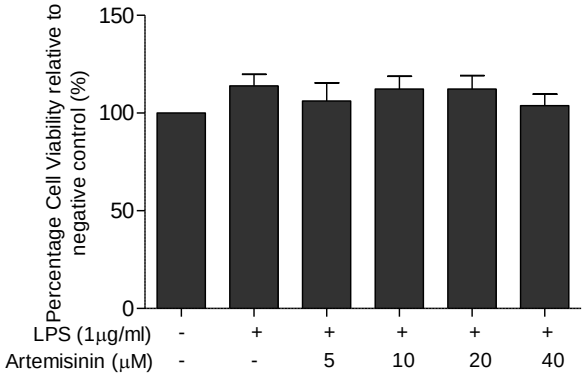

Figure 29: Artemisinin did not affect cell viability of BV2 cells.
BV2 cells were pre-treated with artemisinin (5-40 µM) for 30 minutes and subsequently stimulated with LPS (1 µg/ml) for 24 hours. Thereafter, MTT viability assay was performed. All values are expressed as mean ± SEM for at least 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test.

3.5. Discussion

The anti-inflammatory activity of artemisinin has been explored in many cell types (Ho et al., 2014). However prior to this study, its effect in LPS activated BV2 microglia cells had not been fully explored. A previous study using primary microglia by Zhu et al (2012)

investigated the effect of artemisinin on NF-κB signalling following LPS activation. In this study, the effect of artemisinin on NF-κB and p38 MAPK signalling pathways, which play a huge role in neuroinflammation, was investigated. In addition, the effect of the drug on Nrf2 antioxidant system as well as the role of Nrf2 on the anti-neuroinflammatory properties was studied. Findings from Zhu et al (2012) support the results from this study, which will be discussed in subsequent paragraphs.

Results from the MTT assay showed that the compounds did not affect cell viability. This was taken as a preliminary data to ensure that the results obtained from the study were not dependent on toxicity. Subsequent investigation on nitrite production was carried out and also used as another preliminary data and a pointer to determine whether artemisinin possessed anti-inflammatory properties. From the results obtained artemisinin inhibits nitric oxide production as well as iNOS an important enzyme important for its production in inflammatory conditions. In addition, nitric oxide and iNOS are important participants in nitrosative stress, which is chiefly involved, in the early pathogenesis of neuroinflammation and demyelination (Ghasemi & Fatemi, 2014; Ljubisavljevic & Stojanovic, 2014). Hence, this inhibitory action of artemisinin might be beneficial in situations involving neuroinflammation and demyelination. This observation was also reported in the work of Zhu et al, 2012 in LPS-activated primary microglia. Therefore, validating the results obtained from this study that artemisinin inhibits iNOS mediated NO production.

Artemisinin also suppressed PGE_2 production in LPS activated microglia cells by inhibiting COX-2 and mPGES-1 protein expression. Interestingly, inhibition of mPGES-1 was significant at all concentrations ($p<0.001$). This observation suggests that inhibition of PGE_2 by artemisinin is probably more via inhibition of mPGES-1 in LPS activated BV2 cells. These results suggest that a component of the anti-inflammatory activity of artemisinin involves inhibition of PGE_2/COX-2/mPGES-1. This is the first time artemisinin is shown to inhibit PGE_2/COX-2/mPGES-1 in LPS activated microglia cells.

Furthermore, artemisinin inhibited the production of TNFα and IL-6. With the microglia been the major source of cytokines (Olmos & Llado, 2014) during neuroinflammation, it is significant that artemisinin inhibits the production of these cytokines. This is because microglia-driven production of pro-inflammatory cytokines activates astrocytes, which further generates pro-inflammatory mediators, which can be detrimental to neurons (Glass et al., 2010). Supporting this inhibitory effects are the findings from a closely related study by Zhu et al. (2012) which also showed that artemisinin inhibited proinflammatory cytokines TNFα and IL-6 in primary microglia cells following stimulation with LPS. Taken together, this present study and the study by Zhu et al. (2012) strongly support that artemisinin inhibits proinflammatory cytokine production. This is important because these pro-inflammatory

cytokines contribute to the pathogenesis of various neurodegenerative disorders, including Alzheimer's disease, Huntington's disease, Parkinson's disease, multiple sclerosis (Erta et al., 2012; Frankola et al., 2011).

To understand the molecular mechanism(s) involved in the anti-neuroinflammatory actions of artemisinin, its effects on NF-κB signalling and p38MAPK signalling were investigated. Results showed that artemisinin inhibits nuclear translocation as well as DNA binding of NF-κB.

Following the observation that artemisinin exerted inhibitory effects on NF-κB translocation and DNA binding, the effect of artemisinin on upstream targets of NF-κB signalling was investigated. The compound inhibited both phosphorylation and degradation of IκB, without affecting phosphorylation of IKK. This suggests that the artemisinin targets NF-κB signalling by blocking IκB phosphorylation and degradation. Zhu et al. (2012) also reported that the artemisinin inhibited NF-κB activity in primary microglia following stimulated with LPS.

Artemisinin was shown to inhibit p38MAPK protein expression. This is important, as the p38MAPK pathway has been suggested to play a central role in the expression and activity of pro-inflammatory cytokines. MKK3/6 is a kinase that is responsible for the phosphorylation of p38MAPK. Artemisinin was shown for the first time to inhibit the phosphorylation of this kinase following LPS stimulation. This observation suggested that artemisinin interfere with p38MAPK signalling by inhibition of MKK3/6.

Upon activation of p38MAPK, the active kinase can activate transcription factors such as AP-1 by acting on its substrate MAPKAPK2. Artemisinin also inhibited MAPKAPK2 in LPS activated BV2 cells. This inhibitory action of artemisinin is significant as MAPKAPK2 deficiency has been reported to inhibit pro-inflammatory mediator release in transgenic mouse model of AD (Culbert et al., 2006). This is also the first time artemisinin has been shown to have inhibitory actions against MAPKAPK2 in BV2 cells following LPS stimulation.

After the M1 phase, the microglia progresses to the M2 phase which helps in recovery and restoration of homeostasis. To date, the Nrf2 signalling pathway has been strongly implicated in the activities observed in the M2 phase of the microglia (Cherry et al., 2014). Nrf2 activity controls HO-1, a phase II detoxifying and antioxidant enzyme, which have been implicated in the modulation of inflammatory responses (Zhang et al., 2013). Artemisinin was shown to activate HO-1 protein expression in BV2 cells.

Upon activation, Nrf2 translocate to the nucleus where it binds to ARE promoter region. Hence, the effect to artemisinin on Nrf2 was investigated. Artemisinin activated nuclear translocation of Nrf2. However, it was observed that this activation was at different time

points (15 and 120 minutes). An exact reason as to why this is so is not fully understood. However, this observation suggests that artemisinin activates Nrf2 mediated HO-1 protein expression. This is very important as in most CNS diseases involving neuroinflammation; the cell program is distorted causing the cells to remain in the M1 phase without proper recovery by progression to the M2 phase. Nuclear Nrf2 binds to ARE and transcriptionally regulate a set of protective genes, including HO-1 (Zhang et al., 2013). To further elucidate the effect of artemisinin on Nrf2, the effect on ARE reporter was investigated, and results showed that artemisinin activated ARE luciferase activity. This is the first time artemisinin is reported to activate Nrf2 mediated HO-1 activity. However, further investigations to understand why the compound activated Nrf2 at two time points need to be further examined.

Induction of Nrf2 by compounds has been reported to correlate directly with the inhibition of COX-2 and iNOS expression (Baird & Dinkova-Kostova, 2011). To gain a further understanding as to whether the effect of artemisinin on neuroinflammation was dependent on Nrf2 activity, experiments were carried out in Nrf2-silenced BV2 cells. Results obtained from the analysis of samples from Nrf2-silenced cells showed that Nrf2 did not affect inhibitory effect of artemisinin on of nitrite production. It is important to note that at the concentration investigated (40μM); artemisinin totally blocked NO production in both wild type and Nrf2-silenced BV2 cells. This highlights the possibility of a different outcome at lower concentration, which should be further investigated. On the other hand, the inhibition of TNFα, IL-6 and PGE_2 in LPS-activated BV2 cells by artemisinin was found to be dependent on Nrf2 gene. This is the first time artemisinin is shown to induce Nrf2 activity. It is also the first time a relationship between the inhibitory actions of the compound has been linked to the antioxidant activity.

In summary, artemisinin has been shown in these experiments to exert anti-neuroinflammatory properties and to activate the Nrf2 signalling pathway in BV2 microglial cells. In comparison with the findings of Zhu et al. (2012), the findings of this study is much more elaborate highlighting the signalling pathways (NF-κB, p38MAPK and Nrf2) that are involved in the activity of artemisinin in neuroinflammation.

CHAPTER 4

STUDIES ON ARTEMETHER

4. Studies on Artemether

4.1. Background

Artemether, the lipid-soluble derivative of artemisinin has been shown to have promising anti-inflammatory effects in various cell types (Cuzzocrea et al., 2005; Wu, 2011). Artemether was investigated for possible inhibition of neuroinflammation in BV2 microglia.

4.1.1. Artemether inhibits nitrite production in LPS-activated BV2 microglia

Following activation of the microglia, nitric oxide, an important mediator for regulating chronic inflammation in the CNS is released. Therefore, the effect of artemether on nitrite production was investigated. Results obtained show that nitrite production is significantly increased following LPS stimulation in comparison to the unstimulated cells (Figure 30 a).

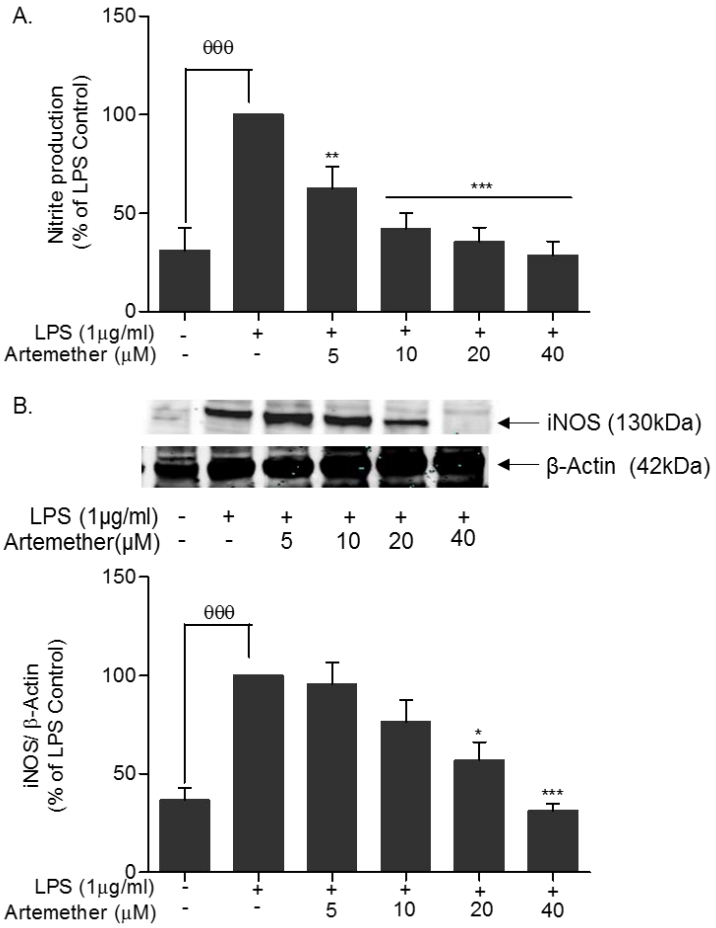

Figure 30: Artemether inhibited iNOS mediated NO production in LPS activated BV2 cells. (A) Artemether inhibited the nitrite production in LPS-activated BV2 cells. Supernatants from 24 hours stimulation were analysed for nitrite production. (B) Artemether (20 and 40 µM) attenuated iNOS protein expression in LPS activated BV2 cells. Whole cell extracts from 24 hours stimulation were analysed for iNOS protein expression using western blot. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman-Keuls test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells).

On the other hand, pre-treatment with artemether (5-40 µM) suppressed nitrite production significantly in a concentration-dependent manner (Figure 30 a). At 10 µM of artemether, nitrite production was 42.3 %. Subsequent increases in concentration to 20 µM and 40 µM resulted in 35.8 % and 28.68 % nitrite production, respectively.

Further investigations to understand the inhibitory effects of artemether on nitrite production showed that at lower concentrations (5 and 10 µM), artemether did not inhibit iNOS protein expression. However, artemether (20 and 40 µM) suppressed iNOS protein expression following LPS stimulation (Figure 30 b). These results suggest that at higher concentrations, artemether inhibited iNOS mediated NO production in LPS-activated BV2.

4.1.2. Artemether suppresses PGE_2 production in LPS stimulated BV2 microglia by inhibiting COX-2 and mPGES-1 proteins

Stimulation of BV2 cells with LPS (1 µg/ml) significantly increased PGE_2 production (Figure 31 a). However, pre-treatment with artemether (5-40 µM) significantly ($p<0.05$) suppressed PGE_2 production in LPS-activated BV2 microglia cells.

Following the observation that artemether could reduce PGE_2 production, the effect of the compound on COX-2 protein expression was then investigated. Figure 31 b shows that at 5 µM, artemether did not significantly inhibit COX-2 protein expression. However, pre-treatment with artemether (10-40 µM) resulted in a significant ($p<0.05$) reduction in COX-2 protein expression (Figure 31 b). At 20 µM and 40 µM, COX-2 protein expression was found to be 60 % and 50 % respectively when compared to the LPS control ($p<0.001$).

It was also observed that pre-treatment with artemether (5-40 µM) significantly ($p<0.001$) suppressed mPGES-1 protein (Figure 31 c). From these observations, it can be concluded that artemether suppressed PGE_2 production in LPS-activated BV2 microglia cells by inhibiting COX-2 and mPGES-1 protein expression.

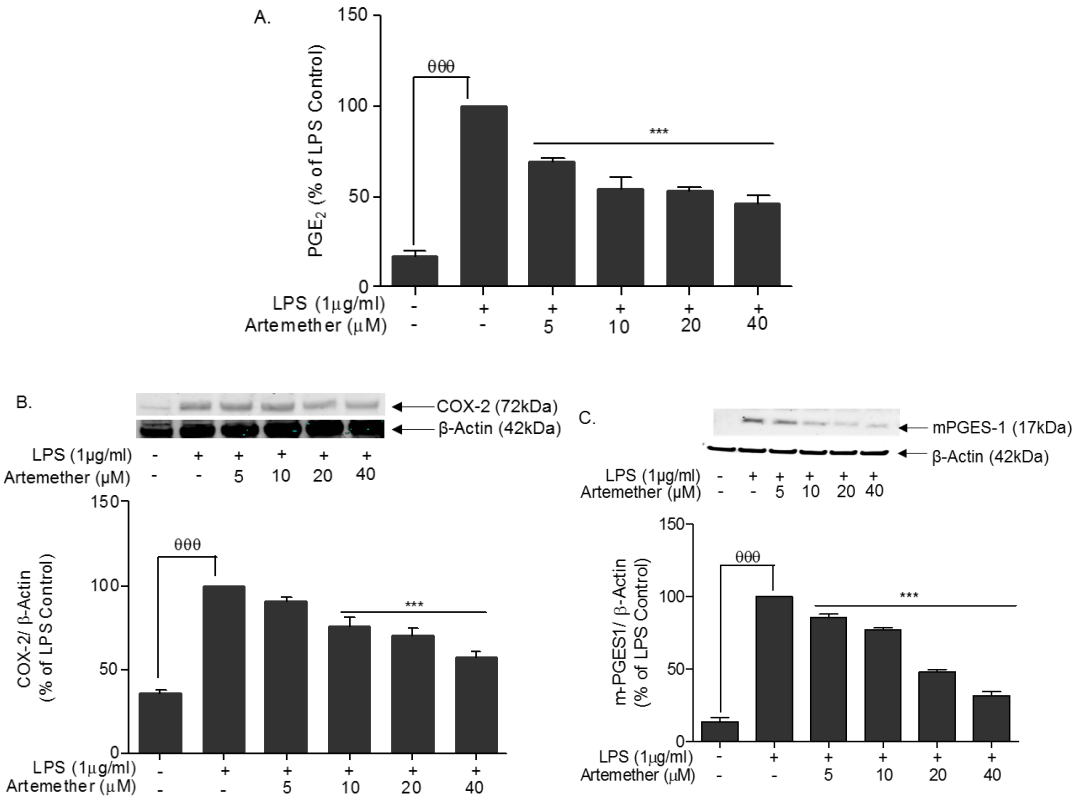

Figure 31: Artemether inhibited PGE_2 production in LPS activated BV2 microglia cells via dual inhibition of COX-2 and mPGES-1 proteins.
(A) Artemether (5-40 µM) suppressed PGE_2 production in LPS-stimulated BV2 microglia cells (B) Artemether (10-40 µM) inhibited COX-2 protein expression in LPS-activated BV2 microglia cells. (C) Artemether inhibited mPGES-1 protein expression in LPS-stimulated BV2 cells. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman-Keuls test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells).

4.1.3. Artemether reduces the production of TNFα and IL-6 in LPS-activated BV2 microglia

Following stimulation with LPS (1 µg/ml), levels of secreted TNFα were significantly increased ($p<0.001$) in culture supernatants of BV2 microglia (Figure 32 a). However, pre-treatment with artemether (10-40 µM) resulted in a significant ($p<0.05$) concentration-dependent reduction of TNFα production. At 40 µM TNFα production was 18 % when compared to the LPS control ($p<0.001$).

Experiments also showed that pre-treatment with artemether (5-40 µM) significantly ($p<0.05$) suppressed IL-6 production in LPS-activated BV2 cells in a concentration-dependent manner (Figure 32 b). At a concentration of 5 µM, artemether produced 63.6 % of IL-6, compared with LPS control. Subsequent increase in concentrations to 10, 20 and 40 µM resulted in 51.4 %, 40.6 % and 26.8 % of IL-6 production, respectively.

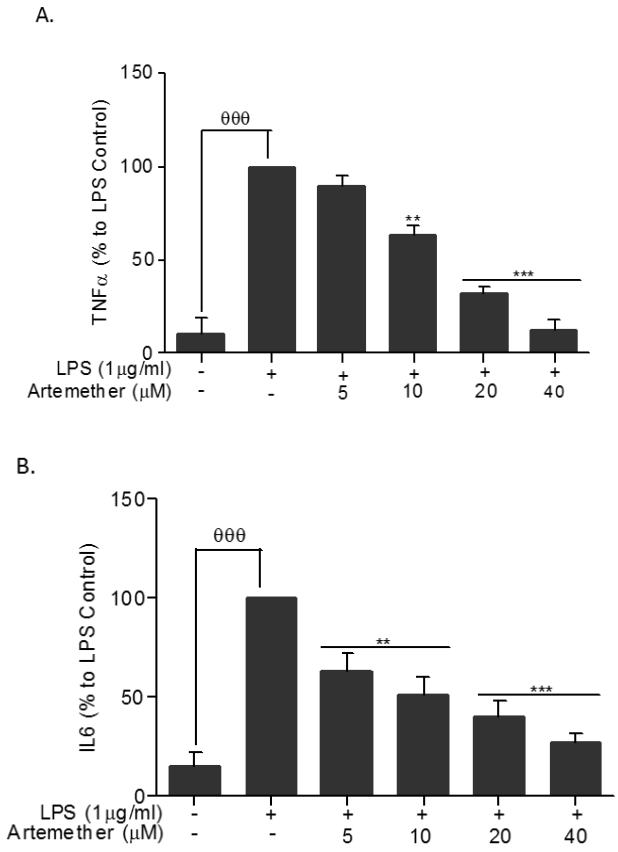

Figure 32: Artemether suppressed the production of proinflammatory cytokines TNFα and IL-6 in LPS-activated BV2 microglia cells.
Culture supernatants from 24hour stimulation were analysed using an ELISA. (A). Artemether (10-40 µM) inhibited TNFα production in LPS-activated BV2 microglia cells. (B). Artemether (5-40 µM) attenuated IL-6 production in LPS-activated BV2 microglia cells. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman-Keuls test. $^{*}p < 0.05$, $^{**}p<0.01$, $^{***}p<0.001$ in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells).

4.1.4. Artemether inhibits neuroinflammation in LPS-activated BV2 microglia by inhibiting IκB/NF-κB signalling.

Regulation of pro-inflammatory genes like iNOS which controls nitrite production, and COX-2 which mediates PGE_2 production in activated microglia is controlled by NF-κB signalling. Based on observations showing that artemether inhibited iNOS/NO, COX-2/mPGES-1/PGE_2 as well as pro-inflammatory cytokines TNFα and IL-6, its effect on NF-κB signalling was investigated.

First, an experiment to determine the effect of artemether on NF-κB luciferase activity was done. Results obtained show that artemether (5-40 µM) inhibited NF-κB regulated luciferase reporter gene expression following stimulation with TNFα (1 ng/ml) (Figure 33). Interestingly,

at all concentrations, the percentage luciferase activity was less than 50 % in comparison to TNFα control. This observation suggests that artemether inhibit NF-κB activity generally.

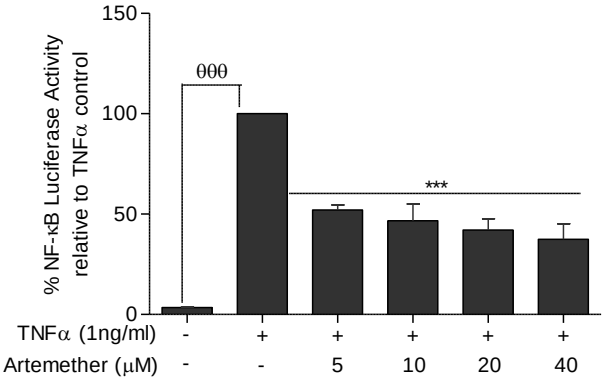

Figure 33: Artemether suppressed NF-κB activity in HEK293 cells transiently transfected with NF-κB construct.
NF-κB transfected HEK293 cells were stimulated with TNFα (1 ng/ml) in the absence or presence of artemether (5-40 μM) for 6 hours. Luminescence measurement was used as a measure of NF-κB activity. 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. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with TNFα control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells).

Under resting conditions, NF-κB dimers are bound in the cytoplasm to inhibitory IκB proteins, which sequester inactive NF-κB complexes. However, following stimulation, the IκB proteins are phosphorylated and degraded. Hence, the effect of artemether on IκB-α phosphorylation and degradation was investigated. Figure 34 a shows that pre-treatment with artemether (5 and 10 μM) did not significantly inhibit IκB-α phosphorylation induced by LPS stimulation. However, treatment with artemether (20 and 40 μM) resulted in significant ($p<0.05$) inhibition of phospho-IκB-α protein expression (Figure 34 a). Interestingly, artemether (10 μM) inhibited the degradation of IκB-α (Figure 34 a). However the effects at 20 and 40 μM were not statistically significant.

Free NF-κB subunits released upon phosphorylation and degradation of IκB translocate to the nucleus where they bind to the DNA and regulate transcriptional activity of pro-inflammatory genes.

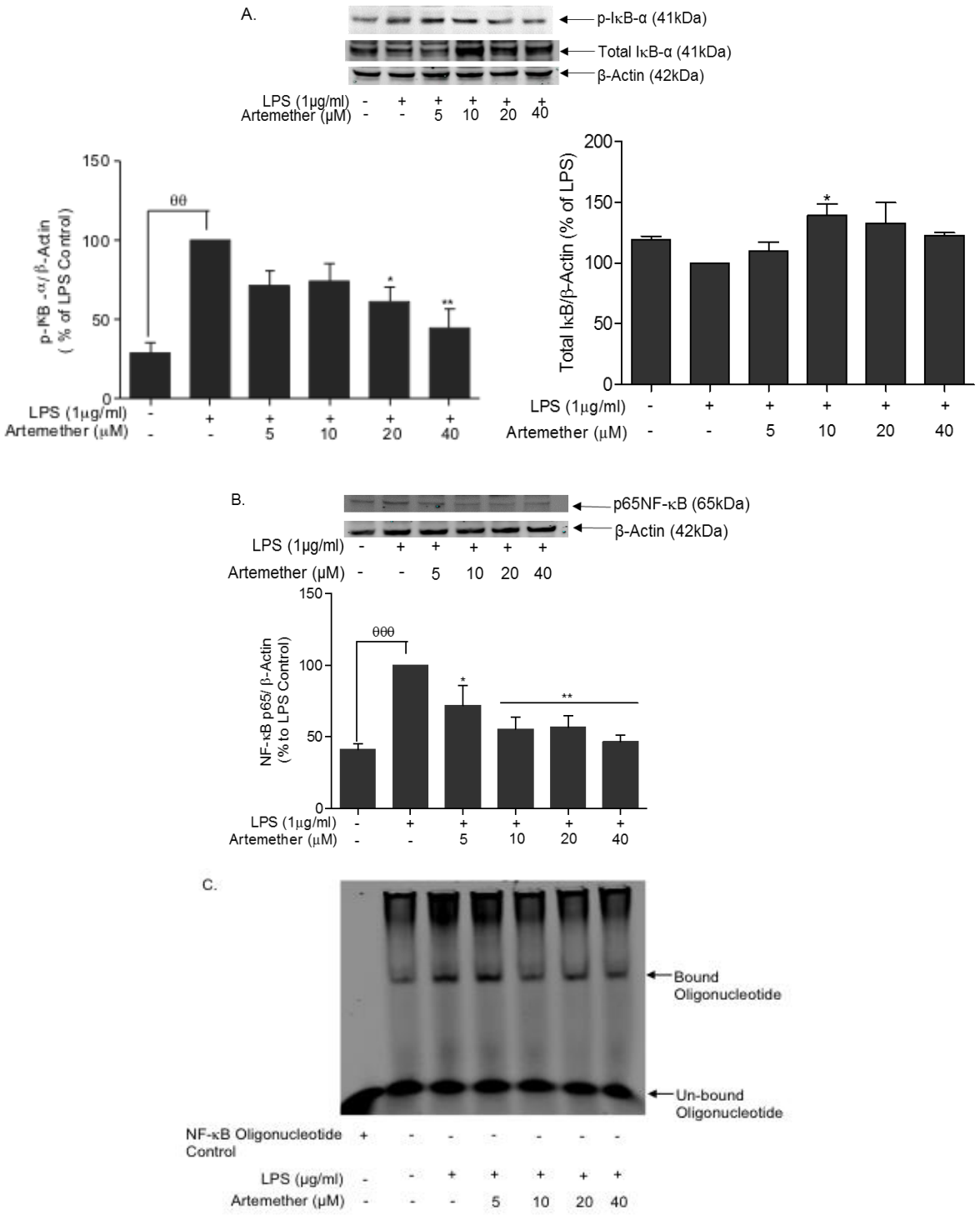

Figure 34: Artemether inhibited IκB/NF-κB signalling in LPS-activated microglia cells. (A) Artemether inhibited IκB-α phosphorylation and degradation in LPS-activated BV2 cells. Whole cell extracts from 1 hour stimulation were analysed for phospho- and total IκB-α protein using western blot. (B) Artemether suppressed p65 translocation in LPS-activated BV2 microglial cells. Nuclear extracts from 1 hour stimulation were analysed for p65 protein expression using western blot. (C) Artemether inhibited NF-κB DNA binding in LPS-activated BV2 microglia cells. 1hour stimulation nuclear extracts were incubated with NF-κB oligonucleotide for 30 minutes and analysed for NF-κB binding using EMSA. 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. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells).

As a result of the observed inhibitory effect of artemether on the phosphorylation and degradation of IκB-α, it was considered worthwhile to determine whether the compound could affect nuclear translocation of p65 subunit. Figure 34 b shows that upon activation with LPS, nuclear expression of phospho-p65NF-κB was significantly increased. The results also show that pre-treatment with artemether (5-40 µM) significantly ($p<0.05$) inhibited protein expression of phospho-p65NF-κB in the nucleus in LPS-activated BV2 cells. This suggests that in LPS-activated BV2 cells that artemether inhibited nuclear translocation of the p65 subunit (Figure 34 b).

Subsequently the effect of artemether on DNA binding of NF-κB was investigated in an EMSA. At 5 µM artemether did not affect DNA binding of NF-κB (Figure 34 c). However, artemether (10-40 µM) inhibited DNA binding of NF-κB to the promoter region in the nucleus. These results suggest that not only does artemether inhibit IκB-α phosphorylation and degradation; it also inhibits p65NF-κB translocation to the nucleus and binding to the DNA.

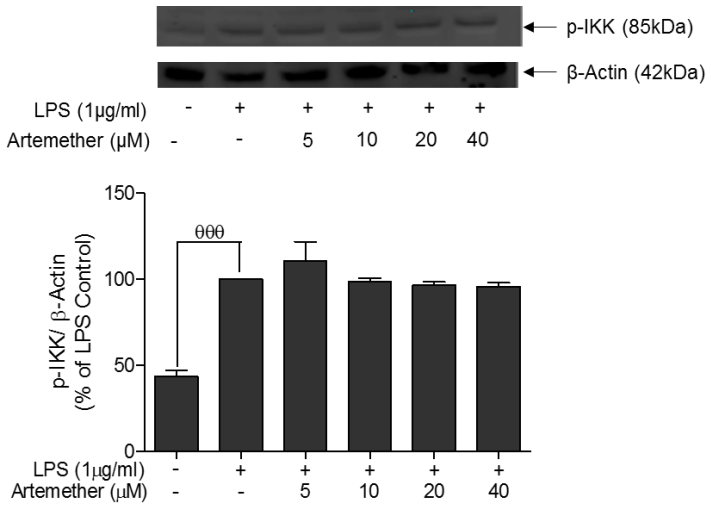

Figure 35: Artemether (5-40 µM) did not inhibit phosphorylation of IKK in LPS-activated BV2 cells significantly.
BV2 microglia cells were stimulated with LPS (1 µg/ml) for 10 minutes in the absence or presence of artemether (5-40 µM). Whole cell extracts were collected and analysed for p-IKK and total IKK protein expressions using western blot. 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. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells).

In NF-κB signalling, stimulus-induced phosphorylation and degradation of IκB proteins is initiated through phosphorylation by the IκB kinase (IKK) complex. Therefore, to further elucidate the molecular mechanisms involved in the anti-neuroinflammatory activity of

artemether, its effect on phospho-IKK protein was investigated. Figure 35 show that artemether did not have significant inhibitory effect on phospho-IKK protein expression in LPS-activated BV2 cells. This result suggests that artemether may interfere with NF-κB signalling by targeting IκB phosphorylation and degradation directly.

4.1.5. Artemether targets p38MAPK signalling to exert anti-neuroinflammatory actions in LPS activated BV2 microglia.

Stimulation of BV2 microglia for 1 hour resulted in a significant ($p<0.001$) increase in phospho-p38 protein expression when compared to the untreated control (Figure 36). However, pre-treatment with artemether (5-40 μM) led to a significant ($p<0.001$) reduction in the expression of this protein (Figure 36).

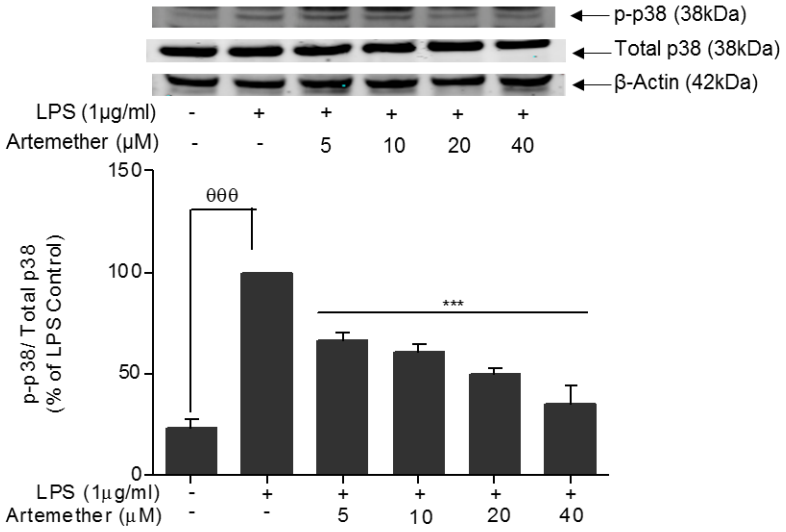

Figure 36: Artemether inhibited phospho-p38 protein expression in LPS-activated BV2 microglia cells. Whole cell extracts from 1 hour stimulation were analysed for phospho-p38 and total p38 protein expression with anti-phospho-p38 and anti-total p38 antibody. All values are expressed as mean ± SEM for 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 LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with Negative control (untreated cells).

To further elucidate the effect of artemether on p38MAPK signalling, its effect on MKK3/6 was investigated. Figure 37a show that stimulation with LPS significantly ($p<0.001$) induced the phosphorylation of MKK3/6. However, pre-treatment with artemether (5-40 μM) significantly suppressed the phosphorylation of MKK3/6 (Figure 37 a). This suggests that artemether inhibited p38MAPK phosphorylation via direct suppression of MKK3/6 phosphorylation.

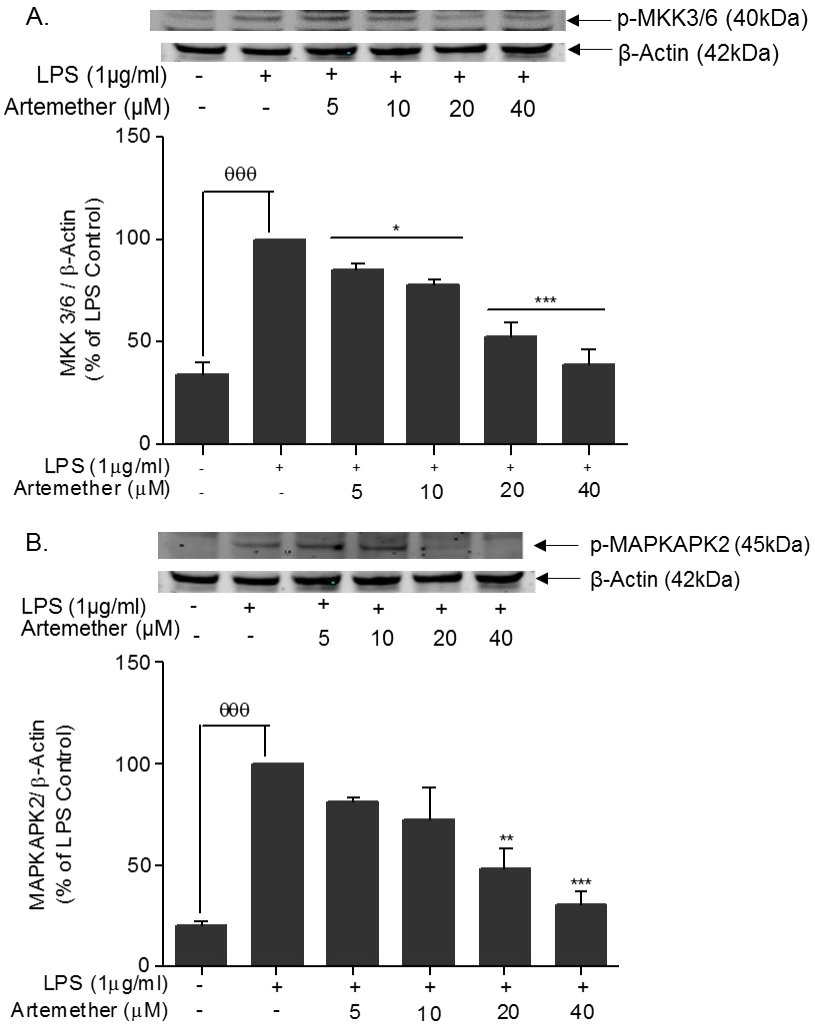

Figure 37: Artemether inhibited phospho-MKK3/6 and phospho-MAPKAPK2 protein expression in LPS-activated BV2 microglia cells.
Whole cell extracts from 1 hour stimulation were analysed for p-MKK3/6 and p-MAPKAPK2 protein expression. (A). Artemether (5-40 µM) suppressed MKK3/6 phosphorylation in LPS-activated BV2 cells. (B) Artemether (20 & 40 µM) inhibited MAPKAPK2 phosphorylation in LPS-activated BV2 microglia cells. All values are expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells). $^{*}p<0.05$, $^{***}p<0.001$ in comparison with LPS control.

Phosphorylated p38MAPK protein activates transcription factors by acting on MAPKAPK2. Therefore, the effect of artemether on the phosphorylation of MAPKAPK2 was investigated. It was observed that there was a marked increase in phospho-MAPKAPK2 protein expression following stimulation of BV2 cells with LPS (Figure 37 b) However, pre-treatment with artemether (20 and 40 µM) caused a reduction in blocked the phospho-MAPKAPK2 protein (Figure 37 b).

4.2. Artemether activates Nrf2/HO-1 antioxidant protective mechanism in BV2 microglia

Following the observation that artemether blocked neuroinflammation by targeting NF-κB and p38MAPK signalling in BV2 microglia, it was decided to explore if the Nrf2/HO1 antioxidant system played any role in the activity.

Firstly, the effect of artemether on HO-1 protein expression was investigated. Results from these experiments showed that in comparison with untreated cells, HO-1 was activated by 40 µM of artemether after 24 hours of treatment (Figure 38).

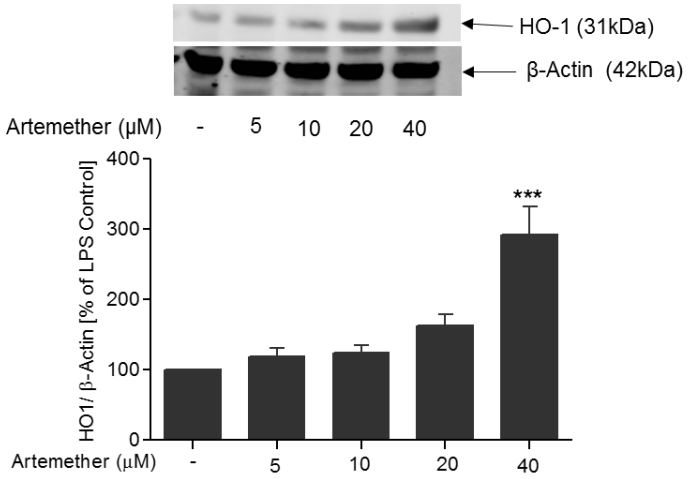

Figure 38: Artemether activated HO-1 in BV2 microglia cells. BV2 cells were treated with artemether (5-40 µM) for 24 hours.
Whole cell extracts were collected and analysed for HO-1 protein expression using western blot. All values are expressed as mean ± SEM for at least 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with control.

Nrf2 activates a battery of antioxidant and cytoprotective genes that have in common a cis-acting enhancer sequence known as ARE. Therefore, to further understand how artemether could be affecting the antioxidant protective mechanisms in the microglia, its effect on the ARE activity was investigated. HEK 293 cells were transiently transfected with ARE construct, followed by treatment with artemether. Results obtained show that treatment with artemether increased ARE luciferase activity in a concentration-dependent manner (Figure 39).

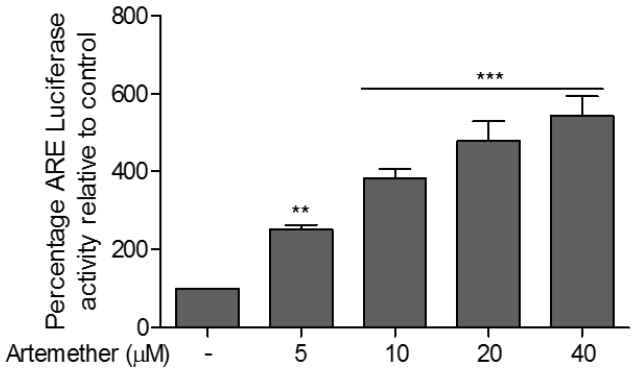

Figure 39: Artemether activated ARE luciferase activity in HEK293 cells transfected with ARE construct.
HEK293 cells were transfected with ARE-reporter construct for 18 hours. Thereafter, cells were treated with artemether (5-40 µM) for 8 hours. Luciferase activity was measured with fluorescence. All 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<0.001 in comparison with control.

Subsequently, the effect of artemether on nuclear Nrf2 protein expression was investigated. BV2 cells were treated with artemether (40 µM) for 15, 30, 60 120 and 180 minutes. At 40 µM artemether produced significant activation of Nrf2 at 15 and 120 minutes of treatment (Figure 40).

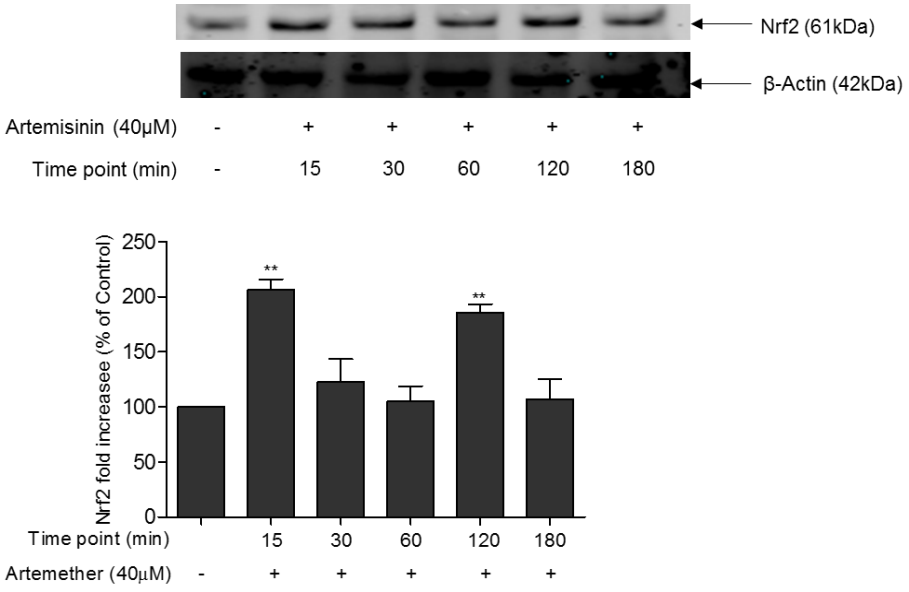

Figure 40: Artemether (40 µM) activates Nrf2 protein expression in BV2 microglia cells.
BV2 microglia cells were treated with artemether (40 µM) for 0 - 180 minutes. Thereafter, nuclear extracts were collected and analysed for Nrf2 protein expression using western blot. All values are expressed as mean ± 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<0.001 in comparison with control.

4.3.1. Inhibition of nitric oxide production by artemether is independent of Nrf2

Nrf2-silenced BV2 cells were pre-treated with artemether prior to LPS stimulation for 24 hours. This was followed by determination of nitrite. Results obtained show that nitrite production was significantly ($p<0.001$) enhanced in Nrf2 silenced cells (Figure 41). However, pre-treatment with artemether did not reverse the inhibitory effect of artemether on nitrite production in Nrf2 knockout cells (Figure 41), suggesting that the inhibitory actions of artemether on nitrite production is independent of Nrf2 activity.

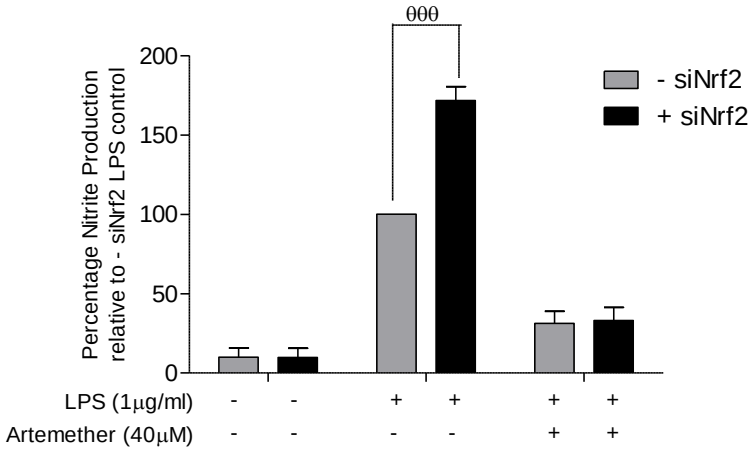

Figure 41: Inhibition of NO in LPS-activated BV2 cells by artemether is independent of Nrf2 activity. Nrf2 silenced BV2 microglia cells were stimulated with LPS (1 µg/ml) for 24 hours after pre-treatment with artemether (40 µM). Subsequently, culture supernatants were collected and analysed for NO production using Griess Assay. 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. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with –siNrf2 LPS control.

4.3.2. Inhibition of TNFα and IL-6 production in LPS-stimulated BV2 cells by artemether is dependent on Nrf2

Nrf2-silenced BV2 cells were pre-treated with artemether and stimulated with LPS for 24 hours. Thereafter, supernatants were analysed for TNFα production. Nrf2 knockout significantly ($p<0.001$) enhance LPS-induced TNFα production. Furthermore, pre-treatment with artemether reversed the inhibitory effects of artemether on TNFα production (Figure 42 a). These results suggest that the inhibitory effect of artemether on TNFα production is dependent of Nrf2 activity.

In addition, the effect of Nrf2 on the inhibitory actions of artemether on IL-6 production was investigated. Stimulation with LPS significantly enhanced IL-6 production in Nrf2-silenced BV2 cells (Figure 42 b). However, pre-treatment with artemether reversed the inhibitory effects of artemether on IL-6 production. This observation shows that the effects of artemether on IL-6 production in BV2 cells are dependent of Nrf2 activity.

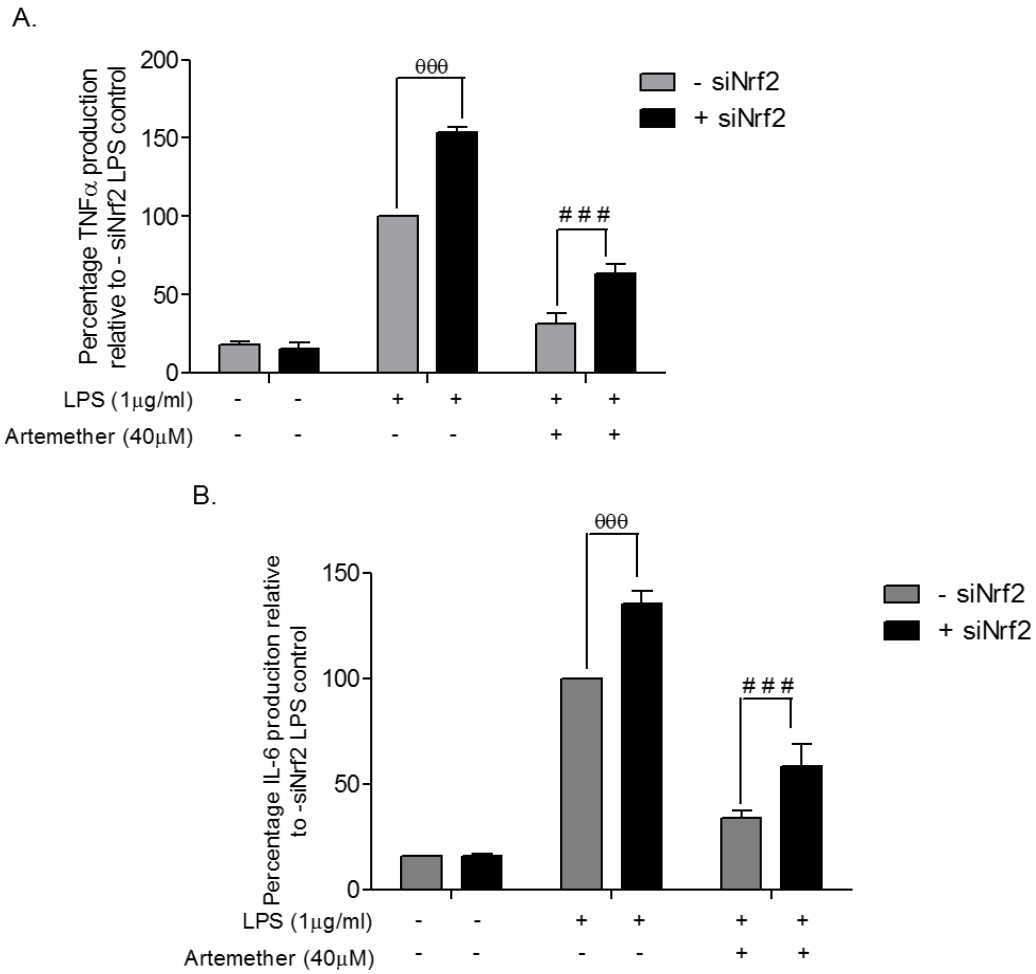

Figure 42: Inhibition of TNFα and IL-6 by artemether in LPS-activated BV2 cells is dependent on Nrf2 activity.

Nrf2-silenced BV2 cells were pre-treated with artemether prior to LPS stimulation for 24 hours. Subsequently culture supernatants were analysed for TNFα and IL-6 production using an ELISA. (A) Inhibition of TNFα production in LPS activated BV2 cells is dependent on Nrf2 activity. (B) Inhibition of IL-6 production in LPS-activated BV2 cells is dependent on Nrf2 activity. 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. $^{\theta}$ $p<0.05$, $^{\theta\theta\theta}p< 0.001$ in comparison with -siNrf2 LPS control. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ in comparison with -siNrf2 40 μM artemether treatment.

4.3.3. Inhibition of PGE_2 production in LPS-stimulated BV2 cells by artemether is dependent on Nrf2

Nrf2-silenced BV2 cells were pre-treated with artemether prior to LPS stimulation for 24 hours, followed by determination of PGE_2 production. Results show that Nrf2 knockout enhanced PGE_2 production on LPS stimulation (Figure 43). However, pre-treatment with artemether (40 μM) reversed the inhibitory effects of artemether on PGE_2 production in Nrf2 knockout cells (Figure 43). This result show that the effects of artemether on PGE_2 production in LPS activated BV2 cells is dependent on Nrf2.

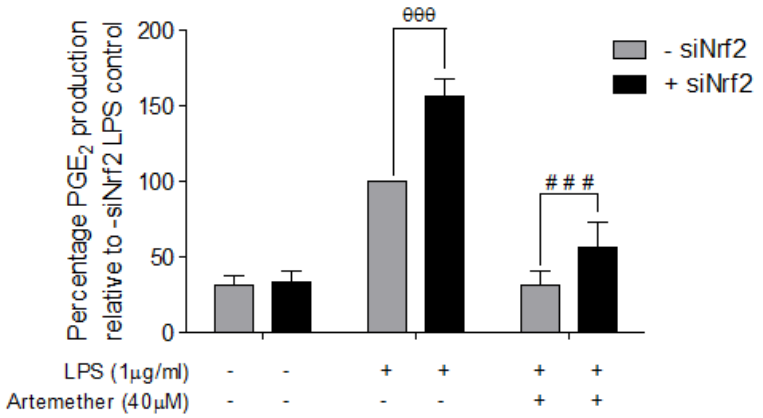

Figure 43: Inhibition of PGE_2 production by artemether in MPS-activated BV2 microglia is dependent on Nrf2 activity.
BV2 microglia cells were transfected with Nrf2 siRNA, pre-treated with artemether (40µM) for 30 minutes and stimulated with LPS (1 µg/ml) for 24 hours. Culture supernatants were collected and analysed for PGE_2 production. 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. $^{\theta}$ $p<0.05$, $^{\theta\theta\theta}p< 0.001$ in comparison with -siNrf2 LPS control. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ in comparison with wild-type 40 µM artemether treatment.

4.4. Artemether (5-40 µM) did not affect cell viability

To ascertain that artemether (5-40 µM) did not affect cell viability, BV2 cells were stimulated for 24 hours in the presence or absence of artemether (5-40 µM). Thereafter, MTT assay was carried out on the cells. Results obtained (Figure 44) show that artemether did not affect cell viability in BV2 microglia cells.

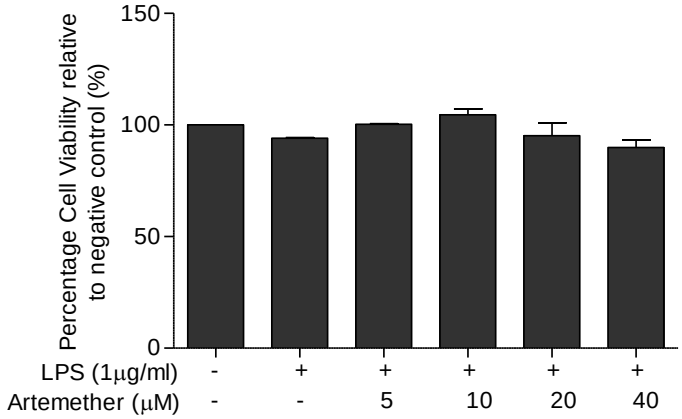

Figure 44: Artemether did not affect cell viability of BV2 cells.
BV2 cells were pre-treated with artemether (5-40 µM) for 30 minutes and subsequently stimulated with LPS (1 µg/ml) for 24 hours. Thereafter, MTT viability assay was performed. All values are expressed as mean ± SEM for at least 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test.

4.5. Discussion

In this study, the anti-neuroinflammatory property of artemether was investigated in LPS-activated BV2 cells. Concentrations used for the study was determined through the MTT assay which showed that chosen concentrations were not toxic. First, the effect of artemether on nitric oxide was evaluated. Result obtained showed that artemether significantly inhibited nitrite production in LPS-activated BV2 cells at all concentrations. However, high concentrations of artemether (20 and 40 µM) significantly decreased iNOS protein expression, suggesting that the compound suppresses NO/iNOS in neuroinflammation. This is the first time artemether is shown to inhibit iNOS mediated NO production in LPS activated BV2 microglia cells. The outcome of the effect of artemether on NO production also served as a preliminary data and pointer that the compound might possess anti-neuroinflammatory properties.

Increased COX-2-mediated PGE_2 production is associated with inflammatory pathophysiological conditions. Therefore, to further understand the possible anti-neuroinflammatory effect of artemether (5-40 µM); the effect on PGE_2 production following LPS-stimulation of BV2 cells was investigated. Artemether (5-40 µM) significantly inhibited PGE_2 production in LPS-activated BV2 cells. To further elucidate the inhibitory actions of artemether on PGE_2, its effect on COX-2 was investigated. Artemether (10-40 µM) also significantly inhibited COX-2 protein expression in LPS-activated BV2 microglia cells. It is noteworthy that at 5µM artemether did not inhibit COX-2 protein expression but inhibited PGE_2 production. Interestingly, artemether significantly inhibited mPGES-1 protein expression in LPS activated BV2 cells at all concentrations investigated. This result suggests that the inhibitory action of artemether on PGE_2 in LPS activated BV2 microglia cells was probably mediated mainly through its inhibition of mPGES-1.

Pro-inflammatory cytokines have been reported to play a significant role in the progression of CNS neuroinflammatory diseases including AD, PD, MS, and ALS. Therefore, the effect of artemether on TNFα and IL-6 secretion was investigated. Artemether (10-40 µM) significantly inhibited TNFα production in LPS-activated BV2 cells. However, it significantly inhibited IL-6 production at all concentrations investigated. These observations suggest that artemether inhibits pro-inflammatory cytokines; TNFα and IL-6, produced in LPS activated BV2 microglia cells.

The NF-κB signalling pathway is known to regulate the genes involved in the production of pro-inflammatory mediators including iNOS, COX-2 and the pro-inflammatory cytokines, TNFα and IL-6. Hence, to further understand the possible mechanism involved in the inhibitory actions of artemether on pro-inflammatory mediators, the NF-κB signalling was

investigated. Artemether inhibited NF-κB luciferase activity. This observation suggests that artemether might possess inhibitory actions against the NF-κB signalling pathway.

To further elucidate the effect of artemether on NF-κB signalling, its effect on IκB, a kinase that holds NF-κB subunits in an inactive state in the cytoplasm, was investigated. Upon activation of NF-κB signalling, IκB is phosphorylated and degraded releasing the NF-κB subunits. Results obtained showed that artemether inhibited IκB phosphorylation and degradation in LPS-activated BV2 cells. In other for IκB phosphorylation to occur, IKK is phosphorylated. Hence, the effect of artemether on IKK phosphorylation was investigated. Results obtained show that artemether did not inhibit IKK phosphorylation. This observation suggests that the inhibitory actions of artemether on NF-κB signalling by directly targeting IκB.

Free NF-κB subunits translocate to the nucleus binds to the DNA and regulate gene transcriptional activity of pro-inflammatory mediators. Subsequently, the effect of artemether on nuclear phospho-p65 was investigated. Artemether significantly inhibited phospho-p65 protein expression at all concentrations, suggesting that artemether inhibits nuclear translocation of p65 subunit. Artemether also inhibited DNA binding of NF-κB. Taken together; it is proposed that artemether interferes with NF-κB signalling by inhibiting IκB-mediated NF-κB translocation and binding to the DNA.

p38MAPK signalling has been suggested to play a role in the expression and activity of pro-inflammatory cytokines. Consequently, artemether was shown to significantly suppress phosphorylation of p38MAPK and its upstream kinase MKK3/6 in LPS activated BV2 cells. Activated p38MAPK activate transcription factors by acting on its substrate MAPKAPK2. Artemether was also shown to inhibit the phosphorylation of its downstream substrate; MAPKAPK2 in LPS activated BV2 microglia cells. The findings appear to suggest that the anti-inflammatory action of artemether in LPS-activated microglia is mediated in part through the interference of p38 MAPK signalling pathway.

The restoration of homeostasis in the microglia following an inflammatory challenge has been reported to involve the Nrf2 pathway (Rojo et al., 2014). Nrf2 activity controls HO-1, a phase II detoxifying and antioxidant enzyme. Artemether significantly activated HO-1 protein expression in BV2 microglia cells, suggesting some antioxidant activity. Upon activation, Nrf2 translocate to the nucleus where it regulates the transcription of HO-1 by binding to ARE. Artemether activated both nuclear Nrf2 and ARE luciferase activity in BV2 microglia. It can thus be concluded that artemether activates ARE reporter-mediated Nrf2/HO-1 antioxidant protective mechanisms

Having shown that artemether exerted inhibitory effects on neuroinflammation, as well as activating the Nrf2 mechanisms in the microglia; it became necessary to explore the relationships between these two activities. Consequently, Nrf2 gene was silenced in BV2 cells, which were then stimulated with LPS and levels of pro-inflammatory factors measured. Results show that the inhibitory action of artemether on nitrite production in LPS activated BV2 cells is independent of Nrf2. However, the inhibition of TNFα, IL-6 and PGE_2 of artemether in LPS-activated BV2 cells is dependent on Nrf2 activity.

In summary, the study on artemether demonstrates that the anti-neuroinflammatory activity of artemether in LPS activated BV2 cells is mediated through the NF-κB and p38MAPK-signalling pathway. The study also demonstrates that artemether activates Nrf2 mediated HO-1 expression, which may account for some of this anti-inflammatory effects. It is important to highlight that this study on artemether provides the first evidence that artemether possess anti-neuroinflammatory actions in LPS activated microglia. It is also the first time that the inhibitory actions on pro-inflammatory cytokines TNFα and IL-6 as well as PGE_2 are linked to the activation of Nrf2.

CHAPTER 5

STUDIES ON ARTESUNATE

5. Studies on Artesunate

5.1. Background

Artesunate, the water-soluble derivate of artemisinin has been reported to show anti-inflammatory effects in various cell types. Although artesunate is one of the most studied derivatives of artemisinin, a detailed study of its mechanism of action in LPS-activated BV2 microglia has not been conducted.

5.1.1. Artesunate suppressed iNOS mediated nitrite production in LPS-activated BV-2 microglia cells.

Stimulation with LPS (1 µg/ml) led to a marked increase in nitrite production in BV2 microglia (Figure 45 a). However, pre-treatment with artesunate (0.5-4 µM) caused a significant ($p<0.05$) reduction in nitrite production in LPS-activated BV2 cells (Figure 45 a).

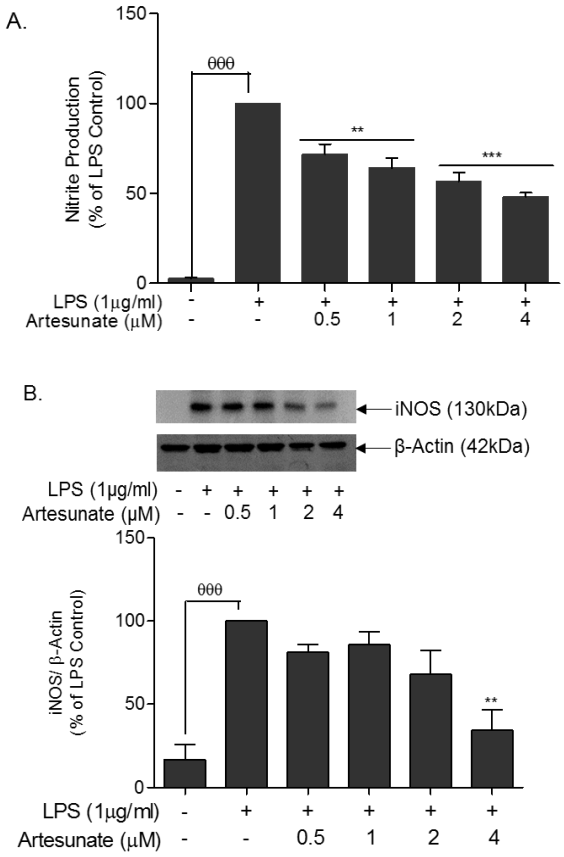

Figure 45: Artesunate inhibited iNOS mediated NO production in LPS-activated BV2 microglia. (A) Artesunate inhibited nitrite production in LPS-activated BV2 cells. Culture supernatants from 24 hour stimulation were analysed for nitrite production. (B) Artesunate attenuated iNOS protein expression in LPS-activated BV2 cells. Whole cell extracts from 24 hour stimulation were analysed for iNOS protein expression. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman-Keuls test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells).

At a concentration of 1 µM, 64 % nitrite was produced in the cells, while 57 % and 48 % nitrite production was measured in LPS-activated BV2 microglia cells treated with 2 and 4 µM of artesunate, respectively. Investigations on iNOS protein levels showed that artesunate (0.5-2 µM) did not significantly inhibit iNOS protein expression (Figure 45 b). However, at 4 µM artesunate was shown to cause a significant decrease in iNOS protein expression, suggesting that artesunate exerted inhibitory actions on iNOS protein at high concentrations.

5.1.2. Artesunate inhibited COX-2 and mPGES-1 mediated PGE_2 production in LPS-stimulated BV2 microglia cells

Experiments showed that LPS stimulation significantly ($p<0.001$) increased PGE_2 production (Figure 46 a). Pre-treatment with 0.5 and 1 µM artesunate did not inhibit PGE_2 production significantly.

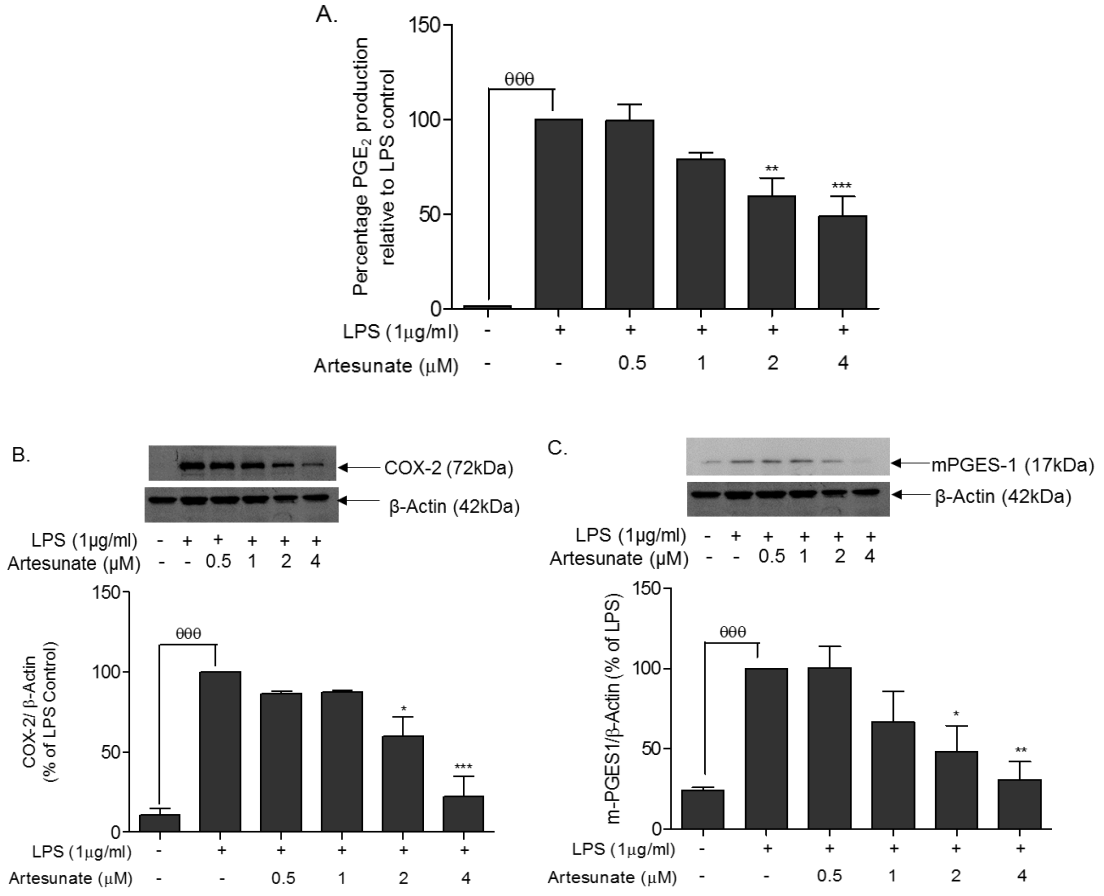

Figure 46: Artesunate inhibited PGE_2 production in LPS-stimulated BV2 microglia cells. BV2 cells were pre-treated with artesunate (0.5-4 µM) for 30 minutes and stimulated with LPS (1 µg/ml) for 24 hours. Cell supernatants were collected and PGE_2 production quantified. Whole cell extracts were analysed for COX-2 and mPGES-1 protein expression (A) Artesunate (2 and 4 µM) inhibited PGE_2 production in LPS-stimulated BV2 microglia cells. (B) Artesunate (2 and 4 µM) suppressed COX-2 protein expression in LPS-activated BV2 microglia cells. (C) Artesunate (2 and 4 µM) suppressed mPGES-1 protein expression in LPS-activated microglia. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for

multiple comparison with post hoc Student Newman-Keuls test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells).

In addition, BV2 cells stimulated with LPS produced marked expression ($p<0.001$) of COX-2 protein. Similar to the observations on PGE_2 production, there was no significant reduction in COX-2 protein expression following pre-treatment with 0.5 and 1 µM artesunate. However, at 2 µM and 4 µM artesunate significantly suppressed COX-2 production (Figure 46 b).

The effect of artesunate on mPGES-1 protein expression in LPS-activated BV2 cells was also investigated (Figure 46 c). At lower concentrations (0.5 and 1 µM), artesunate did not inhibit mPGES-1 protein expression. However, pre-treatment with artesunate (2 and 4 µM) produced significant inhibition of mPGES-1 protein expression (Figure 46 c). From the results obtained it can be inferred that artesunate (2-4 µM) suppressed PGE_2 production in BV2 microglia cells via dual inhibition of COX-2 and mPGES-1 protein expression.

5.1.3. Artesunate inhibited pro-inflammatory cytokines TNFα and IL-6 in LPS-stimulated BV2 microglia cells

TNFα is a potent pro-inflammatory mediator produced during chronic inflammation, thus an attractive target for inhibiting neuroinflammation (McCoy & Tansey, 2008). Previous study on the anti-inflammatory properties of artesunate in experimental colitis by Yang et al (2012) showed that artesunate inhibited the production of pro-inflammatory cytokines including TNFα. In the present study, stimulation with LPS significantly increased TNFα production in LPS-activated BV2 cells (Figure 47 a). At a concentration of 0.5 µM, artesunate did not produce a significant effect on TNFα production in LPS-activated BV2 cells. However, at 1-4 µM, artesunate significantly ($p<0.05$) suppressed TNFα production following LPS stimulation of BV2 cells (Figure 47 a). This observation is important as the presence of this potent inflammatory factor at sites of injury implicates it as a mediator of neuronal damage and disease pathogenesis.

In addition, LPS stimulation of BV2 cells produced a marked increase in IL-6 production (Figure 47 b). At 0.5 µM, artesunate did not decrease IL-6 production significantly. Subsequent increase in concentrations to 1, 2 and 4 µM resulted in 60 %, 51.1 % and 33 % of IL-6 production (Figure 47 b), respectively; suggesting a concentration-dependent relationship.

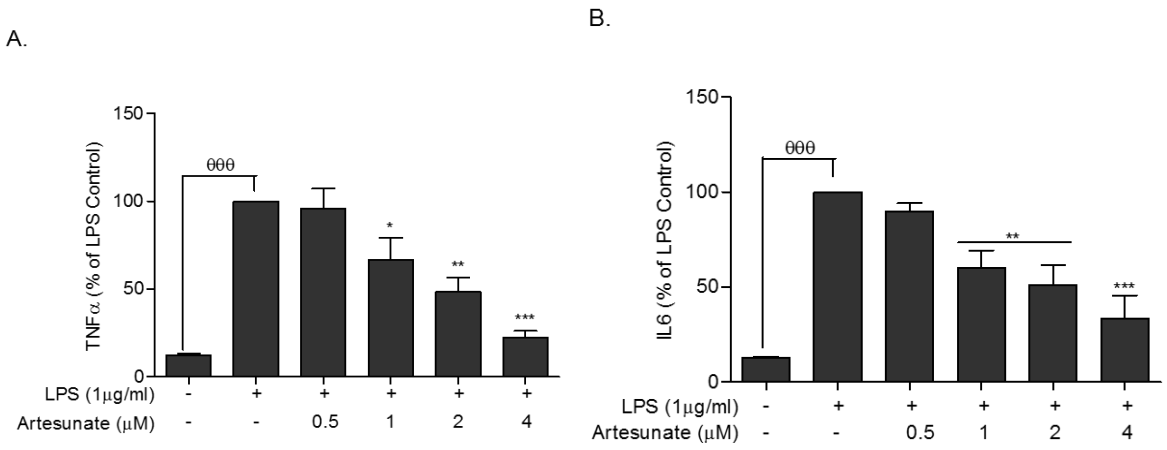

Figure 47: Artesunate suppressed the production of pro-inflammatory cytokines in LPS-activated BV2 cells.
BV2 cells were stimulated with LPS (1 µg/ml) in the presence or absence of artesunate (0.5-4 µM) for 24 hours. Culture supernatants were collected and analysed for TNFα and IL-6 production. (A) Artesunate (1-4 µM) inhibited TNFα production in LPS-activated BV2 microglia cells. (B) Artesunate (1-4 µM) suppressed IL-6 production in LPS-activated BV2 microglia cells. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman-Keuls test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells).

5.1.4. Artesunate inhibits NF-κB signalling in LPS-activated BV-2 microglia cells by targeting IκB/NF-κB signalling

Following the observation that artesunate inhibited iNOS/NO, COX-2/mPGES-1/PGE_2 and pro-inflammatory cytokines (TNFα and IL-6) in LPS-activated BV2 microglia, its effect on NF-κB-mediated gene transcription was investigated. TNFα stimulation of NF-κB-transfected HEK 293 cells resulted in significant activation of NF-κB-driven luciferase expression ($p<0.001$). The result obtained also show that pre-treatment with artesunate (0.5-4 µM) significantly ($p<0.001$) inhibited NF-κB-regulated luciferase gene expression (Figure 48).

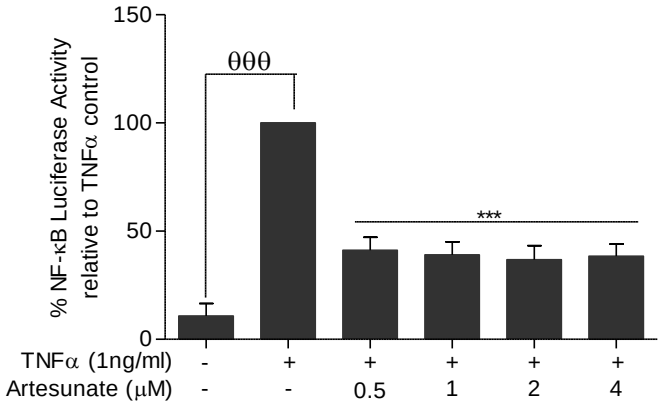

Figure 48: Artesunate (0.5-4 μM) suppressed NF-κB activity in HEK293 cells transfected with NF-κB luciferase construct.
NF-κB transfected HEK-293 cells were stimulated with TNFα (1 ng/ml) in the absence or presence of artesunate (0.5-4 μM) for 6 hours. Luminescence measurement was used as a measure of NF-κB activity. 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. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with TNFα control.

Following an exposure to an inflammatory stimulus (e.g. LPS), phosphorylation and degradation of IκB (an inhibitory protein which sequester inactive NF-κB complexes in the cytoplasm) occur, releasing NF-κB subunits. The inhibitory actions of artesunate on phosphorylation and degradation of IκB-α was therefore investigated. Following LPS activation, there was a marked increase in the phosphorylation of IκB-α (Figure 49 a). Pre-treatment with artesunate (0.5-4 μM) caused a significant and concentration-dependent inhibition of phospho-IκB-α (Figure 49 a). Pre-treatment with artesunate also inhibited IκB-α degradation induced with stimulation of BV2 microglia with LPS. Interestingly, the latter effect was not concentration-dependent and significant inhibition of degradation was observed at 0.5, 1 and 4 μM (Figure 49 a).

The phosphorylation and degradation of IκB is followed by translocation of p65NF-κB subunit to the nucleus. Experiments on nuclear protein expression of p65NF-κB show that pre-treatment with artesunate at 0.5 μM did not affect nuclear levels of phospho-p65NF-κB. However, at 1-4 μM, pre-treatment with artesunate inhibited phospho-p65NF-κB protein expression in a concentration-dependent fashion (Figure 49 b).

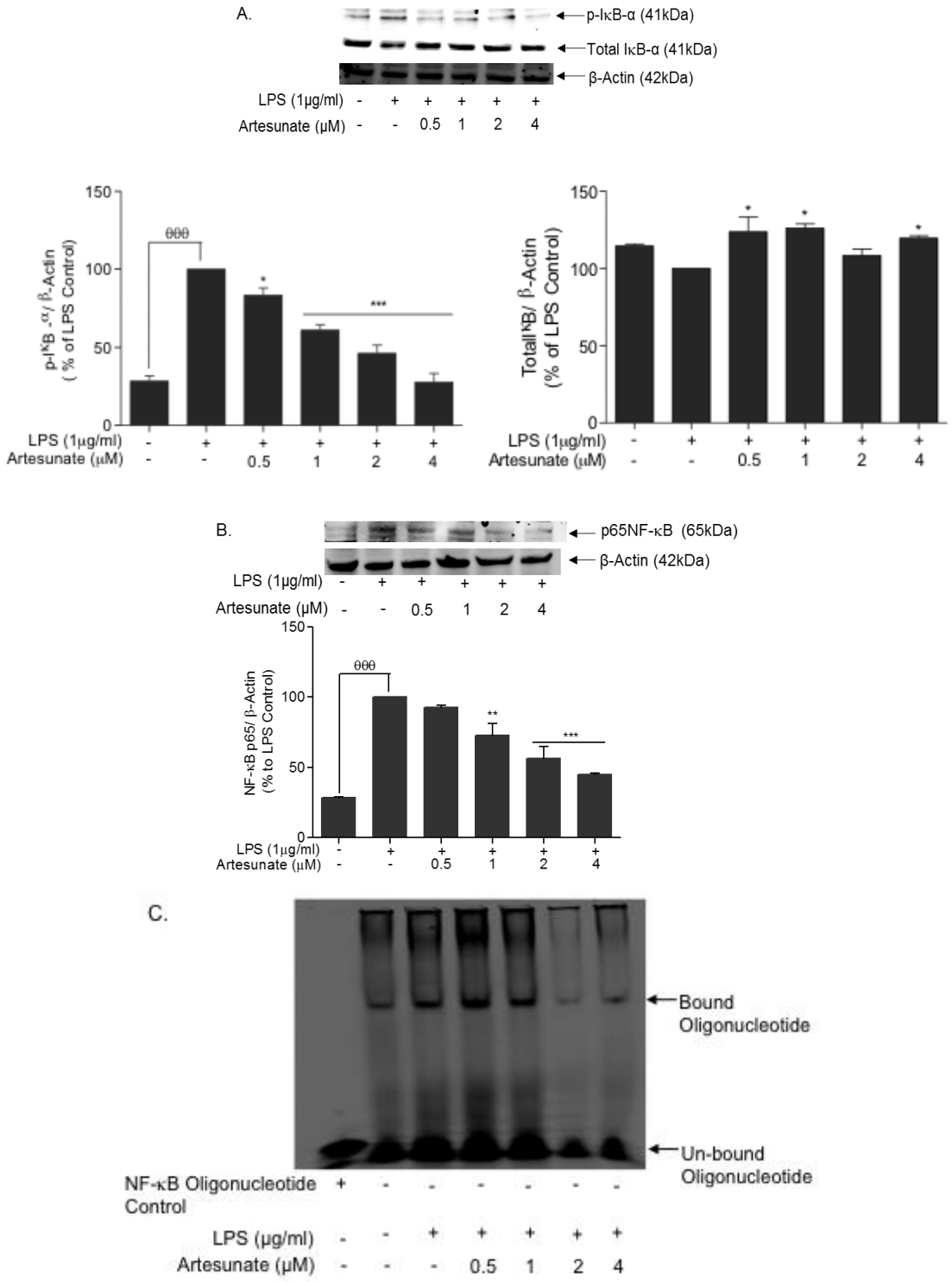

Figure 49: Artesunate inhibited NF-κB signalling in LPS-activated BV2 cells by interfering with IκB/NF-κB.

Whole cell extracts from 1 hour stimulation were analysed for IκB-α. Nuclear extracts were analysed for p65NF-κB phosphorylation and DNA binding. (A) Artesunate suppressed phosphorylation and degradation of IκB-α in LPS-activated BV2 microglia cells. (B) Artesunate (1-4 µM) inhibited p65NF-κB nuclear translocation in LPS-activated BV2 cells. (C) Artesunate inhibited p65NF-κB DNA binding in LPS-activated 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. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells).

Following translocation to the nucleus, p65NF-κB subunit binds to the DNA and regulates the transcription of various genes regulating inflammatory mediators. Figure 49 c shows that artesunate inhibited the binding of p65NF-κB to the DNA in LPS-activated BV2 microglia cells.

To further elucidate the effect of artesunate on NF-κB signalling, its effect on IKK phosphorylation in LPS-activated BV2 cells was evaluated. Results obtained show that there was no significant inhibition of IKK phosphorylation by artesunate (Figure 50).

Taken together the results obtained, it can be inferred that artesunate may have inhibited iNOS/NO, COX-2/mPGES-1/PGE_2 and pro-inflammatory cytokines by inhibiting IκB/NF- κB signalling in LPS-activated BV2 cells.

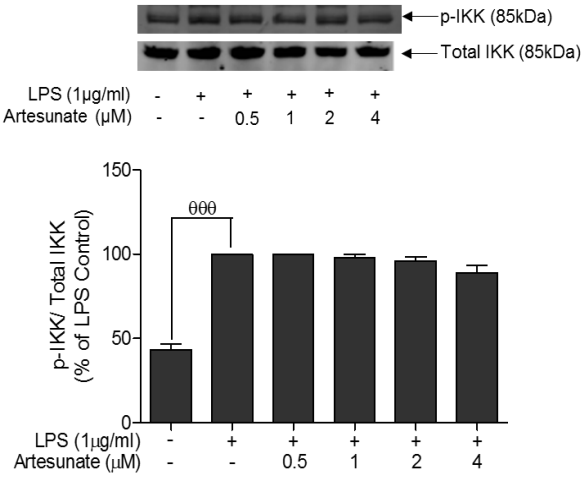

Figure 50: Artesunate did not inhibit phosphorylation of IKK in LPS-activated BV2 cells. BV2 microglia cells were stimulated with LPS (1 µg/ml) for 30 minutes in the absence or presence of artesunate (0.5-4 µM). Whole cell extracts were collected and analysed for p-IKKα and total IKK protein expressions using western blot. 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. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells).

5.1.6. Anti-neuroinflammatory activity of artesunate is mediated partly via suppression of phosphorylation of p38 signalling.

p38MAPK cascade has been shown to contribute to the transcriptional and post-translational regulation of iNOS and TNFα in LPS activated glial cells (Bhat, et al., 1998). Artesunate has been shown in this research to inhibit iNOS/NO, pro-inflammatory cytokines TNFα and IL-6. Therefore, its effect on phospho-p38MAPK protein expression was investigated. Results obtained showed that there was a marked increase in the expression of phopho-p38 protein in BV2 cells following stimulation with LPS (1 µg/ml) (Figure 51). The result also shows that

pre-treatment with artesunate (0.5-4 µM) significantly ($p<0.05$) inhibited protein expression of phospho-p38 (Figure 51).

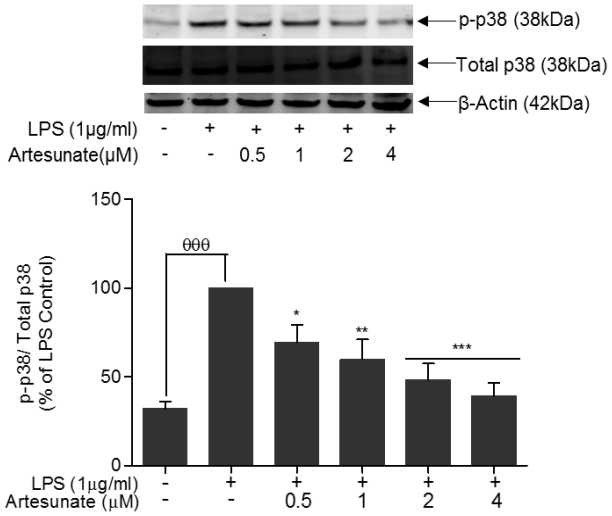

Figure 51: Artesunate inhibited phospho-p38 protein expression in LPS-activated BV2 microglia cells. BV2 microglia cells were pre-treated with artesunate (0.5-4 µM) for 30 minutes and then activated by LPS (1 µg/ml) for 1 hour. Whole cell extracts were obtained and analysed for phospho-p38 and total p38 protein with anti-phospho-p38 and anti-total p38 antibody using western blot. All values are expressed as mean ± SEM for 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 LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells).

The activation of the MKK3/6 represents one distinct pathway through which p38MAPK is activated. Hence, the effect of artesunate on phospho-MKK3/6 protein was also investigated. Figure 5.1.6 a shows that there was a marked increase ($p<0.001$) in protein expression of phospho-MKK3/6 in BV2 cells stimulated with LPS (1 µg/ml). At 0.5 and 1 µM artesunate did not show any significant inhibition of phospho-MKK3/6 protein expression. However, at 2µM and 4µM artesunate significantly inhibited phospho-MKK3/6 (Figure 52 a).

Following the phosphorylation of p38MAPK, p38MAPK activate transcription factors by acting on MAPKAPK2. The effect of artesunate (0.5-4 µM) on phospho-MAPKAPK2 was investigated in LPS-activated BV2 microglia. Figure 52 b shows that phospho-MAPKAPK2 protein expression was significantly increased following stimulation with LPS (1 µg/ml). Pre-treatment with 1, 2 and 4 µM artesunate resulted in 69.3 %, 55.2 % and 41.3 % phospho-MAPKAPK2 respectively when compared with LPS control (Figure 52 b).

From the results obtained from the study of the effect of artesunate (0.5-4 µM) on p38MAPK signalling it can be inferred that artesunate suppressed p38MAPK signalling through inhibition of MKK3/6 phosphorylation.

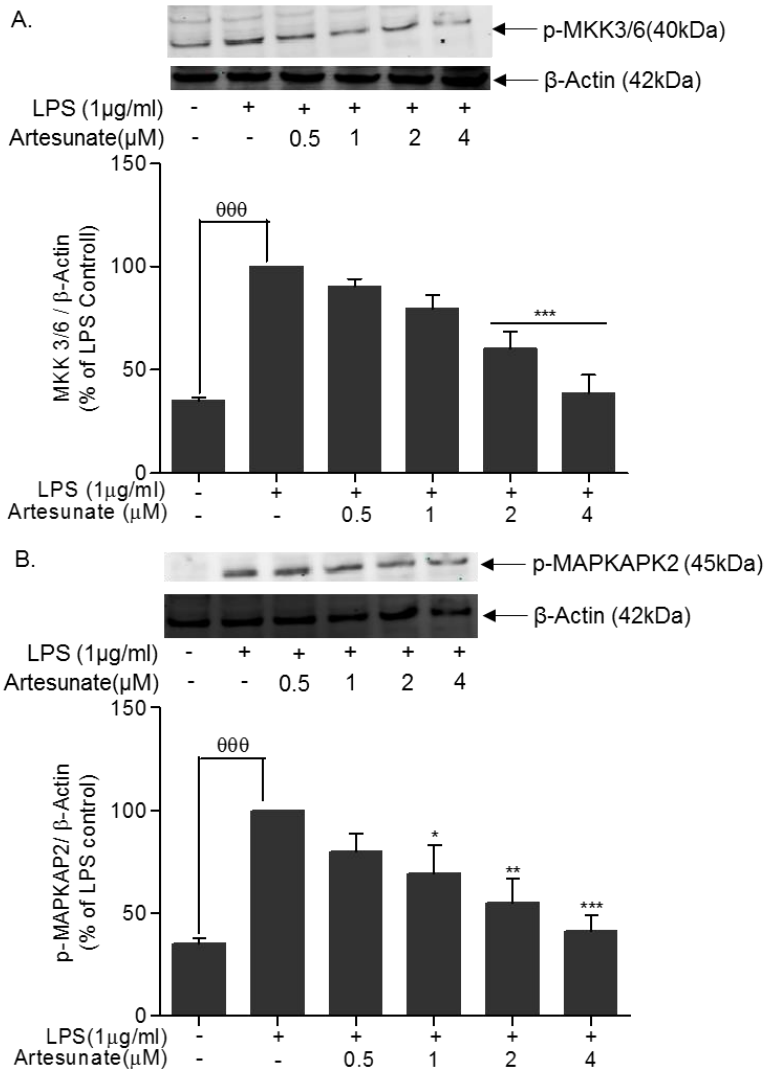

Figure 52: Artesunate inhibited phospho-MKK3/6 and phospho-MAPKAPK2 in LPS-activated BV2 microglia cells.
(A) Artesunate inhibited phospho-MKK3/6 protein expression in LPS-activated BV2 microglia cells. (B) Artesunate suppressed phospho-MAPKAP2 protein expression in LPS-activated BV2 microglia cells. All values are expressed as mean ± SEM for 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 LPS control. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with negative control (untreated cells).

5.2. Artesunate activated Nrf2/HO-1 antioxidant protective system in BV2 microglia.

First, the effect of artesunate on HO-1 protein expression was investigated. In control cells, basal level of HO-1 expression was observed. However, treatment with artesunate (4 µM) significantly ($p<0.001$) increased the expression of HO-1 protein in BV2 microglia cells, when compared with untreated control (Figure 53).

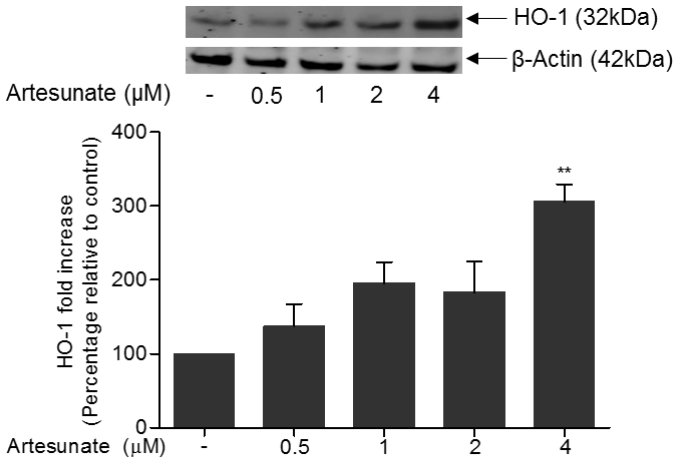

Figure 53: Artesunate (4 μM) activated HO-1 in BV2 microglia cells.
BV2 cells were treated with artesunate (0.5-4 μM) for 24 hours. Whole cell extracts were collected and analysed for HO-1 protein expression using western blotting. All values are expressed as mean ± SEM for at least 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with control.

Next, the effect of the artesunate (0.5-4 μM) on ARE activation was investigated by treating ARE transfected HEK 293 cells with artesunate (0.5-4 μM) for 8 hours. Results obtained show that artesunate increased ARE luciferase activity in a concentration-dependent fashion (Figure 54 a). This result suggests that artesunate may be able to activate Nrf2 antioxidant pathway. ARE genes are activated by the transcription factor Nrf2. This result also suggests that artesunate may have effects on Nrf2 activity.

Following the observation that artesunate activated ARE luciferase activity in transfected HEK 293 cells, the effect of artesunate (4 μM) on nuclear Nrf2 protein expression was investigated in BV2 cells for 15, 30, 60, 120 and 180 minutes. Experimental analysis of nuclear extracts obtained showed that artesunate (4 μM) activated Nrf2 after 15, 30, 120 and 180 minutes of treatment (Figure 54 b). However, the most significant activation was observed at 180 minutes.

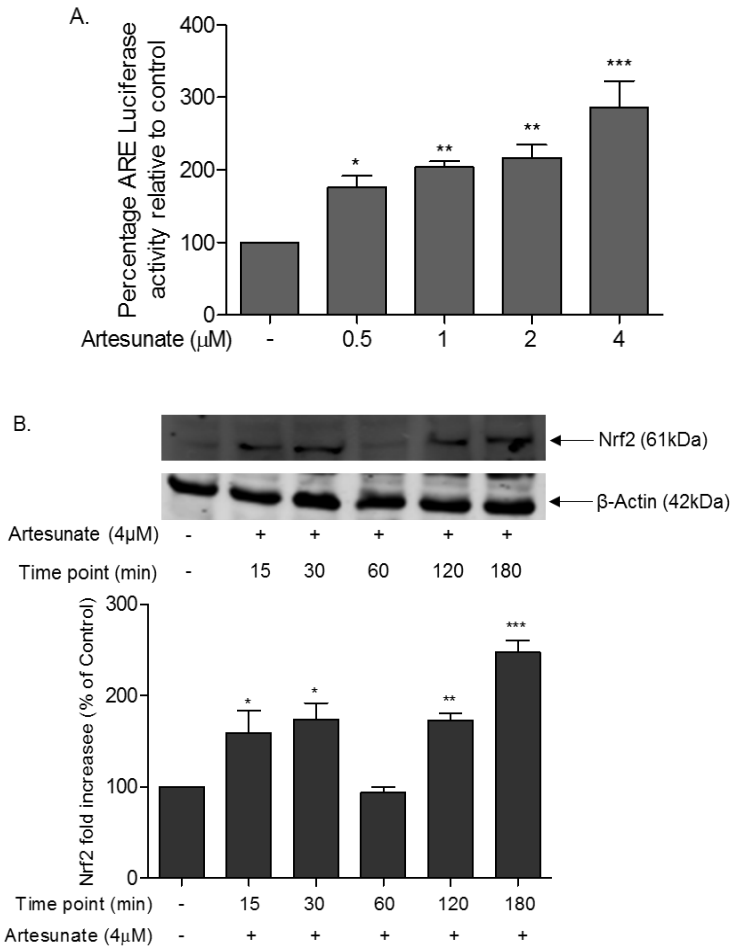

Figure 54: Artesunate activated ARE luciferase activity in HEK cells and Nrf2 protein expression in BV2 microglia cells.
(A) Artesunate activated ARE luciferase activity in HEK 293 cells transfected with ARE construct. (B) Artesunate activated Nrf2 in BV2 cells. BV2 cells were treated with artesunate (4 μM) for 15, 30, 60, 120 and 180 minutes. Nuclear extracts were analysed for Nrf2 protein expression using western blotting. 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. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with control

5.3.1. Inhibition of nitric oxide production in LPS-activated BV2 cells by artesunate is independent of the Nrf2 signalling pathways.

Nrf2-silenced BV2 cells were pre-treated with artesunate (4 μM) for 30 minutes prior to LPS stimulation for 24 hours. Subsequently, nitrite production was investigated. Results obtained show that Nrf2 silencing in BV2 microglia significantly ($p<0.001$) enhanced nitrite production following LPS stimulation (Figure 55). Pre-treatment with artesunate (4 μM) did not reverse the inhibitory effect of artesunate on nitrite production in Nrf2 knockout cells, suggesting that the inhibitory effects of artesunate on nitrite production was independent of Nrf2 activity.

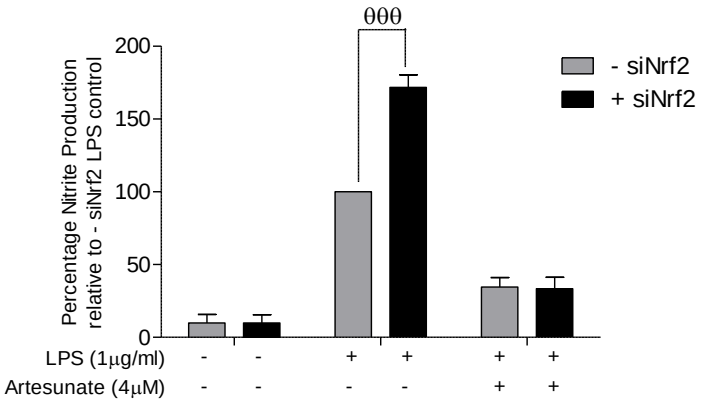

Figure 55: Inhibition of NO production in LPS-activated BV2 cells by artesunate is independent of Nrf2 activity.
BV2 cells transfected with Nrf2 siRNA were stimulated with LPS for 24 hours following pre-treatment with artesunate (4 μM). Subsequently, NO production was measured. All values are expressed 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. $^{\theta}p<0.05$, $^{\theta\theta\theta}p<0.001$ in comparison with –siNrf2 LPS control.

5.3.2. Inhibition of TNFα and IL-6 production in LPS-stimulated BV2 cells by artesunate is dependent on Nrf2.

Stimulation of Nrf2-silenced BV2 cells with LPS enhanced TNFα production (Figure 56 a). Pre-treatment with artesunate (4 μM) significantly ($p<0.001$) reversed the inhibitory actions of artesunate following LPS stimulation.

The effect of Nrf2 silencing on the inhibitory actions of artesunate on IL-6 production was also investigated. Pre-treatment with artesunate (4 μM) significantly ($p<0.001$) reversed the inhibitory actions of artesunate on IL-6 production in Nrf2 knockout cells (Figure 56 b).

It therefore appears that the inhibitory effects of artesunate on TNFα and IL-6 production in LPS-activated BV2 cells were dependent on the Nrf2 activity.

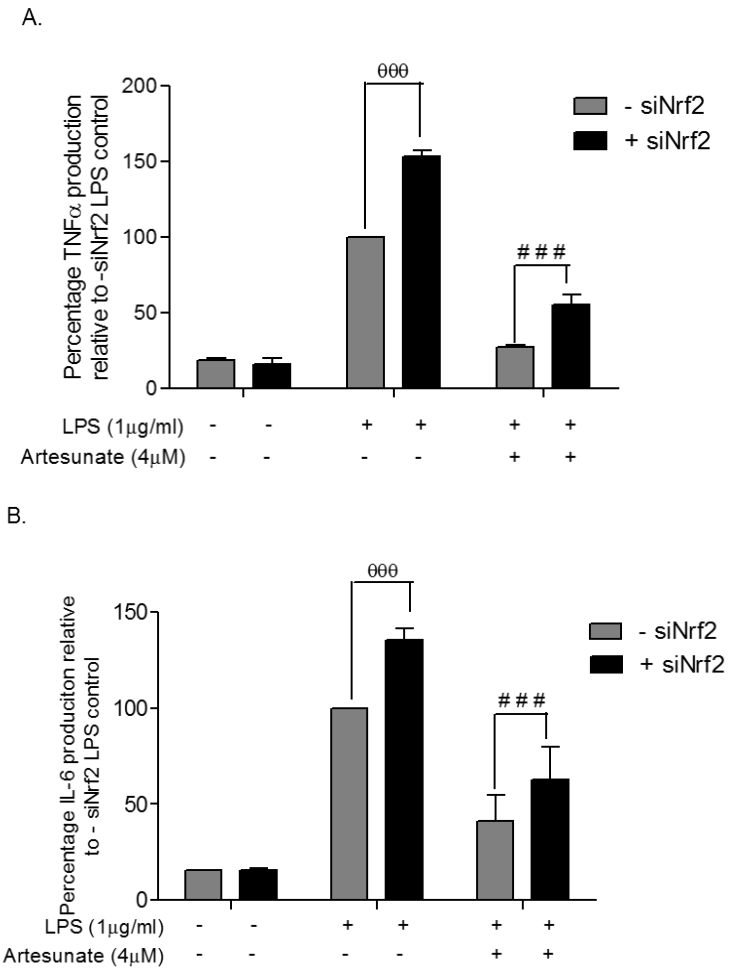

Figure 56: Inhibition of pro-inflammatory cytokines TNFα and IL-6 in LPS activated BV2 microglia cells by artesunate is dependent on Nrf2 activity.
BV2 cells were transfected with Nrf2 siRNA, treated with artesunate (4 µM) for 30 minutes and stimulated with LPS (1 µg/ml) for 24 hours. Thereafter, culture supernatants were analysed using ELISA. (A) Inhibitory effect of artesunate on TNFα production in LPS-stimulated BV2 cell is dependent on Nrf2 activity. (B) Inhibitory action of artesunate on LPS-activated BV2 cell is dependent on Nrf2 activity. 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. $^{\theta}p<0.05$, $^{\theta\theta\theta}p< 0.001$ in comparison with -siNrf2 LPS control. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ in comparison with -siNrf2 4 µM artesunate treatment.

5.3.3. Inhibition of PGE_2 production in LPS-activated BV2 cells by artesunate is dependent on Nrf2 activity.

Nrf2-silenced BV2 cells were stimulated with LPS (1 µg/ml) for 24 hours following pre-treatment with artesunate. Culture supernatants were collected and analysed for PGE_2 production. Figure 57 show that Nrf2 knockout significantly ($p<0.001$) enhanced PGE_2 production following LPS stimulation. However, pre-treatment of Nrf2 knockout cells with artesunate (4 µM) significantly reversed the inhibitory actions of artesunate on PGE_2

production; suggesting that the inhibitory actions of artesunate on PGE_2 production is dependent on Nrf2 activity.

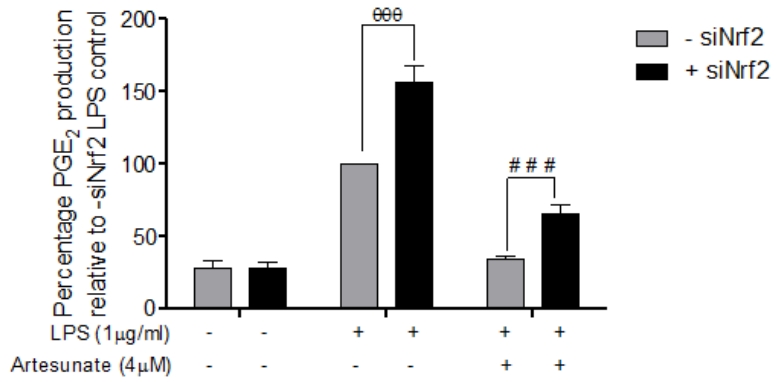

Figure 57: Inhibition of PGE_2 production by artesunate in LPS-activated BV2 cells is dependent on Nrf2 activity.
Nrf2 knockout BV2 microglia cells were stimulated with LPS (1 µg/ml) for 24 hours following pre-treatment with artesunate (4 µM). Thereafter, culture supernatants were collected and analysed for PGE_2 production using PGE_2 EIA Assay. 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<0.001 in comparison with negative control. $^{\theta}$p<0.05, $^{\theta\theta\theta}$p< 0.001 in comparison with -siNrf2 LPS control. $^{\#}$p<0.05, $^{\#\#\#}$p<0.001 in comparison with -siNrf2 4 µM artesunate treatment.

5.4. Artesunate (0.5-4 µM) did not affect cell viability

To ascertain that artesunate (0.5-4 µM) did not affect cell viability, BV2 cells were stimulated for 24 hours in the presence or absence of artesunate (0.5-4 µM). Subsequently, MTT assay was carried out on the cells. Results obtained (Figure 58) show that artesunate did not affect cell viability in BV2 microglia cells.

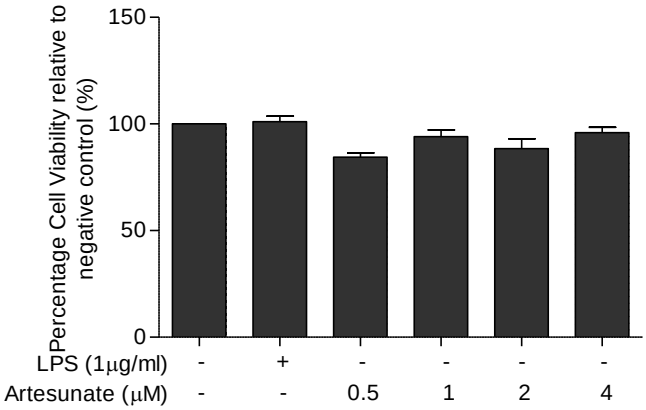

Figure 58: Artesunate did not affect cell viability of BV2 microglia cells.

BV2 cells were pre-treated with artesunate (0.5-4 µM) for 30 minutes and subsequently stimulated with LPS (1 µg/ml) for 24 hours. Thereafter, MTT viability assay was performed. 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.

5.5. Discussion

Concentrations used for study was determined following MTT assay. Result obtained showed that artesunate did not affect cell viability. Therefore, the possible anti-neuroinflammatory effects of artesunate were investigated.

Elevated levels of NO production within the CNS are associated with the pathogenesis of neuroinflammatory diseases. Glial cells express inducible iNOS and produce a high level of NO in response to a pro-inflammatory stimulus suggesting the need to inhibit iNOS/NO during neuroinflammation. In this study artesunate inhibited NO production in LPS-activated BV2 microglia as well as iNOS protein. In a study by Konkimalla et al (2008), artesunate was reported to inhibit nitric oxide-related signalling pathway in RAW 264.7 mouse macrophages. In another study by Xu et al (2007), artesunate also inhibited NO/iNOS in human rheumatoid arthritis fibroblast-like synoviocytes following TNFα stimulation. In a closely related study by Lee et al (2012), artesunate was also reported to inhibit iNOS mediated nitrite production. These reports are consistent with the outcome of this study, which showed that artesunate suppressed NO production via inhibition of iNOS protein expression.

Artesunate has also been shown to inhibit PGE_2 production in LPS-activated BV2 microglia cells. In addition, artesunate inhibited COX-2 and mPGES-1 protein levels in LPS activated BV2 cells. Recent studies on LPS/IFNγ activated BV2 cells have also shown that artesunate inhibited PGE_2/COX-2 (Okorji & Olajide, 2013). Lee et al (2012) also reported that artesunate inhibited COX-2 in LPS activated BV2 cells. It therefore follows that in LPS-activated microglia, artesunate blocks PGE_2 production via dual inhibition of COX-2 and mPGES-1 protein expression. It is important to highlight that this is the first time artesunate is shown to inhibit COX-2/mPGES1 mediated PGE_2 production in LPS activated BV2 microglia.

Pro-inflammatory cytokines are known to play a crucial role in neuronal cell death following excessive production by activated microglia cells. In this study, experiments on pro-inflammatory cytokines demonstrate that artesunate significantly reduced the increased production of TNFα and IL-6 following stimulation of BV2 microglia with LPS. Previous study on the anti-inflammatory properties of artesunate in experimental colitis by Yang et al (2012) shows that artesunate inhibited the production of pro-inflammatory cytokines. Studies by Lee et al (2012) showed that artesunate inhibited another pro-inflammatory cytokine IL-1β. These reports are consistent with the outcome of this study, which showed that artesunate may possess inhibitory actions against pro-inflammatory mediators in neuroinflammation

To further understand the molecular mechanism (s) involved in the inhibition of NO/iNOS, PGE_2/COX-2/mPGES-1 and the pro-inflammatory cytokines (TNFα and IL-6), the effect of artesunate on the NF-κB signalling was investigated. Artesunate inhibited NF-κB luciferase activity in HEK 293 cells suggesting that artesunate may possess inhibitory effects on NF-κB signalling. Further experiments showed that artesunate produced inhibitory actions on the phosphorylation and degradation of IκB. Recent study by Okorji and Olajide (2013) also showed that artesunate inhibited IκB phosphorylation in LPS/IFNγ activated BV2 microglia cells. Artesunate also inhibited nuclear translocation of NF-κB subunit. It is therefore proposed that artesunate might be inhibiting neuroinflammation by targeting NF-κB signalling.

p38 MAPKs have been shown to be crucial signal transduction pathways that contribute to glia-induced neuronal death. It has also been suggested to play an important role in the expression and activity of pro-inflammatory cytokines. Therefore, the effect of artesunate on p38MAPK signalling in LPS-activated BV2 cells was explored. Results obtained show that artesunate inhibited p38MAPK signalling by blocking the phosphorylation of MKK3/6, a kinase that regulates p38MAPK phosphorylation. Artesunate also inhibited the phosphorylation of p38MAPK substrate, MAPKAPK2. The results obtained show that the inhibition of p38MAPK signalling by artesunate is through the suppression of MKK3/6. It therefore suggests that artesunate exerts its anti-neuroinflammatory effects partly through inhibition of p38MAPK signalling. This is the first time artesunate is shown to block p38MAPK signalling following LPS activation of BV2 microglia.

Further to the observations that artesunate inhibits neuroinflammation in BV2 cells by interfering with NF-κB and p38MAPK signalling, its effect on Nrf2 mediated HO-1 activity was investigated. Artesunate activated HO-1 expression in BV2 microglia cells. In addition, artesunate activated Nrf2 protein expression, suggesting that artesunate activate Nrf2 mediated HO-1 production. Experimental investigations on ARE activity, suggests that artesunate activates the general activity of ARE. Previously, Lee et al. (2012) showed that artesunate activates Nrf2-ARE system. This report confirms the findings of this study that artesunate activates Nrf2 mediated HO-1 production and ARE activity.

Previous studies have suggested that the activation of Nrf2 by compounds is correlated with the inhibition of iNOS and COX-2 directly (Baird & Dinkova-Kostova, 2011). Therefore the effect of Nrf2 knockout on the inhibitory actions of artesunate on pro-inflammatory mediators was explored. Inhibition of nitric oxide in LPS-activated BV2 cells was shown to be independent of Nrf2 activity. However, the inhibitory actions of artesunate on TNFα, IL-6 and PGE_2 production in LPS-activated BV2 cells are dependent on Nrf2 activity. Therefore, the

inhibitory actions of artesunate on pro-inflammatory mediators may be partly dependent on Nrf2 activity. It is important to highlight that this is the first time artesunate inhibitory actions against TNFα, IL-6 and PGE_2 is dependent on Nrf2 activity.

In summary, artesunate has been shown in these experiments to exert anti-neuroinflammatory properties by interfering with NF-κB and p38MAPK signalling. Artesunate also activate Nrf2 mediated HO-1 expression in BV2 microglia cells.

CHAPTER 6

GENERAL DISCUSSION

6. General Discussion

6.1. Discussion

Neuroinflammation has been implicated in the initiation and/or progression of some neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis and Multiple sclerosis. Previously, artemisinin a sesquiterpene lactone and its derivatives artemether and artesunate have been reported to have promising anti-inflammatory activity in various cell types and models of inflammation (Ho et al., 2014; Pahl, 1999, Wu, 2011). However, a detailed investigation of these compounds in LPS-induced neuroinflammation in BV2 microglia cells had not been conducted. In this study, artemisinin and its derivatives artemether and artesunate have been studied independently of each other.

Aberrant iNOS induction influences the pathophysiology of several neurological diseases, leading to detrimental consequences. For example, toxicity in neurological diseases can occur through the overproduction of NO, which leads to the production of toxic reactive nitrogen species (RNS), especially $ONOO^-$ (Ghasemi & Fatemi, 2014). In addition, iNOS-derived NO regulates prostaglandin formation by COX-2 through the protein cysteine residues (S-nitrosylation) (Ghasemi & Fatemi, 2014; Kim, 2011; Kim et al., 2005). These suggest the importance of modulation of NO/iNOS production in chronic neuroinflammation.

In this study, it has been demonstrated that artemisinin, artemether and artesunate inhibit iNOS mediated NO production in LPS-activated BV2 microglia. Previous studies have reported the inhibition of NO/iNOS production by these compounds. In a study on RAW 264.7 macrophages, an artemisinin derivative SM905 was reported to profoundly inhibit NO production following activation with LPS (Wang et al., 2009). In another study, artemisinin was reported to inhibit NO/iNOS production in LPS activated primary microglia (Zhu et al., 2012).

Pro-inflammatory cytokines have been reported to play vital roles in the initiation and/or progression of various CNS neuroinflammatory conditions. Excessive production of these pro-inflammatory cytokines exacerbates deleterious effects in the CNS, which subsequently result in neuronal death and chronicity of the disease. TNFα produced during neuroinflammation plays a central role in initiating and regulating cytokine cascade during an inflammatory response (Rubio-Perez & Morillas-Ruiz, 2012). In addition, its production during neuroinflammation leads to toxicity in neurons (Olmos & Llado, 2014). IL-6 production during neuroinflammation has also been reported to play a role in neurodegeneration and cognitive impairment (Rojo et al., 2008). Hence, modulation of these cytokines proves to be a major contributing factor in suppressing neuroinflammation. Inhibition of pro-inflammatory

cytokines in microglia has been shown to attenuate the severity of Alzheimer's disease, Parkinson's disease, trauma, multiple sclerosis and cerebral ischemia (Koning et al., 2007; Krause & Muller, 2010; Qian et al., 2010).

Artemisinin, artemether and artesunate blocked the production of the pro-inflammatory cytokines; TNFα and IL-6 production in LPS activated BV2 cells. Previously, artemisinin has been shown to suppress TNFα and IL-6 production upon stimulation with LPS in primary microglia cells (Zhu et al., 2012), thus confirming the results from this research Artesunate has also been reported to inhibit TNFα-induced production of pro-inflammatory cytokines in human rheumatoid arthritis fibroblast-like synoviocytes (Xu et al., 2007). The outcome of these previous studies and the current research seem to suggest that artemisinin and derivatives are able to inhibit production of pro-inflammatory cytokines in various cells and in diverse inflammatory responses.

This study has shown that artemisinin, artemether and artesunate reduced PGE_2 production in LPS-activated BV2 cells. PGE_2 produced during neuroinflammation act on EP receptors expressed in the microglia and neurons. Activation of microglial EP2 receptors enhances neurotoxic activities (Schlachetzki et al., 2010). Therefore, inhibition of PGE_2 generated during neuroinflammation is important. Previously, artesunate has been reported to exert an anti-immunosuppressive effect in part on cervical cancer by inhibiting PGE_2 production (Zhang et al., 2014). Artemisinin has also been shown to inhibit PGE_2 production in TNFα induced vascular smooth muscle cells (Cao et al., 2014). These studies confirm the inhibitory effects of artemisinin, artemether and artesunate on PGE_2 production following stimulation.

Artemisinin, artemether and artesunate also inhibited COX-2 protein expression. This infers that the compounds inhibit PGE_2 production through inhibition of COX-2 in LPS activated microglia cells. COX-2 enzyme catalyses the rate limiting step in the formation of prostaglandins from arachidonic acid. Therefore, its regulation is crucial in the control of prostaglandin synthesis. Previous studies have shown that artesunate inhibited COX-2 protein expression in activated BV2 microglia cells (Okorji & Olajide, 2014). The study reported by Okorji & Olajide also suggested that artesunate inhibited PGE_2 production through inhibition of COX-2.

mPGES1 is an inducible terminal enzyme for PGE_2 biosynthesis (de Oliveira et al., 2008; Kudo & Murakami, 2005). In addition mPGES-1 is functionally coupled with COX-2 (de Oliveira et al., 2008; Lazarus et al., 2002). In this research, artemisinin, artemether and artesunate were shown to inhibit mPGES-1 protein expression. This suggests that these

compounds inhibit PGE_2 production via dual inhibition of COX-2 and mPGES-1 in LPS activated BV2 cells.

NF-κB is a ubiquitous transcription factor which regulates the expression of many inflammation-related genes, including TNFα, IL-6, COX-2 and iNOS. The blockage of NF-κB transcriptional activity can therefore suppress the expression of these mediators (Li & Verma, 2002; Olajide et al., 2007; Olajide et al., 2014). Results from this study have demonstrated that artemisinin; artemether and artesunate suppressed TNFα, IL-6, nitric oxide, iNOS and COX-2. Consequently, it was decided to investigate whether the inhibitory actions of the compounds were due to interference with NF-κB signalling.

In response to an inflammatory stimulus, IκB is phosphorylated and degraded; p65NF-κB is then released and subsequently translocate to the nucleus (Kanaarek & Ben-Neriah, 2012). Artemisinin, artemether and artesunate significantly inhibited the phosphorylation and degradation of IκB in LPS-activated BV2 microglia cells. Artemisinin has been reported to inhibit IκB phosphorylation and degradation in PMA-induced THP-1 monocytes (Wang et al., 2011). Recent study by Okorji and Olajide (2013) also showed that artesunate inhibited IκB phosphorylation in LPS/IFNγ activated BV2 microglia cells. Wang et al. (2009) also reported that a derivative of artemisinin, SM905, inhibited IκB phosphorylation and degradation in LPS activated macrophages. These reports are consistent with the outcome of the current study, and suggest that these compounds are potent inhibitors of IκB phosphorylation and degradation. Artemisinin is a sesquiterpene lactone. Several reports have demonstrated that this group of natural products are potent inhibitors IκB phosphorylation and degradation in several cell types (Zingarelli et al., 2002; Siedle et al. 2004; Shin et al., 2005; Nam et al., 2015). Specifically, Hehner et al. (1998) have suggested that the isoprenoide ring system and a lactone ring are the two structural features of sesquiterpene lactones, which confer inhibitory activity on NF-κB activation pathways. These evidences might explain the observed effects of artemisinin and its derivatives on IκB phosphorylation and degradation following LPS stimulation in BV2 microglia.

Degradation of IκB is initiated through phosphorylation by IκB kinase (IKK) complex. However, artemisinin, artemether and artesunate did not inhibit IKK phosphorylation in LPS-activated BV2 microglia cells. This suggests these compounds might be acting directly by interfering with IκB or there might be an involvement of another signalling node(s) that might be involved in the inhibition of IκB phosphorylation and degradation. For example, protein kinase C (PKC) and Ca^{2+}–dependent signal transduction pathways can regulate IκB phosphorylation (Steffan et al., 1995). Steffan et al (1995) showed that Ca^{2+}-dependent pathways, including the phosphatase calcineurin, participate in the regulation of NF-κB in a

cell specific fashion and synergizes with PKC-dependent and independent pathways at the level of IκBα phosphorylation and degradation.

Phosphorylation and degradation of IκB results in nuclear translocation of p65NF-κB subunits, followed by binding to the DNA, and regulation of transcriptional activity of pro-inflammatory mediators including COX-2, iNOS and pro-inflammatory cytokines. This study shows that artemisinin, artemether and artesunate inhibited nuclear translocation of p65 NF-κB. Artemisinin, artemether and artesunate also inhibit DNA binding. It appears that artemisinin, artemether and artesunate interfere with NF-κB signalling by inhibiting IκB phosphorylation and degradation, as well as NF-κB binding in LPS activated BV2 cells.

Studies by Xu et al. (2007) have suggested that artemisinin and its derivatives may exert anti-inflammatory and immunoregulatory effects by inhibiting NF-κB activation. In addition, Habtemariam (2000) reported that natural products with anti-inflammatory actions inhibit inflammation by interfering with NF-κB signalling and subsequent inhibition of transcriptional activation of TNF gene. In RAW 264.7 macrophages, an artemisinin derivative, SM905, inhibited pro-inflammatory NO and pro-inflammatory cytokine production in part by suppressing nuclear translocation of NF-κB subunit (Wang et al., 2009). Another study reported that artemisinin inhibited neuroinflammation in APPswe/PS1dE9 transgenic mice, which was attributable in part to inhibition of NF-κB by inhibiting nuclear p65NF-κB translocation (Shi et al., 2013). Shi et al (2013) also showed that artemisinin inhibited IκB phosphorylation in APPswe/PS1dE9 transgenic mice. In addition, artesunate has been shown to inhibit PGE_2 production in LPS/IFNγ-activated BV2 microglia in part via inhibition of NF-κB (Okorji & Olajide, 2014). Consistent with the results from this research, in primary microglia, artemisinin was reported to inhibit NF-κB translocation and DNA binding following activation with LPS. These evidences confirm that artemisinin, artemether and artesunate inhibit neuroinflammation through interference with NF-κB signalling.

In microglia-driven neuroinflammation, p38MAPK cascade has been shown to contribute to the transcriptional and post-translational regulation of iNOS and TNFα gene expression in LPS activated glial cells (Bhat et al., 1998). Furthermore, the p38MAPK is known to regulate pro-inflammatory cytokine production in degenerative diseases of the CNS (Bachstetter et al., 2011). In primary rat and human microglia, LPS stimulated p38MAPK signalling mediated production of NO and TNFα (Lee et al., 2010; Munoz & Ammit, 2010). Therefore, the effects of artemisinin, artemether and artesunate on p38MAPK signalling were investigated in LPS-activated BV2 microglia. Results show that the three compounds prevented phosphorylation of p38MAPK following activation of BV2 cells with LPS.

LPS stimulation of microglia is known to activate p38MAPK-signalling pathway, resulting in phosphorylation of p38 by MKK3/6. Interestingly, artemisinin, artemether and artesunate blocked phosphorylation of MKK3/6, suggesting that these compounds affect p38 activity by targeting these upstream kinases.

Activated p38 is known to further activate transcription factors by acting on its substrate, MAPKAPK2 (Bachstetter & Eldik, 2010). Therefore in order to gain a better understanding of the mechanisms involved in the targeting of p38MAPK signalling by the compounds, they were investigated for possible inhibitory effects on MAPKAPK2 phosphorylation following LPS stimulation in BV2 microglia. Artemisinin, artesunate and artemether showed a similar trend in suppressing the increased phospho-MAPKAPK2 protein expression induced by LPS in BV2 microglia. These observations suggest that the three compounds target p38MAPK signalling by blocking MKK3/6 phosphorylation. This outcome provides the first evidence demonstrating that artemisinin and its derivatives artemether and artesunate inhibited neuroinflammation in LPS-activated BV2 microglia cells by targeting p38 signalling.

Nrf2 activity has been reported to be relevant in down-regulation of neuroinflammation by restoring homeostasis (Innamorato et al., 2008; Rojo et al., 2014). Upon activation, Nrf2 binds to ARE sites in promoter regions of genes encoding phase II detoxifying and antioxidant enzymes, such as HO-1. HO-1 activity is implicated in the modulation of inflammatory responses. Recent studies have shown that up-regulation of HO-1 blocks the expression of iNOS and COX-2, consequently suppressing NO and PGE_2 production (Dilshara et al., 2014; Surh et al., 2009). In addition, pharmacological induction of HO-1 has been shown to suppress inflammation-related brain injuries (Lee et al., 2012; Pamplona et al., 2007). Consequently, it was sought to determine if artemisinin, artesunate and artemether could modulate HO-1 expression in BV2 microglia. Interestingly, all the compounds showed a similar profile by causing an increase in the expression of HO-1, suggesting that activation of this enzyme might contribute to their anti-neuroinflammatory activity.

Nrf2 tightly controls HO-1 expression via direct binding of Nrf2 to AREs. Consequently, the effects of artemisinin, artemether and artesunate were investigated in BV2 microglia cell. These compounds activated HO-1 protein expression in BV2 cells. Consistent with some of the results of this study, Lee et al (2012) showed that artesunate increased the levels of HO-1 protein in BV2 microglia cells.

Nrf2 is a pivotal regulator of endogenous defence systems that function via the activation of a set of protective genes, including HO-1. Therefore, the effects of artemisinin, artemether and artesunate on nuclear expression of Nrf2 were investigated in BV2 microglia cells. The

compounds produced a consistent activation of nuclear Nrf2. It is important to note that each of the compound activated Nrf2 at different time points. For example, artemisinin activated Nrf2 at 15 minutes and 120 minutes. There is no known explanation as to why this might be so. However, it is known that different compounds activate Nrf2 at different time points. For example, resveratrol has been reported to activate Nrf2 at 3, 6, 9 and 12 hours in PC12 cells (Chen et al., 2005). In an earlier study by Lee et al. (2012), artesunate was shown to activate nuclear Nrf2 protein expression at 24 hours. These reports suggest that compounds can activate Nrf2 at different time points. Although the reasons why this might be so is uncertain, activation of Nrf2 provides an effective approach to activate the M2 phenotype of the microglia which helps in restoration of homeostasis. Upon translocation of Nrf2 to the nucleus, Nrf2 forms a heterodimer with small Maf proteins, recognises and then binds to ARE promoter site. Reporter gene assay showed that artemisinin, artemether and artesunate induced ARE activation.

The induction of Nrf2 by compounds of various chemical classes is directly correlated to the inhibition of COX-2 and iNOS expression (Baird & Dinkova-Kostova, 2011). In addition, Nrf2 activity has been suggested to modulate the activity of NF-κB. Having established that artemisinin, artemether and artesunate produced activation of Nrf2/HO-1 system, as well as the ARE reporter, it was thought to confirm whether some of the anti-neuroinflammatory effects of these compounds were dependent on Nrf2. This hypothesis was tested in Nrf2 knockout BV2 microglia stimulated with LPS after treatment with the compounds. Analyses of samples from these cells showed that inhibition of nitrite production by the three compounds at the highest concentrations studies was unaffected by silencing the Nrf2 gene. Further investigations on the effect of Nrf2 knockout on nitrite production at lower concentrations of the compounds were explored and a similar trend was observed. However, this investigation was not done up to three times to ensure statistical significance. Therefore, the outcomes have not been included in the thesis. The results obtained from the investigations on the effect of artemisinin and its derivatives on nitrite production suggest that the effect of all compounds on nitrite production were independent on Nrf2. This observation suggests that other pathway(s) are involved in the nitrite production. Further investigation (s) to determine other pathway (s), which might be influencing this observation, should be carried out.

Interestingly, inhibition of TNFα, IL-6 and PGE_2 were reversed to varying degrees by the three compounds in Nrf2-silenced BV2 cells stimulated with LPS. This was the first observation demonstrating a relationship between Nrf2 activation and inhibition of TNFα, IL-6 and PGE_2 by artemisinin, artemether and artesunate in the microglia. Recent studies on BV2 microglia cells suggest that Nrf2 has an inverse effect on NF-κB (Cuadrado et al.,

2014). This probably explains the reversal in the inhibitory effect of artemisinin, artemether and artesunate on PGE_2, TNFα and IL-6 production in Nrf2-silenced BV2 microglia cells activated with LPS. It however remains unclear why the effects of these compounds on nitrite production were unaffected in Nrf2-silenced cells. The proposed actions of artemisinin, artemether and artesunate on neuroinflammation in BV2 microglia are shown in figure 59.

It is also important to highlight that these compounds did not affect cell viability and hence are not toxic to the cells. Therefore, the results obtained on the inhibitory actions of the compounds were not related to any cytotoxic effect observed on cell viability. This observation is important as artemisinin and its derivatives have been reported to be neurotoxic at high concentrations *in vivo* (Classen et al., 1999). It also highlights the need to consider lower concentrations of the drug for indications like neuroinflammation.

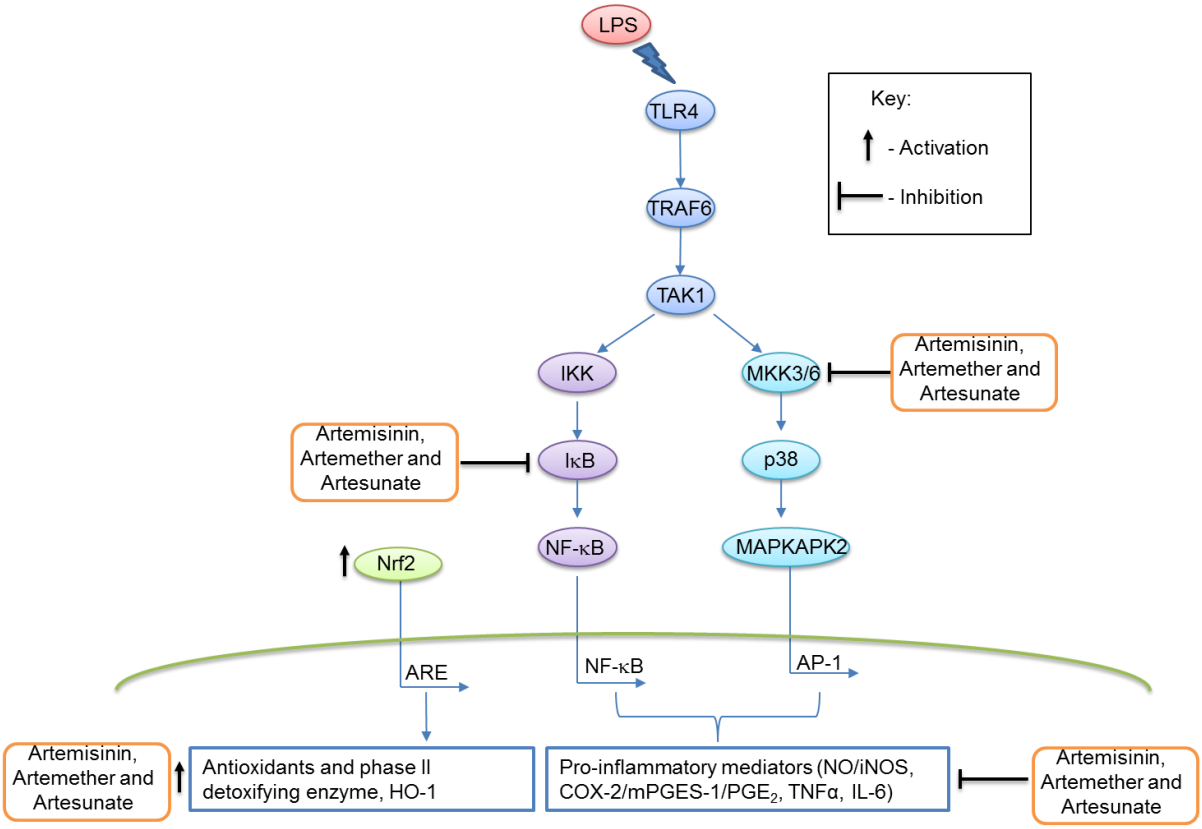

Figure 59: Proposed actions of artemisinin, artemether and artesunate on neuroinflammation in BV2 microglia cells.

Artemisinin, artemether and artesunate are currently used as anti-malarial drugs. They have also been proven very efficient in the treatment of cerebral malaria (Shakir et al., 2011). However, new pharmacological applications have been reported for these compounds. Artemisinin and its derivatives have been shown to have wide range of activity other than malaria ranging from anti-viral activity, to antifungal, to anti-inflammatory and anti-cancer

activity (Ho et al., 2014). This present study has also shown that artemisinin, artemether and artesunate possess anti-neuroinflammatory activity. Therefore, the remarkable activities of these compounds suggest their possible development for new indications. This will be highly beneficial as drug repositioning presents a new approach for the identification of new compounds in drug discovery.

6.2. Comparison of the activity of artemisinin and its derivatives artemether and artesunate

From this study, artemisinin and its derivatives have been shown to have anti-neuroinflammatory actions in LPS activated BV2 microglia. However, it is important to note that the compounds have had these effects at various concentration and percentages. Artesunate showed much more inhibitory actions in all parameters investigated because the concentration investigated was 10 times smaller when compared to the concentrations investigated for artemisinin and artemether. Studies on artemisinin and artemether used the same concentrations but produced different effects. Investigations of inhibitory actions on phosphorylation of p65 showed that artemether is more effective than artemisinin the parent compound. At 40 μM, pre-treatment with artemisinin produced 50 % p65 protein expression while artemether produced 45 % p65 protein expression. With p38 phosphorylation, results obtained also suggest that artemether is more effective in inhibiting p38 protein expression than artemisinin at the highest concentration studied. These observations suggest that artemether might be more potent than artemisinin. This observation is probably because the two compounds are metabolised differently. It is also important to note that these compounds were studied independently and the comparisons made are based on the results obtained from the individual studies. Therefore, highlighting the need to further investigate the compounds together to determine relative IC50 values to ensure adequate and accurate comparison between the compounds. In addition, a positive control compound with known activity should also be included in the study. In addition, weaknesses in the comparison also highlight the need to further investigate the active metabolite DHA which artemisinin derivatives are converted to. This might provide insights as to why artemether and artesunate seem to be more effective than artemisinin the parent compound.

It is also important to highlight that the activity of these compounds differ from each other due to their pharmacokinetics properties and metabolism. As mentioned in section 1.6.2, these drugs have different bioavailability and elimination half-life. Comparison of artemisinin and artemether suggested that artemether might be more effective. This could be due to its higher bioavailability. This could also be attributed to the fact that 80% of artemisinin is bound to proteins with only 8-10 % unbound. On the other hand, the activity of artesunate at

very low concentrations when compared to artemisinin and artemether could be attributed to its higher bioavailability (82 %). However, it might be worth nothing that artesunate is more rapidly metabolised when compared to the other derivative artemether. These pharmacokinetics properties of artemisinin and its derivatives artemether and artesunate should be considered in further investigations of these compounds.

Conclusions

This study has shown that artemisinin, artemether and artesunate have shown an exceptional similarity in their pharmacological profile in the inhibiting neuroinflammation in LPS activated BV2 microglia cells. All the compounds inhibited neuroinflammation in LPS activated BV2 cells through marked interference with both NF-κB and p38MAPK signalling. The compounds also produced consistent activation of the Nrf2/HO-1 antioxidant protective system in the microglia. This research has also provided the first evidence that the anti-neuroinflammatory activity of these compounds is possibly dependent, at least in part, to their ability to activate the Nrf2 transcription factor.

Recommendations

The study has answered a lot of questions regarding the anti-neuroinflammatory potentials of artemisinin, artemether and artesunate. However, further studies are required to further explore the actions of the compounds in neuroinflammation. To this end, the following follow-up studies are strongly recommended:

a. Artemisinin, artesunate and artemether have shown an extraordinary consistency in the similarity of their pharmacological actions in LPS-activated BV2 microglia. It would be interesting to investigate these compounds together to determine relative IC50 values to ensure adequate and accurate comparison between the compounds.
b. The three compounds showed consistent lack of activity on IKK phosphorylation. It would be useful to determine if their effects on IκB was due to direct inhibition on the phosphorylation of this inhibitor or through interference with another signalling pathway, which cross talks with NF-κB through IκB.
c. Further experiments are needed to explore detailed effects of these compounds on Nrf2/KEAP-1 signalling and its implications on their ability to enhance the antioxidant status of the microglia.
d. In terms of drug discovery, it is recommended that experiments need to be done to test the neuroprotective effects of these compounds in neuronal mono-cultures, as well as neuron-microglia co-cultures. Considering their significant effects on NF-κB, it would be interesting to explore possible impact on critical targets of amyloidogenesis and neuronal apoptosis, especially those that have been shown to be transcriptionally controlled by NF-κB.
e. Artemisinin derivatives are metabolised to DHA. Further investigations into the anti-neuroinflammatory properties of DHA in LPS-activated BV2 microglia should be carried out. In addition, studies relating to the permeability of DHA into the CNS compared to the parent compound artemisinin should also be explored.

References

Abraham, M.H. & Acree, W.E. (2013). Descriptors for artemisinin and its derivatives; estimation of physicochemical and biochemical data. *Eur. Chem. Bull.*, 2 (12), 1027-1037.

Aid, S., & Bosetti, F. (2011). Targeting cyclooxygenases-1 and -2 in neuroinflammation: Therapeutic implications. *Biochimie*, 93(1), 46-51. doi: 10.1016/j.biochi.2010.09.009.

Aid, S., Silva, A., Candelario-Jalil, E., Choi, S., Rosenberg, G., & Bosetti, F. (2010). Cyclooxygenase-1 and -2 differentially modulate lipopolysaccharide-induced blood-brain barrier disruption through matrix metalloproteinase activity. *Journal of Cerebral Blood Flow Metabolism, 30*, 370-380.

Ajroud-Driss, S., & Siddique, T. (2015). Sporadic and hereditary amyotrophic llateral sclerosis (ALS). *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1852*(4), 679-684. doi: 10.1016/j.bbadis.2014.08.010

Alzheimer's-Association. (2014). 2014 Alzheimer's disease facts and figures. *Alzheimer's and Dementia, 10*(2), e47-e92. doi: 10.1016/j.jalz.2014.02.001

Aungst, S., Kabadi, S., Thompson, S., Stoica, B., & Faden, A. (2014). Repeated mild traumatic brain injury causes chronic neuroinflammation, changes in hippocampal synaptic plasticity, amd associated cognitive deficits. *Journal of Cerebral Blood Flow & Metabolism, 34*, 1223-1232. doi: doi:10.1038/jcbfm.2014.75

Avery, M.A., Bonk, J.D., Chong, W.K, Mehrotra, S., Miller, R., Milhous, W., Goins, D.K., Venkatesan, S., Wyandt, C., Khan, I (1995). Structure activity relationships of the antimalarial agent artemisinin.2. Effect of heteroatom substitution at O-11: synthesis and bioassay of N-alkyl-11-aza-9-desmethylartemisinins. *J Med Chem*, 38 (26), 5038 - 5044.

Bachstetter, A., & Eldik, L. V. (2010). The p38 MAP kinase family as regulators of proinflammatory cytokine production in degenerative diseases of the CNS. *Aging and Disease, 1*(3), 199-211.

Bachstetter, A., Xing, B., Almeida, L. d., Dimayuga, E., Watterson, D., & Eldik, L. V. (2011). Microglial p38alpha MAPK is a key regulator of proinflammatory cytokine up-regulation induced by toll-like receptor (TLR) ligands or beta-amyloid (Abeta). *J Neuroinflammation, 8*, 79.

Baird, L., & Dinkova-Kostova, A. (2011). The cytoprotective role of the Keap1–Nrf2 pathway. *Archives of Toxicology, 85*(4), 241-272. doi: 10.1007/s00204-011-0674-5

Balint, G.A. (2001). Artemisinin and its derivatives - an important new class of antimalarial agents. *Pharmacology & Therapeutics*, 90, 261-265.

Banuls, C., Rocha, M., Rovira-Llopis, S., Falcon, R., Castello, R., Herance, J., . . . Victor, V. (2014). The pivotal role of nitric oxide: effects on the nervous and immune systems. *Current pharmaceutical design, 20*(29), 4679-4689. doi: 10.2174/1381612820666140130213510

Banzhaf-Strathmann, J., Benito, E., May, S., Arzberger, T., Tahirovic, S., Kretzschamar, H., . . . Edbauer, D. (2014). microRNA-125b induces tau hyperphosphorylation and cognitive deficits

in Alzheimer's disease. *The EMBO Journal, 33*(15), 1667-1680. doi: 10.15252/embj.201387576

Baune, B.T., Ponath, G., Rothermundt, M., Roesler, A., & Berger, K. (2009). Association between cytokines and cerebral MRI changes in the aging brain. *Journal of Geriatic Psychiatry and Neurobiology,* 4. doi:10.1177/0891988708328216

Bellezza, I., Mierla, A., & Minelli, A. (2010). Nrf2 and NF-κB and Their Concerted Modulation in Cancer Pathogenesis and Progression. *Cancers, 2*(2), 483-497. doi: 10.3390/cancers2020483

Bhaskar, K., Maphis, N., Xu, G., Varvel, N., Kokiko-Cochran, O., Weick, J., . . . lamb, B. (2013). Microglial derived tumour necrosis factor alpha drives Alzheimer's disease-related neuronal cell cycle events. *Neurobiology of Disease, 62*, 273-285. doi: 10.1016/j.nbd.2013.10.007

Bhat, N., Zhang, P., Lee, J., & Hogan, E. (1998). Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumour necrosis factor-alpha gene expression in endotoxin-stimulated primary glia cultures. *Journal of Neuroscience, 18*(5), 1633-1641.

Breman, J.G., Alilio, M.S., & White, N.J. (2007). Defining and Defeating the intolerable burden of malaria III: progress and perspectives. *American Journal of Tropical Medicine and Hygiene*, Supplement to vol 77(6)

Breydo, L., Wu, J., & Uversky, V. (2012). alpha-Synuclein misfolding and Parkinson's disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1822*(2). doi: 10.1016/j.bbadis.2011.10.002

Brunssen, S., Moy, S., Toews, A., McPherson, C., & Harry, J. (2013). Interleukin-6 (IL-6) receptor/IL-6 fusion protein (Hyper IL-6) effects on the neonatal mouse brain: Possible role for IL-6 trans-signaling in brain development and functional neurobehavioral outcomes. *Brain, Behavior, and Immunity, 27*(0), 42-53. doi: 10.1016/j.bbi.2012.08.017

Buckley, C. D., Gilroy, D. W., Serhan, C. N., Stockinger, B., & Tak, P. P. (2013). The resolution of inflammation. *Nat Rev Immunol, 13*(1), 59-66. doi: 10.1038/nri3362

Buggia-Prevot, V., Sevalle, J., Rossner, S., & Checler, F. (2008). NF-kappaB dependent control of BACE1 promoter transactivation by Abeta42. *J Biol Chem,* 283 (15), 10037 - 10047. doi: 10.1074/jbc.M706579200.

Butovsky, O., Jedrychowski, M., Moore, C., Cialic, R., Lanser, A., Gabriely, G., . . . Weiner, H. (2014). Identification of a unique TGF-beta-dependent molecular and functional signature in microglia. *Nat Neurosci, 17*, 131 - 143.

Butterfield, D. A., Reed, T., & Sultana, R. (2011). Roles of 3-nitrotyrosine- and 4-hydroxynonenal-modified brain proteins in the progressio and pathogenesis of Alzheimer's disease. *Free Radical Research, 45*, 59-72. doi: 10.3109/10715715762.2010.520014

Cao, Q., Jiang, Y., Shi, J., Xu, C., Liu, X., Yang, T., . . . Niu, T. (2014). Artemisinin inhibits the proliferation, migration, and inflammatory reaction induced by tumor necrosis factor-alpha in vascular smooth muscle cells through nuclear factor kappa B pathway. *Journal of Surgical Research*. doi: 10.1016/j.jss.2014.12.013

Cespuglio, R., Amrouni, D., Meiller, A., Buguet, A., & Gautier-Sauvigné, S. (2012). Nitric oxide in the regulation of the sleep-wake states. *Sleep Medicine Reviews, 16*(3), 265-279. doi: 10.1016/j.smrv.2012.01.006

Chami, L., Buggia-Prevot, V., Duplan, E., Delprete, D., Chami, M., Peyron, J-F, & Checler, F. (2012). Nuclear factor-kappa B regulates betaAPP and beta- and gana-secretases differently at physiological and supraphysiological Abeta concentrations. *J Biol Chem,* 2012 doi: 10.1074/jbc.M111.333054

Chaudhry, U., Zhuang, H., Crain, B., & Dore, S. (2008). Elevated microsomal prostaglandin-E synthase-1 in Alzheimer's disease. *Alzheimers Dement, 4*(1), 6-13.

Chen, C.H., Zhou, W., Liu, S., Deng, Y., Cai, F., Tone, M., Tone, Y., Song, W. (2012). Increased NF-kappaB signalling up-regulates BACE1 expression and its therapeutic potential in Alzheimer's disease. *Int J Neurophychopharmacol*. 15(1), 77-90. doi: 10.1017/S1461145711000149.

Chen, C.Y., Jang, J.H., Li, M.H., & Surh, Y.J. (2005). Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. *Biochem Biophy Res Commun*, 331 (4), 993 - 1000.

Cherry, J., Olschowka, J., & O'Banion, M. (2014). Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. *Journal of Neuroinflammation, 11*(1), 98.

Chinta, S., & Andersen, J. (2011). Nitrosylation and nitration of mitochondrial complex I in Parkinson's disease. *Free Radical Research, 45*, 53-58. doi: 10.3109/10715762.2010.509398

Choi, S., Aid, S., Caracciolo, L., Minami, S., Niikura, T., Matsuoka, Y., . . . Bosetti, F. (2013). Cyclooxygenase-1 inhibition reduces amyloid pathology and improves memory deficits in a mouse model of Alzheimer's disease. *J Neurochem, 124*(1), 59-68. doi: 10.1111/jnc.12059

Classen, W., Altmann, B., Gretener, P., Souppart, C., Skelton-Stroud, P., & Krinke, G. (1999). Differential effects of orally versus parentally adminisered qinghaosu derivative artemether in dogs. *Exp Toxicol Pathol*, 51(6), 507-516

Clement, A., Nguyen, M., Roberts, E., Gracia, M., Boillee, S., Rule, M., . . . Cleveland, D. (2003). Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science Translational Medicine, 302*, 113-117.

Coghi, P., Basilico, N., Taramelli, D., Chan, W.C., Haynes, R.K., & Monti, D. (2009). Interaction of artemisins with oxyhemoglobin Hb-FeII, Hb-FeII, carboxyHb-FeII, heme-FeII, and carboxyheme FeII: significance for mode of action and implications for therapy of cerebral malaria. *Chem Med Chem*, 4 (12), 2045-2053. doi: 1002/cmdc.200900342

Corrêa, S., & Eales, K. (2012). The Role of p38 MAPK and Its Substrates in Neuronal Plasticity and Neurodegenerative Disease. *Journal of Signal Transduction, 2012*, 12. doi: 10.1155/2012/649079

Coulthard, L., White, D., Jones, D., McDermott, M., & Burchill, S. (2009). p38MAPK: stress responses from molecular mechanisms to therapeutics. *Trends in Molecular Medicine, 15*(8), 369-379. doi: 10.1016/j.molmed.2009.06.005

Cuadrado, A., Martin-Moldes, Z., Ye, J., & Lastres-Becker, I. (2014). Transcription factors Nrf2 and NF-kB are coordinated effectors of the RHO family, GTP binding protein rac1 during inflammation. *The Jounal od Biological Chemistry, 289*(22), 15244-15258. doi: 10.1074/jbc.M113.540633

Culbert, A., Skaper, S., Howlett, D., Evans, N., Facci, L., Soden, P., . . . Richardson, J. (2006). MAPK-activated protein kinase 2 deficieny in microglia inhibits pro-inflammatory mediator release and resultant neurotoxicity: relevance to neuroinflammation in a transgenic mouse model of Alzheimer's disease. *Journal of Biological Chemistry, 281*(33), 23658-23667. doi: 10.1074/jbc.M513646200

Cuzzocrea, S., Saadat, F., Paola, R. D., & Mirshafiey, A. (2005). Artemether: a new therapeutic strategy in experimental rheumatoid arthritis. *Immunopharmacology and Immunotoxicology, 27*, 615-630.

D'Acquarica, I., Gasparrini, F., Kotoni, D., Pierini, M., Villani, C., Cabri, W., Di Mattia, & M., Giorgi, F. (2010). Stereodynamic investigation of labile stereogenic centres in dihydroartemisinin. *Molecules*, 15, 1309 - 1323. doi: 10.3390/molecules15031309.

Dalakas, M. (2008). Invited article: Inhibition of B cell functions. *Neurology, 70*(23), 2252-2260. doi: 10.1212/01.wnl.0000313840.27060.bf

Dargahi, L., Nasiraei-Moghadam, S., Abdi, A., Khalaj, L., Moradi, F., & Ahmadiani, A. (2011). Cyclooxygenase (COX)-1 Activity Precedes the COX-2 Induction in Aβ-Induced Neuroinflammation. *Journal of Molecular Neuroscience, 45*(1), 10-21. doi: 10.1007/s12031-010-9401-6

Davis, T.M., Binh, T.Q., IIett, K.F., Batty, K.T., Phuong, H.L., Chiswell, G.M., Phuong, V.D., & Agus, C. (2003). Penetration of dihydroartemisinin into cerebrospinal fluid after dministration of intravenous artesunate in severe falciparum malaria. A*ntimicrob Agents Chemother*. 47(1), 368-370

deOliveira, A., Candelario-Jalil, E., Bhatia, H., Lieb, K., Hull, M., & Fiebich, B. (2008). Regulation of prostaglandin E2 synthase expression in activated primary rat microglia: evidence for uncoupled regulation of mPGES-1 and COX-2. *Glia, 56*(8), 844-855. doi: 10.1002/glia.20658

Dilshara, M., Lee, K., Jayasooriya, R., Kang, C., Park, S., Choi, Y., . . . Kim, G. (2014). Downregulation of NO and PGE2 in LPS-stimulated BV2 microglia cells by trans-isoferulic

acid via suppression of PI3K/Akt-dependent NF-kappaB and activation of Nrf2-mediated HO-1. *International Immunopharmacology, 18*, 203-211. doi: 10.1016/j.intimp.2013.11.020

DiVirgilio, F., Ceruti, S., Bramanti, P., & Abbracchio, M. (2009). Purinergic signalling in inflammation of the central nervous system. *Trends in Neuroscience, 32*(2), 79-87. doi: 10.1016/j.tins.2008.11.003

Drechsel, D., Estévez, A., Barbeito, L., & Beckman, J. (2012). Nitric Oxide-Mediated Oxidative Damage and the Progressive Demise of Motor Neurons in ALS. *Neurotoxicity Research, 22*(4), 251-264. doi: 10.1007/s12640-012-9322-y

Efferth, T., Romero, M.R., Wolf, D.G., Stamminger, T., Martin, J.J.G., & Marschall, M. (2008). The antiviral activities of artemisinin and artesunate. *Clinical Infectious Diseases*. 47(6), 804-811. doi: 10.1086/591195

Ellwardt, E., & Zipp, F. (2014). Molecular mechanisms linking neuroinflammation and neurodegeneration in MS. *Experimental Neurology, 262, Part A*(0), 8-17. doi: 10.1016/j.expneurol.2014.02.006

Erta, M., Quintana, A., & Hidalgo, J. (2012). Interleukin-6, a major cytokine in the central nervous system. *International Journal of Biological Sciences, 8*(9), 1254-1266. doi: 10.7150/ijbs.4679

Escott-Price, V., Bellenguez, C., Wang, L., Choi, S., Harold, D., Jones, L., . . . Williams, J. (2014). Gene-Wide Analysis Detects Two New Susceptibility Genes for Alzheimer's Disease. *PLoS ONE, 9*(6), e94661. doi: 10.1371/journal.pone.0094661

Fan, Z., Aman, Y., Ahmed, I., Chetelat, G., Landeau, B., Chaudhuri, K. R., . . . Edison, P. (2014). Influence of microglial activation on neuronal function in Alzheimer's and Parkinson's disease dementia. *Alzheimers Dement, 14*, S1552-5260. doi: 10.1016/j.jalz.2014.06.016

Fiebich, B., Akter, S., & Akundi, R. (2014). The two-hit hypothesis for neuroinflammation: role of exogenous ATP in modulation inflammation in the brain. *Front Cell Neurosci, 8*, 1-11. doi: 10.3389/fncel.2014.00260

Förstermann, U., & Sessa, W. (2012). Nitric oxide synthases: regulation and function. [10.1093/eurheartj/ehr304]. *European Heart Journal, 33*(7), 829-837.

Frankola, K., Greig, N., Luo, W., & Tweedie, D. (2011). Targeting TNF-alpha to elucidate and ameliorate neuroinflammation in neurodegenerative diseases. *CNS and Neurological disorders - Drug Targets, 10*(3), 391-403. doi: 10.2174/187152711794653751

Gandhi, S., & Wood, N. (2010). Genome-wide association studies: the key to unlocking neurodegeneration? *Nature Neuroscience, 13*, 789-794. doi: 10.1038/nn.2584

Gaudet, A., Popovich, P., & Ramer, M. (2011). Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury. *Journal of neuroinflammation, 8*, 110-123. doi: 10.1186/1742-2094-8-110

Gerondakis, S., Fulford, T.S., Messina, N.L., & Grumont, R.J. (2014). NF-kappa B control of T cell development. *Nature Immunology*. 15, 15-25. doi: 10.1038/ni.2785

Ghasemi, M., & Fatemi, A. (2014). Pathologic role of glial nitric oxide in adult and pediatric neuroinflammatory diseases. *Neuroscience & Biobehavioral Reviews, 45*(0), 168-182. doi: 10.1016/j.neubiorev.2014.06.002

Glass, C., Saijo, K., Winner, B., Marchetto, M., & Gage, F. (2010). Mechanisms underlying inflammation in neurodegeneration. *Cell, 140*(6), 918-934. doi: 10.1016/j.cell.2010.02.016

Godinez-Rubi, M., Rojas-Mayorquin, A., & Ortuno-Sahagun, D. (2013). Nitric oxide donors as neuroprotective Agent after an Ischemic Stroke-related inflammatory reaction *Oxidative Medicine and Cellular Longevity, 2013*, 1-16. doi: 10.1155/2013/297357

Golenser, J., Waknine, J., Krugliak, M., Hunt, N., & Grau, G. (2006). Current perspectives on the mechanism of action of artemisinins. *International Journal of Parasitology, 36*(14), 1427-1441. doi: 10.1016/j.ijpara.2006.07.011

Gomez-Nicola, D., & Perry, V.H. (2015). Microglial Dynamics and Role in the Healthy and Diseased Brain: A Paradigm of Functional Plasticity. *The Neuroscientist*. doi: 10.1177/1073858414530512

Graeber, M., & Streit, W. (2010). Microglia: biology and pathology. *Acta Neuropathologica, 119*(1), 89-105. doi: 10.1007/s00401-009-0622-0

Gresa-Arribas, N., Vieitez, C., Dentesano, G., Serratosa, J., Saura, J., & Sola, C. (2012). Modelling neuroinflammation in vitro: a tool to test the potential neuroprotective effect of anti-inflammatory agents. *Plos one*. 7(9), e45227. doi: 10.1371/journal.pone.0045227.

Griess, P. (1879). Bemerkungen zu der abhandlung der H.H.Weselsky und Benedikt 'Ueber einige azoverbindungen'. *Chem Ber*. 12, 426-428

Gruol, D. (2014). IL-6 regulation of synaptic function in the CNS. *Neuropharmacology*(0). doi: 10.1016/j.neuropharm.2014.10.023

Habtemariam, S. (2000). Natural inhibitors of tumour necrosis factor-α production, secretion and function. *Planta Medica, 66*, 303-314

Hald, A., & Lotharius, J. (2005). Oxidative stress and inflammation in Parkinson's disease: is there a causal ink? *Experimental Neurology, 193*(2), 279-290. doi: 10.1016/j.expneurol.2005.01.013

Hall, I.H., Lee, K.H., Starenes, C.O., Sumida, Y., Wu, R.Y., Waddell, T.G., Cochran, W., & Gerhart, K.G. (1979). Anti-inflammatory activity of sesquiterpene lactones and related compounds. *Journal of Pharmaceutical Sciences*, 68 (5), 537 -542. doi: 10.1002/jps.2600680505

Hall, I.H., Starnes Jr, C.O., Lee, K.H., & Waddell, T.G. (1980). Mode of actio of sesquiterpene lactones as anti-inflammatory agents. *Journal of Pharmaceutical Sciences*, 69 (5), 537-543. doi: 10.1002/jps.2600690516

Hanisch, U. (2002). Microglia as a source and target of cytokines. *Glia, 40*(2), 140-155. doi: 10.1002/glia.10161

Hanisch, U., & Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. [10.1038/nn1997]. *Nat Neurosci, 10*(11), 1387-1394.

Harari, O., & Liao, J. (2010). NF-kB and innate immunity in ischemic stroke. *Ann N Y Acad Sci, 1207*, 32-40. doi: 10.1111/j.1749-6632.2010.05735.x

Hayes, J., & Dinkova-Kostova, A. (2014). The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends in Biochemical Sciences, 39*(4), 199-218. doi: 10.1016/j.tibs.2014.02.002

Hefendehl, J., Neher, J., Suhs, R., Kohsaka, S., Skodras, A., & Jucker, M. (2014). Homeostatic and injury-induced microglia behaviour in the aging brain *Aging Cell, 13*(1), 60-69. doi: 10.1111/acel.12149

Hehner, S.P., Heinrich M., Bork, P.M., Vogt, M., Ratter, F., Lehmann, V., Schulze-Osthoff, K., Droge, W., Schmitz, M.L. (1998). Sesquiterpene lactones specifically inhibit activation of NF-kappaB by preventing the degradation of IκB-α and IκB-β. *Journal of Biological chemistry.* 273, 1288-1297. doi: 10.1074/jbc.273.3.1288

Hehner, S.P., Hofmann, T.G., Droge, W., & Schmitz, M.L. (1999). The antiinflammatory sesquiterpene lactone parthenolide inhibits NF-kappa B by targeting the I kappa B kinase complex. *Journal of Immunology*, 163 (10): 5617-5623.

Hernández-Romero, M., Delgado-Cortés, M., Sarmiento, M., Pablos, R. d., Espinosa-Oliva, A., Argüelles, S., . . . Machado, A. (2012). Peripheral inflammation increases the deleterious effect of CNS inflammation on the nigrostriatal dopaminergic system. *NeuroToxicology, 33*(3), 347-360. doi: 10.1016/j.neuro.2012.01.018

Hickman, S.E., Kingery, N.D., Ohsumi, T.K., Borowsky, M.L., Wang, L., Means, T.K., & Khoury, J.E. (2013). The microglial sensome revealed by direct RNA sequencing. *Nature Neuroscience*. 16, 1896-1905. doi: 10.1038/nn.3554.

Hien, T.T., Arnold, K., Vinh, H., Cuong, B.M., Phu, N.H., Chau, T.T., Hoa, H.T., Chuong, L.V., Mai, N.T., Vinh, N.N., *et al* (1992). Comparison of artemisinin suppositories with intravenous artesunate and intravenous quinine in the treatment of cerebral malaria. *Trans R Soc Trop Med Hyg,* 86 (6), 582 - 583

Ho, W., Peh, H., Chan, T., & Wong, W. (2014). Artemisinins: pharmacological actions beyond anti-malarial. *Pharmacol Ther, 142*(1), 126-139. doi: 10.1016/j.pharmthera.2013.12.001

Holtzman, D., Morris, J., & Goate, A. (2011). Alzheimer's disease: the challenge of the second century. *Science Translational Medicine, 3*(77), 77sr71. doi: 10.1126/scitranslmed.3002369

Hong, S., & Banks, W. (2014). Role of the immune system in HIV-associated neuroinflammation and neurocognitive implications. *Brain, Behavior, and Immunity*(0). doi: 10.1016/j.bbi.2014.10.008

Hooten, K., Beers, D., Zhao, W., & Appel, S. (2015). Protective and toxic neuroinflammation in Amyotrophic Lateral Sclerosis. *Neurotherapeutics, 2015*. doi: 10.1007/s13311-014-0329-3

Hoozemans, J., Rozemuller, J., vanHaastert, E., Veerhuis, R., & Eikelenboom, P. (2008). Cyclooxygenase-1 and -2 in the different stages of Alzheimer's disease pathology. *Curr Pharm Des, 14*, 1419-1427.

Hoozemans, J., Veerhuis, R., Rozemuller, J., & Eikelenboom, P. (2011). Soothing the inflamed brain: Effect of Non-Steroidal Anti-inflammatory drugs on Alzheimer's disease pathology. *CNS and Neurological disorders - Drug Targets, 10*, 57-67.

Hosmane, S., Tegenge, M., Rajbhandari, L., Uapinyoying, P., Kumar, N., Thakor, N., & Venkatesan, A. (2012). Toll/interl;eukin-1 receptor domain-containing adapter inducing interferon-beta mediates microglial phagocytosis of degenerating axons. *Journal of Neuroscience, 32*(22), 7745-7757.

Hu, X., Leak, R., Shi, Y., Suenaga, J., Gao, Y., Zheng, P., & Chen, J. (2015). Microglial and macrophage polarization- new prospects of brain repair. *Nature Reviews Neurology, 11*, 56-64. doi: 10.1038/nrneurol.2014.207

Hunot, S., Boissiere, F., Faucheux, B., Brugg, B., Mouatt-Prigent, A., Agid, Y., & Hirsch, E. (1996). Nitric oxide synthase and neuronal vulnerability in Parkinson's disease. *Neuroscience, 72*, 355-363.

Huuskonen, J., Suuronen, T., Miettinen, & vanGroen, T. (2005). A refined in vitro model to study inflammatory responses in organotypic membrane culture of postnatal rat hippocampal slices. *Journal of Neuroinflammation.* 2,25. doi: 10.1186/1742-2094-2-25.

Ikeda-Matsuo, Y., Ikegaya, Y., Matsuki, N., Uematsu, S., Akiri, S., & Sasaki, Y. (2005). Microglia-specific expression of microsomal prostaglandin E2 synthase-1 contributes to lipopolysaccharide-induced prostaglandin E2 production. *Jounal of Neurochem, 94*(6), 1546-1558.

Innamorato, N., Rojo, A., & Garcia-Yague, A. (2008). The transcription factor Nrf2 is a therapeutic target against brain inflammation. *The Journal of Immunology, 181*, 680-689. doi: 10.4049/jimmunol.181.1.680

Jakobsson, P.-J., Thoren, S., Morgenstern, R., & Samuelsson, B. (1999). Identification of human prostaglandin E synthase: A microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc Natl Acad Sci U S A, 96*, 7220-7225.

Jha, M., Jeon, S., & Suk, K. (2012). Glia as a link between neuroinflammation and neuropathic pain. *Immune Network, 12*(2), 41-47.

Ji, R., Xu, Z., & Gao, Y. (2014). Emerging targets in neuroinflammation-driven chronic pain. *Nature Reviews Drug Discovery, 13*(7), 533-548. doi: 10.1038/nrd4334

Jin, W., Wang, H., Yan, W., Xu, L., Wang, X., Zhao, X., . . . Ji, Y. (2008). Disruption of Nrf2 Enhances Upregulation of Nuclear Factor-B Activity, Proinflammatory Cytokines, and Intercellular Adhesion Molecule-1 in the Brain after Traumatic Brain Injury. *Mediators of Inflammation, 2008*, 7. doi: 10.1155/2008/725174

Joshua, M., & Thomas, A. (2014). Nitric oxide synthase: non-canonical expression patterns. [Perspective]. *Frontiers in Immunology, 5*. doi: 10.3389/fimmu.2014.00478

Kakalacheva, K., & Lunemann, J. (2011). Environmental triggers of multiple sclerosis. *FEBS Letters, 585*(23), 3724-3729. doi: 10.1016/j.febslet.2011.04.006

Kaltschmidt, B., & Kaltschmidt, C. (2009). NF-kappaB in the nervous system. *Cold Spring Harb Perspect Biol, 1*(3), a001271. doi: 10.1101/cshperspect.a001271

Kaltschmidt, B., Uherek, M., Volk, B., Baeuerle, P.A., & Kaltschmidt, C. (1997). Transcription factor NF-kappaB is activated in primary neurons by amyloid beta peptides and in neurons surrounding early plaques frol patients with ALzheimer disease. *Proc Natl Acad Sci USA,* 94 (6), 2642 - 2647.

Kaminska, B., Gozdz, A., Zawadzka, M., Ellert-Miklaszewska, A., & Lipko, M. (2009). MAPK signal transduction underlying brain inflammation and gliosis as therapeutic target. *Anatomical Record, 292*(12), 1902-1913. doi: 10.1002/ar.21047

Kanarek, N., & Ben-Neriah, Y. (2012). Regulation of NF-kappaB by ubiquitination and degradation of the IkappaBs. *Immunological Reviews, 246*, 77-92.

Kang, J., Park, K., Lee, H., Han, S., Lee, K., Park, S., & Kin, H. (2010). Artemisinin inhibits lipopolysaccharide-induced nitric oxide production by blocking IFN-beta production and STAT-1 signalling in macrophages. *The Journal of Immunologw, 184*, 142.142.

Kettenmann, H., Hanisch, U., Noda, M., & Verkhratsky, A. (2011). *Physiology of Microglia* (Vol. 91).

Kim, H.G., Yang, J.H., Han, E.H., Choi, J.H., Khanal, T., Jeong, T.C., & Jeong, H.G. (2013). Inhibitory effect of dihydroartemisinin against phorbol ester-induced cyclooxygenase-2 expression in macrophages. *Food Chem Toxicol*, 56: 93-99. doi: 10.1016/j.fct.2013.02.017.

Kim, S. (2011). The role of nitric oxide in prostaglandin biology; update. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society, 25*(3), 255-264. doi: 10.1016/j.niox.2011.07.002

Kim, S., Huri, D., & Snyder, S. (2005). Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. *Science Translational Medicine, 310*, 1966-1970.

Kim, T., & Eberwine, J. (2010). Mammalian cell transfection: the present and the future. *Analytical and Bioanalytical Chemistry, 397*(8), 3173-3178. doi: 10.1007/s00216-010-3821-6

Kimura, H., Yoshizumi, M., Ishii, H., Oishi, K., Ryo, A. (2013). Cytokine production and signalling pathways in respiratory virus infection. *Frontiers Microbiol*. 4, 276, 1-9, doi: 10.3389/fmicb.2013.00276

Koh, K., Cha, Y., Kim, S., & Kim, J. (2009). tBHQ inhibits LPS-induced microglial activation via Nrf2-mediated suppression of p38 phosphorylation. *Biochemical and Biophysical Research Communications, 380*(3), 449-453. doi: 10.1016/j.bbrc.2009.01.082

Koning, N., Bo, L., Hoek, R., & Huitinga, I. (2007). Downregulation of macrophage inhibitory molecules in multiple sclerosis lesions. *Ann Neurol, 62*, 504-514. doi: 10.1002/ana.21220

Konkimalla, V.B., Blunder, M., Korn, B., Soomro, S.A., Jansen, H., Chang, W., Posner, G.H., Bauer, R., & Efferth, T. (2008). Effect of artemisinins and other endoperoxides on nitric oxide-related signaling pathway in RAW 264.7 mouse macrophage cells. *Nitric Oxide, 19 (2)*, 184-191. doi:10.1016/j.niox.2008.04.008

Korn, T., Bettelli, E., Oukka, M., & Kuchroo, V. (2009). IL-17 and Th17 cells *Annu Rev Immunol, 27*, 485-517.

Krause, D., & Muller, N. (2010). Neuroinflammation, microglia and implications for anti-inflammatory treatment in Alzheimer's disease. *Int J Alzheimers Dis, 14*, 732806. doi: 10.4061/2010/732806

Krementsov, D., Thornton, T., Teuscher, C., & Rincon, M. (2013). The Emerging Role of p38 Mitogen-Activated Protein Kinase in Multiple Sclerosis and its models. *Molecular and Cellular Biology, 33*, 3728-3734. doi: 10.1128/MCB.00688-13

Kudo, I., & Murakami, M. (2005). Prostaglandin E Synthase, a terminal enzyme for prostaglandin E_2 biosynthesis. *Jounal of Biochemistry and Molecular Biology, 38*(6), 633-638.

Lawrence, T. (2009). The nuclear factor NF-kB pathway in inflammation. *Cold Spring Harbor Perspectives in Biology, 1*(6), a001651. doi: 10.1101/cshperspect.a001651

Lazarus, M., Kubata, B., Equchi, N., Fujitani, Y., Urade, Y., & Hayaishi, O. (2002). Biochemical characterization of mouse microsomal prostaglandin E synthase-1 and its colocalization with cyclooxygenase-2 in peritoneal macrophages. *Arch Biochem Biophys, 397*(2), 336-341.

Leclerc, P., Pawelzik, S., Idborg, H., Spahiu, L., Larsson, C., Stenberg, P., . . . Jakobsson, P. (2013). Characterization of a new mPGES-1 inhibitor in rat model of inflammation. *Prostaglandins & Other Lipid Mediators, 102-103*, 1-12. doi: 10.1016/j.prostaglandins.2013.03.005

Lee, I., Ryu, D., Lim, J., Cho, S., Kang, B., & Choi, H. (2012). Artesunate activates Nrf2 pathway-driven anti-inflammatory potential through ERK signaling in microglial BV2 cells. *Neurosci Lett, 509*(1), 17-21. doi: 10.1016/j.neulet.2011.12.034

Lee, Y., Schrader, J., & Kim, S. (2000). p38 map kinase regulates TNF-alpha production in human astrocytes and microglia by multiple mechanisms. *Cytokine, 12*(7), 874-880.

Levesque, S., Surace, M., MsDonald, J., & Block, M. (2011). Air pollution and the brain: subchronic diesel exhaust exposure causes neuroinflammation and elevates early markers of neurodegenerative disease. *Jounal of Neuroinflammation, 8*(1), 105-115. doi: 10.1186/1742-2094-8-105

Li, B., Li, J., Pan, X., Ding, G., Cao, H., Jiang, W., . . . Zhou, H. (2010). Artesunate protects sepsis model mice challenged with Staphylococcus aureus by decreasing TNF-α release via inhibition TLR2 and Nod2 mRNA expressions and transcription factor NF-κB activation. *International Immunopharmacology, 10*(3), 344-350. doi: 10.1016/j.intimp.2009.12.006

Li, G.Q., Guo, X.B., Fu, L.C., Jian, H.X., & Wang, X.H. (1994). Clinical trials of artemisinin and its derivatives in the treatment of malaria in China. *Trans R Soc Trop Med Hyg*, 88, Supl 1:S5-S6

Li, J., & Zhou, B. (2002). Review biological actions of artemisinin: insights from medicinal chemistry studies. *Molecules*. 15, 1378-1397. doi: 10.3390/molecules15031378

Li, Q., & Verma, I. (2002). NF-kappaB regulation in the immune system. *Nature Reviews Immunology, 2*, 725-734. doi: 10.1038/nri910

Li, X., Afif, H., Cheng, S., Martel-Pelletier, J., Pelletier, J., Ranger, P., & Fahmi, H. (2005). Expression and regulation of microsomal prostaglandin E synthase-1 in human osteoarthritic cartilage and chrondrocytes. *J Rheumatol, 32*(5), 887-895.

Lim, A., Krajina, K., & Marsland, A. (2013). Peripheral Inflammation and Cognitive Aging. *Mod Trends Pharmacopsychiatry, 28*, 175-187. doi: 10.1159/000346362

Lima, I., Bastos, L., Limborco-Filho, M., Fiebich, B., & Oliveira, A. d. (2012). Role of prostaglandins in neuroinflammatory and neurodegenerative diseases. *Mediators Inflamm, 2012*, 946813. doi: 10.1155/2012/946813

Liu, A., New, D., Lo, R., & Wong, Y. (2009). Reporter Gene Assays. In P. A. Clemons, N. J. Tolliday & B. K. Wagner (Eds.), *Cell-Based Assays for High-Throughput Screening* (Vol. 486, pp. 109-123): Humana Press.

Liu, G., Qu, J., & Shen, X. (2008). NF-kappaB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochim Biophys Acta, 1783*, 713-727.

Liu, S., Liu, Y., Hao, W., Wolf, L., Kiliaan, A., Penke, B., . . . Fassbender, K. (2012). TLR2 is a primary receptor for Alzheimer's Amyloid beta peptide to trigger neuroinflammatory activation. *Journal of Immunology, 188*(3), 1098-1107. doi: 10.4049/jimmunol.1101121

Ljubisavljevic, S., & Stojanovic, I. (2014). Neuroinflammation and demylination from the point of nitrosative stress as a new target for neuroprotection. *Reviews in the Neurosciences, 25*. doi: 10.1515/revneuro-2014-0060

Lull, M.E., & Block, M.L. (2010). Microglial activation and chronic neurodegeneration. *Neurotherapeutics,* 7 (4), 354 - 365. doi: 10.1016/j.nurt.2010.05.014

Lyman, M., Lloyd, D., Ji, X., Vizcaychipi, M., & Ma, D. (2014). Neuroinflammation: The role and consequences. *Neuroscience Research, 79*(0), 1-12. doi: 10.1016/j.neures.2013.10.004

Magenta, D., Sangiovannia, E., Basilicob, N., Haynes, R.K., Parapinia, S., Colomboa, E., Bosisioa, E., Taramellia, D., & Dell'Agli, M. (2014). Inhibition of metalloproteinase-9 secretion and gene

expression by artemisinin derivatives. *Acta Tropica*. 140, 77-83. doi: 10.1016/j.actatropica.2014.08.008.

Maniecka, Z., & Polymenidou, M. (2015). From nucleation to widespread propagation: A prion-like concept for ALS. *Viral Research*. doi: 10.1016/j.virusres.2014.12.032

McCoy, M., & Tansey, M. (2008). TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. *J Neuroinflammation, 5*, 45.

Miljkovic, D., Timotijevic, G., & Mostarica, S. (2011). Astrocytes in the tempest of multuple sclerosis. *FEBS Lett, 585*(23), 3781-3788. doi: 10.1016/j.febslet.2011.03.047

Miller, L.H., & Su, X. (2011). Artemisinin: Discovery from the chinese herbal garden. *Cell*, 146 (6): 855 - 858. doi: 10.1016/j.cell.2011.08.024

Mimche, P., Taramelli, D., & Vivas, L. (2011). The plant-based immunodulator curcumin as a potential candidate for the development of an adjunctive therapy for cerebral malaria. *Malaria Journal,* 10(Suppl 1), S10.

Ming, G., & Song, H. (2011). Adult Neurogenesis in the Mammalian Brain: Significant Answers and Significant Questions. *Neuron, 70*(4), 687-702. doi: 10.1016/j.neuron.2011.05.001

Mir M, T. L., Asensio VJ, Llado J and Olmos G. (2008). Complementary roles of tumor necrosis factor alpha and interferon gamma in inducible microglial nitric oxide generation. *Journal of Neuroimmunology, 204*, 101-109.

Moilanen, E. (2014). Two Faces of Inflammation: An Immunopharmacological View. *Basic & Clinical Pharmacology & Toxicology, 114*(1), 2-6. doi: 10.1111/bcpt.12180

Moreno-Gonzalez, I., Estrada, L., Sanchez-Mejias, E., & Soto, C. (2013). Smoking exacerbates amyloid pathology in a mouse model of Alzheimer's disease. [10.1038/ncomms2494]. *Nat Commun, 4*, 1495.

Morley, J., Farr, S., Sell, R., Hileman, S., & Banks, W. (2011). Nitric oxide is a central component in neuropeptide regulation of appetite. *Peptides, 32*(4), 776-780. doi: 10.1016/j.peptides.2010.12.015

Munoz, L., & Ammit, A. (2010). Targeting p38 MAPK pathway for the treatment of Alzheimer's disease. *Neuropharmacology, 58*, 561 - 568.

Myers, R., & Shubayev, V. (2011). The ology of neuropathy: an integrative review of the role of neuroinflammation and TNF-alpha axonal transport in neuropathic pain. *Journal of Peripheral Nervous System, 16*(4), 277-286. doi: 10.1111/j.1529-8027.2011.00362.x

Naegele, M., & Martin, R. (2014). The good and the bad of neuroimnflammation in multiple sclerosis. *Handb Clin Neurol, 122*, 59-87. doi: 10.1016/B978-0-444-52001-2.00003-0

Nam, Y.J., Lee, D.H., Lee, M.S., Lee, C.S. (2015). Sesquiterpene lactone parthenolide attenuates production of inflammatory mediators by suppressing the Toll-like receptor-4-mediated

activation of Akt, mTOR and NF-kappaB. *Naunyn-Schmiedeberg's Arch Pharmacol.* doi: 10.1007/s00210-015-1132-3

Neill, U.S. (2011). From branch to beadside: Youyou Tu is awarded the 2011 Laskker~Debakey Clinical medical research award for discovering artemisinin as a treatment for malaria. *J Clin Invest.*, 121 (10): 3768 - 3773. doi: 10.1172/JCI60887

Niture, S., Khatri, R., & Jaiswal, A. (2014). Regulation of Nrf2—an update. *Free Radical Biology and Medicine, 66*(0), 36-44. doi: 10.1016/j.freeradbiomed.2013.02.008

Nordberg, A. (2014). Dementia in 2014: Towards early diagnosis in Alzheimer disease. *nature Reviews Neurology, 11*, 69-70. doi: 10.1038/nrneurol.2014.257

Oeckinghaus, A., Hayden, M., & Ghosh, S. (2011). Crosstalk in NF-kB signalling pathways. *Nature Immunology, 12*(8), 695-708. doi: 10.1038/mi.2065

Okorji, U., & Olajide, O. (2014). A semi-synthetic derivative of artemisinin, artesunate inhibits prostaglandin E2 production in LPS/IFNgamma-activated BV2 microglia. *Bioorg Med Chem, 22*(17), 4726-4734. doi: 10.1016/j.bmc.2014.07.007

Olajide, O., Bhatia, H.S., de Oliveira, A.C.P., Wright, C.W., & Fiebich, B.L. (2013). Inhibition of Neuroinflammation in LPS-activated microglia by cryptolepine. *Evidence-based Comp Altern Med,* 2013 (459723). doi: 10.1155/2013/459723

Olajide, O., Heiss, E., Schachner, D., Wright, C., Vollmar, A., & Dirsch, V. (2007). Synthetic cryptolepine inhibits DNA binding of NF-kappaB. *Bioorg Med Chem, 15*(1), 43-49. doi: 10.1016/j.bmc.2006.10.018

Olajide, O., Kumar, A., Velagapudi, R., Okorji, U., & Fiebich, B. (2014). Punicalagin inhibits neuroinflammtion in LPS-activated rat primary microglia. *Mol. Nutr. Food Res., 00*, 1-9. doi: 10.1002/mnfr.201400163

Olmos, G., & Llado, J. (2014). Tumor Necrosis Factor alpha - a link between neuroinflammation and excitotoxicity. *Mediators of Inflammation, 2014*, 1-12.

ONeil, P., & Posner, G. (2004). A medicinal chemistry perspective on artemisinin and related endoperoxides. *Jounal of Medicinal Chemistry, 47*(12), 2945-2965. doi: 10.1021/jm030571c

Pahl, H. (1999). Activators and target genes of Rel/NF-kB transcription factors. *Oncogene, 18*, 6853-6866.

Pamplona, A., Ferreira, A., Balla, J., Jeney, V., Balla, G., Epiphanio, S., . . . Mota, M. (2007). Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. *Nat. Med, 13*, 703-710.

Pascual, O., Achour, S., Roastaing, P., Triller, A., & Bessis, A. (2012). Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. *PNSA, 109*, E197-E205. doi: 10.1073/pnas.1111098109

Pasvol, G. (2005). The treatment of complicated and severe malaria. *Br Med Bull*, 75-76 (1): 29 - 47. doi: 10.1093/bmb/ldh059

Perry, V., & O'Connor, V. (2010). The role of microglia in synaptic stripping and synaptic degeneration: a revised perspective. *ASN Neuro, 2*(5), e00047. doi: 10.1042/AN20100024

Perry, V., & Teeling, J. (2013). Microglia and macrophages of the central nervous system: the contribution of microglia priming and systemic inflammation to chronic neurodegeneration. *Semin Immunopathol, 35*, 601 - 612.

Pizza, V., Agresta, A., D'Acunto, C., Festa, M., & Capasso, A. (2011). Neuroinflammation and ageing: current theories and an overview of the data. *Reviews on Recent Clinical Trials, 6*(3), 189-203. doi: doi:10.2174/157488711796575577

Prato, M., Gallo, V., Giribaldi, G., Aldieri, E., & Arese, P. (2010). Role of the NF-kappaB transcription pathway in the haemozoin- and 15-HETE-mediated activation of matris metalloproteinase-9 in human adherent monocytes. *Cell Microbiol, 12*, 1780-1791. doi: 10.1111/j.1462-5822.2010.01508.x

Qian, L., Flood, P., & Hong, J. (2010). Neuroinflammation is a key player in Parkinson's disease and a prime target for therapy. *J Neural Transm, 117*(971-979). doi: 10.1007/s00702-010-0428-1

Ramesh, G., MacLean, A., & Philipp, M. (2013). Cytokines and Chemokines at the Crossroads of Neuroinflammation, Neurodegeneration, and Neuropathic Pain. *Mediators of Inflammation, 2013*, 20. doi: 10.1155/2013/480739

Rao, J., Kellom, M., Kim, H., Rapoport, S., & Reese, E. (2012). Neuroinflammation and synaptic loss. *Neurochemical Research, 37*, 903-910. doi: 10.1007/s11064-012-0708-2

Ricciotti, E., & FitzGerald, G. (2011). Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol, 31*, 986-1000. doi: 10.1161/ATVBAHA.110.207449

Rojo, A., McBean, G., Cindric, M., Egea, J., Lopez, M., Rada, P., . . . Cuadrado, A. (2014). Redox control of microglial function: Molecular mechanisms and functional significance. *Antioxidants and Redox Signaling, 21*(13), 1766-1801. doi: 10.1089/ars.2013.5745

Rojo, L., Fernandez, J., Maccioni, A., Jimenez, J., & Maccioni, R. (2008). Neuroinflammation: implications for the pathogenesis and molecular diagnosis of Alzheimer's disease. *Arch Med Res, 39*(1), 1-16. doi: 10.1016/j.arcmed.2007.10.001

Rubio, N., Cerciat, M., Unkila, M., Garcia-Segura, L.M., & Arevalo, M-A. (2011). An in vitro experimental model of neuroinflammation: the induction of inter-leukin-6 in murine astrocytes infected with Theiler's murine encephalomyelitis virus, and its inhibition by oestreogenic receptor modulators. *Immunology*. 133(3): 360-369. doi: 10.1111/j.1365-2567.2011.03448.x

Rubio-Perez, J., & Morillas-Ruiz, J. (2012). A Reviw: Inflammatory process in Alzheimers disease, role of cytokines. *The scientific world jounal, 2012*. doi: 10.1100/2012/756357

Sabio, G., & Davis, R. (2014). TNF and MAP kinase signalling pathways. *Seminars in Immunology, 26*(3), 237-245. doi: 10.1016/j.smim.2014.02.009

Sagi, Y., Heiman, M., Peterson, J., Musatov, S., Kaplitt, M., Surmeier, D., . . . Greengard, P. (2014). Nitric oxide regulates synaptic transmission between spiny projection neurons. *PNAS, 111*(49), 17636-17641. doi: 10.1073/pnas.1420162111

Salter, M., & Beggs, S. (2014). Sublime Microglia: Expanding Roles for the Guardians of the CNS. *Cell, 158*(1), 15-24. doi: 10.1016/j.cell.2014.06.008

Samuelsson, B., Morgenstern, R., & Jakobsson, P. J. (2007). Membrane prostaglandin E synthase-1: a novel therapeutic target. *Pharmacol Rev, 59*(3), 207-224. doi: 10.1124/pr.59.3.1

Schafer, D., Lehrman, E., Kautzman, A., Koyama, R., Mardinly, A., Yamasaki, R., . . . B, S. (2012). Microglia sculpt post-natal neural circuits in an activity and complement-dependent manner. *Neuron, 74*(4), 691-705.

Scheller, J., Chalaris, A., Schmidt-Arras, D., & Rose-John, S. (2011). The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1813*(5), 878-888. doi: 10.1016/j.bbamcr.2011.01.034

Schlachetzki, J., Fiebich, B., Haake, E., deOliveira, A., Candelario-Jalil, E., heneka, M., & Hull, M. (2010). Norepinephrine enhances the LPS-induced expression of COX-2 and secretion of PGE2 in primary rat microglia. *Journal of neuroinflammation, 7*(2). doi: 10.1186/1742-2094-7-2

Schlagenhauf-Lawlor, P. (2007) Travelers' malaria. PMPH-USA

Schmuck, G., Roehrdanz, E., Haynes, R.K., & Kahl, R. (2002). Neurotoxic mode of action of artemisinin. *Antimicrobial agents and Chemotherapy*. 46(3), 821-827. doi: 10.1128/aac.46.3.821-827.2002

Schofield, L., & Grau, G. (2005). Immunological processes in malaria pathogenesis. *Nature Reviews Immunology, 5*, 722-735.

Shakir, L., Hussain, M., Javeed, A., Ashraf, M., & Riaz, A. (2011). Artemisinins and immune system. *European Journal of Pharmacology, 668*(1–2), 6-14. doi: 10.1016/j.ejphar.2011.06.044

Shastri, A., Bonifati, D., & Kishore, U. (2013). Innate Immunity and Neuroinflammation. *Mediators of Inflammation,* 2013, 19. doi: 10.1155/2013/342931

Sheridan, G.K. & Murphy K.J. (2013). Neuron-glia crosstalk in health and disease: fractalkine and CX_3CR1 take centre stage. *Royal Society Open Biology,* 3: 130181. doi: 10.1098/rsob.130181

Shi, J., Zhang, C., Sun, X., Cheng, X., Wang, J., Zhang, Y., . . . Zou, H. (2013). Antimalarial drug artemisinin extenuates amyloidogenesis and neuroinflammation in APPswe/PS1dE9 transgenic mice via inhibition of nuclear factor kappaB and NLRP3 inflammasome activation. *CNS Neurosci Ther, 19*(2013), 262-268. doi: /cns.12066

Shin, S.G., Kang, J.K., Lee, K.R., Lee, H.W., Han, J.W., & Choi, W.S. (2005). Suppression of inducible nitric oxide and cyclooxygenase-2 expression in Raw 264.7 macrophages by sesquiterpene lactones. *J Tox Environ Health,* 68 (23-24), 2119-2131. doi: 10.1080/15287390591009506

Shulman, J., DeJager, P., & Feany, M. (2010). Parkinson's Disease: Genetics and Pathogenesis. *Annual Review of Pathology: Mechanisms of Disease, 6*, 193-222. doi: 10.1146/annurev-pathol-011110-130242

Siedle, B., Garcia-Pineres, A..J., Murillo, R., Schulte-Monting, J., Castro, V., Rungeler, P., Klaas, C.A., Da Costa, F.B., Kisiel, W., & Merfort, I. (2004). Quantitative structure-activity relationship of sesquiterpene lactones as inhibitors of the transcription factor NF-kappaB. *J Med Chem,* 47 (24), 6042 - 6054. doi: 10.1021/jm049937r

Sierra, A., Encinas, J., Deudero, J., hancey, J., Enikolopov, G., Overstreet-Wadiche, L., . . . Maletic-Savatic, M. (2010). Microglia shapes adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell Stem Cell, 7*(4), 483-495.

Sierra, A., Abiega, O., Shahraz, A., & Neumann, H. (2013). Janus-faced microglia: beneficial and detrimental consequences of microglial phagocytosis. *Frontiers in cellular neuroscience.* 7 (6), 1-22, doi: 10.3389/fncel.2013.00006.

Siljehav, V., Hofstetter, A., Jakobsson, P., & Kerlenius, E. (2012). mPGES-1 and prostaglandin E2: vital role in inflammation, hypoxic response, and survival. *Pediatric Research, 72*(5), 460-468. doi: 10.1038/pr.2012.119

Singh, A., & Sarin, R. (2010). *Artemisia scoparia* - A new source of artemisinin. *Bangladesh J Pharmacol.* 5, 17-20

Song, M., Kim, E., Moon, W., Park, J., Kim, H., So, H., . . . Park, B. (2009). Sulforaphane protects against cytokine- and streptozotocin-induced β-cell damage by suppressing the NF-κB pathway. *Toxicology and Applied Pharmacology, 235*(1), 57-67. doi: 10.1016/j.taap.2008.11.007

Srinivasan, M., & Lahiri, D. (2015). Significance of NF-kappaB as a pivotal therapeutic target in the neurodegenerative pathologies of Alzheimer's disease and multiple sclerosis. *Expert Opinion Therapeutic Targets*, 1-17.

Steffan, N., Bren, G., Frantz, B., Tocci, M., O'Neill, E., & Paya, C. (1995). Regulation of IkB alpha phosphorylation by PKC- and Ca(2+)-dependent signal transduction pathways. *J Immunol, 155*(10), 4685-4691.

Steglich, B., Mahringer, A., Li, Y., Posner, G.H., Fricker, G.,& Efferth T. (2012). Inhibition of p-glycoprotein by two artemisinin derivatives. *Natural Products and Bioprospecting*. 2(2), 59-64. doi: 10.1007/s13659-012-0006-3

Surh, Y., Kundu, J., Li, M., Na, H., & Cha, Y. (2009). Role of Nrf2-mediated heme oxygenase-1 upregulation in adaptive survival response to nitrosative stress. *Archives of Pharmacal Research, 32*(8), 1163-1176. doi: 10.1007/s12272-009-1807-8

Tambuyzer, B., Ponsaerts, P., & Nouwen, E. (2009). Microglia: gatekeepers of central nervous system immunology. *Journal of Leukocyte Biology, 85*(3), 352-370. doi: 10.1189/jlb.0608385

Tansey, M., & Goldberg, M. (2010). Neuroinflammation in Parkinson's disease: Its role in neuronal death and implications for therapeutic interventiion. *Neurobiology of disease, 37*(3), 510-518. doi: 10.1016/j.nbd.2009.11.004

Tao, R., Ji, Y., Lu, Y., Fukunaga, K., & Han, F. (2012). Targeting Nitrosative stress for neurovascular protection: new implications in brain diseases. *Current Drug Targets, 13*, 272-284. doi: 10.2174/138945012799201649

Tripathi, A.K., Sullivan, D.J., & Stins, M.F. (2007). Plasmodium falciparum-infected erythrocytes decrease the integrity of human bllo-brain barrier endothelial cell monolayer. *Infect Dis*. 195 (7), 942 - 950. doi: 10.1086/512083

vanMeerloo, J., Kaspers, G.J.L., & Cloos, J. (2011). Cell sensitivity assays: the MTT assay. *Methods in Molecular Biology.* 731, 237-245.

Virarkar, M., Alappat, L., Bradford, P., & Awad, A. (2012). L-Arginine and Nitric Oxide in CNS Function and Neurodegenerative Diseases. *Critical Reviews in Food Science and Nutrition, 53*(11), 1157-1167. doi: 10.1080/10408398.2011.573885

Wang, J., Hou, L., Yang, Y., Tang, W., Li, Y., & Zuo, J. (2009). SM905, an artemisinin derivative, inhibited NO and pro-inflammatory cytokine production by suppressing MAPK and NF-kappaB pathways in RAW 264.7 macrophages. *Acta Pharmacologica Sinica, 30*, 1428-1435. doi: 10.1038/aps.2009.138

Wang, J., Zhou, H., Zheng, J., Cheng, J., Liu, W., Ding, G., . . . Zhang, L. (2006). The antimalarial artemisinin synergizes with antibiotics to protect against lethal live Escherischia coli challenge by decreasing proinflammatory cytokine release. *Antimicrobial Agents and Chemotherapy, 50*, 2420-2427. doi: 10.1128/AAC.01066-05

Wang, N., Liang, H., & Zen, K. (2014). Molecular Mechanisms That Influence the Macrophage M1–M2 Polarization Balance. *Frontiers in Immunology, 5*, 614. doi: 10.3389/fimmu.2014.00614

Wang, Y., Huang, Z., Wang, L., Meng, S., Fan, Y., Chen, T., . . . Wang, C. (2011). The anti-malarial artemisinin inhibits pro-inflammatory cytokines via the NF-kB canonical signaling pathway in PMA-induced THP-1 monocytes. *Int J Mol Med, 27*(2), 233-241. doi: 10.3892/ijmm.2010.580

Welser-Alves, J., & Milner, R. (2013). Microglia are the majour source of TNF-alpha and TGF-beta1 in postnatal glial cultures, regulation by cytokines, lipopolysaccharide, and vitronectin. *Neurochemistry Internationall, 63*(1), 47-53. doi: 10.1016/j.neuint.2013.04.007

Westman, M., Korotkova, M., Klint, E. a., Stark, A., Audoly, L., Klareskog, L., . . . Kakobsson, P. (2004). Expression of microsomal prostaglandin E synthase 1 in rheumatoid arthritis synovium. *Arthritis Rheum, 50*(6), 1774-1780. doi: 10.1002/art.20286

White, N.J. (2008). Qinghaosu (Artemisinin): The price of success. *Science*, 320, 330-334. doi: 10.1126/science.1155165

Whitney, N.P., Eidem, T.M., Peng, H., Huang, Y., & Zheng, J.C. (2009). Inflammation mediates varying effects in neurogenesis: relevance to the pathogenesis of the brain injury and neurodegenerative disorders. *J Neurochem.* 108(6), 1343-1359. doi: 10.1111/j.1471-4159.2009.05886.x

Whalley, K. (2014). Neuroinflammation: Transport disruption in multiple sclerosis. *Nature Reviews Neuroscience, 16*(1), 2. doi: 10.1038/nrn3892

Woodrow, C., Haynes, R.K., & Krishna, S. (2005). Artemisinins. *Postgrad Med J,* 81, 71 - 78. doi: 10.1136/pgmj.2004.028399

Wu. (2011). Investigation of anti-inflammtory effect of artemether in mouse model of colitis. *Inflammatory Bowel diseases, 17*, S18-S19. doi: 10.1002/ibd.21581

Xiang, M., Namani, A., Wu, S., & Wang, X. (2014). Nrf2: bane or blessing in cancer? *Journal of Cancer Research and Clinical Oncology, 140*(8), 1251-1259. doi: 10.1007/s00432-014-1627-1

Xing, B., Bachstetter, A., & Eldik, L. (2011). Microglial p38alpha MAPK is critical for LPS-induced neuron degeneration, through a mechanism involving TNFalpha. *Mol Neurodegener, 6*, 84.

Xing, B., Bachstetter, A., & Eldik, L. V. (2014). Inhibition of neuronal p38alpha, but not p38beta MAPK, provide4s neuroprotection against three different neurotoxic insults. *J Mol Neurosci*. doi: 10.1007/s12031.014-0372-x

Xu, H., He, Y., Yang, X., Liang, L., Zhan, Z., Ye, Y., . . . Sun, L. (2007). Anti-malarial agent artesunate inhibits TNF-alpha-induced production of proinflammatory cytokines via inhibition of NF-kappaB and PI3 kinase/Akt signal pathway in human rheumatoid arthritis fibroblast-like synoviocytes. *Rheumatology (Oxford), 46*(6), 920-926. doi: 10.1093/rheumatology/kem014

Yamanaka, K., Chun, S., Boillee, S., Fujimori-Tonou, N., Yamashita, H., Gutmann, D., . . . Cleveland, D. (2008). Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nature Neuroscience, 11*, 251-253.

Yang, Y., Ding, J., Yang, C., Gao, Y., Li, X., Chen, X., Peng, Y., Fang, J., & Xiao, S. (2012). Immunomodulatory and anti-inflammatory properties of artesunate in experimental colitis. *Current Med Chem,* 19 (26), 4541 - 4551. doi: 10.2174/092986712803251575

Ydens, E., Lornet, G., Smits, V., Goethals, S., Timmerman, V., & Janssens, S. (2013). The neuroinflammatory role of Schwann cells in disease. *Neurobiology of Disease, 55*, 95-103. doi: 10.1016/j.nbd.2013.03.005

Yu, W.Y., Kan, W.J., Yu, P.X., Li, M.M., Song, J.S., & Zhao, F. (2012). Anti-inflammatory effect and mechanism of artemisinin and dihydroartemisinin. *Zhongguo Zhong Yao Za Zhi,* 37 (17), 2618-2621

Zhang, L., Liu, Z., Ye, J., Sha, M., Qian, H., Bu, X., . . . Ye, L. (2014). Artesunate exerts an anti-immunosuppressive effect on cervical cancer by inhibiting PGE2 production and Foxp3 expression. *Cell Biology International, 38*(5), 639-646. doi: 10.1002/cbin.10244

Zhang, M., An, C., Gao, Y., Leak, R., Chen, J., & Zhang, F. (2013). Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. *Progress in Neurobiology, 100*(1), 30-47.

Zhang, W., Wang, T., Pei, Z., Miller, D., Wu, X., Block, M., . . . Zhang, J. (2005). Aggregated alpha-synuclein activates microglia: a process leading to disease progression in Parkinson's disease. *Faseb J, 19*, 533-542.

Zhao, C., Ling, Z., Newman, M., Bhatia, A., & Carvey, M. (2007). TNF-alpha knockout and minocycline treatment attenates blood-brain barrier leakage in MPTP-treated mice. *Neurobiology of Disease, 26*, 36-46. doi: 10.1016/j.nbd.2006.11.012

Zhu, C., Xiong, Z., Chen, X., Peng, F., Hu, X., Chen, Y., & Wang, Q. (2012). Artemisinin attenuates lipopolysaccharide-stimulated proinflammatory responses by inhibiting NF-kappaB pathway in microglia cells. *PLoS One, 7*(4), e35125. doi: 10.1371/journal.pone.0035125

Zingarelli, B., Hake, P.W., Denenberg, A., (2002). Sesquiterpene lactone parthenolide, an inhibitory of IkB kinase complex and nuclear factor-kappaB, exerts beneficial effects in mycocardial reperfusion injury. *Shock*. 17: 127

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EDUCATION AND QUALIFICATIONS

University of Huddersfield PhD. Pharmacology Jan. 2012 – July 2015
Completion date: July 2015

University of Bradford MSc. Drug Discovery Sept. 2009 – Dec. 2010

University of Aberdeen BSc. (Hons) Pharmacology Sept. 2005 – July 200
Result obtained: 2.1

British Pharmacological Society/ Physiological Society Short Course April and June 2008
University of Glasgow
Personal Licence Holder Training and Investigation of Physiological Function *in vivo* using rodents and rabbits

University of Port Harcourt, Port Harcourt, Nigeria Sept. 2004 – June 2005
BSc Pharmacy (Left after foundation year to study at University of Aberdeen for BSc. Pharmacology)

Dority International Secondary School, Aba, Nigeria Sept. 2001 – July 2004
*O Level*s: Mathematics (A), Chemistry (A), Biology (B), Physics (B), English Language (B), Further Mathematics (A)

RESEARCH EXPERIENCE

Doctoral Research Jan. 2012 – July 2015
PhD VIVA – done, Completion date: 13 July 2015
Project title: Inhibition of neuroinflammation by artemisinin and its derivatives

Postgraduate Masters Research May 2010 – Sept. 2010
Project title: Validation and optimization of novel CXCR4 antagonist, ICT5040

Honours Research Project Jan. 2009 – May 2009
Project title: GPR55 mediated Modulation of Adhesion and Angiogenesis in Breast Cancer cell

PUBLICATIONS

1) Velagapudi R, Baco G, Khela S, Okorji UP, Olajide O (2015) Pomegranate inhibits neuroinflammation and amyloidogenesis in IL-1β- stimulated SK-N-SH cells. Eur Journal of Nutrition. doi: 10.1007/s00394-015-0984-0
2) Okorji UP, Olajide OA (2014) A semi-synthetic derivative of artemisinin, artesunate inhibits prostaglandin E2 production in LPS/IFNγ-activated BV-2 microglia. Bioorganic and Medicinal Chemistry, Jul 11. pii: S0968-0896(14)00510-0. doi: 10.1016/j.bmc.2014.07.007
3) Olajide OA, Kumar A, Velagapudi R, Okorji UP, Fiebich BL (2014) Punicalagin inhibits neuroinflammation in LPS-activated rat primary microglia. Mol Nutr Food Res, Jul 28. doi: 10.1002/mnfr.201400163
4) Olajide, OA, Velagapudi, R, Okorji, UP, Sarker, SD, Fiebich, BL (2014) *Picralima nitida* seeds suppress PGE_2 production by interfering with multiple signaling pathways in IL-1β-stimulated SK-N-SH neuronal cells *Journal of Ethnopharmacology* pii:S0378-8741 (14) 00074-9. doi: 10.1016/j.jep.2014.01.027
5) Olajide, OA, Aderogba, MA, Okorji, UP, Fiebich, BL (2012) Bridelia ferruginea produces antineuroinflammatory activity through inhibition of Nuclear Factor-kappa B and p38 MAPK signaling. *Evidence-based Complementary and Alternative Medicine* 2012: 546873. doi: 10.1155/2012/546873

CONFERENCE POSTERS

Okorji UP, Olajide OA (2014) NF-κB and p38 signalling-dependent inhibition of neuroinflammation by artemether. British Pharmacology Society Conference Proceeding
Okorji UP, Olajide OA (2013) Artesunate inhibits prostaglandin E_2 (PGE_2) production in LPS + IFN-γ activated BV-2 microglia cells. British Pharmacology Society Conference Proceeding

Okorji UP, Olajide OA (2012) Artemisinin inhibits nitrite and prostaglandin production from Lipopolysaccharide (LPS)-stimulated BV-2 microglia. APS PharmSci 2012 Conference Proceeding

CONFERENCE PRESENTATION

Okorji, UP, Olajide, OA (2013) The Antimalarial drug artemisinin inhibits neuroinflammation in LPS-activated BV-2 microglia. West African Society for Pharmacology 2013 Conference Proceeding

COMPETENCY PROFILE

Research Skills

- Competent in cell culture, biochemical and molecular biology including ELISA, SDS-PAGE, western blot, gene transfection, reporter assays, EMSA, RNA interference, qPCR, immunocytochemistry, flow cytometry and functional assays as well as *in vivo* techniques (personal licence in rodents and rabbit). Good experience in HPLC and Mass Spectrometer.

Teaching Skills

- Support laboratory classes and tutorials for undergraduate and master's students as well as supervision of their research projects.

IT/Computing

- Proficient in the use of a wide range of IT applications including Microsoft Word, PowerPoint, Publisher and Excel Packages.

Presentation Skills

- Competent in preparation and presentation of research works, articles and posters.

Legislative Awareness

- Good knowledge of legislative requirements of COSHH. I equally have a good understanding of GLP, GMP and GCP regulations.

Leadership / Administrative/ Organisational Skills

- Managed current research laboratory and organised group meetings.
- Represented postgraduate students as School of Applied Science School Board - 2013/2014.
- Successfully led a group of 6 in carrying out a research review project on the involvement of genetics in breast cancer during undergraduate degree.

Languages

- English and Basic French

PROFESSIONAL AFFILIATIONS

- British Pharmacology Society 2008 to Present
- British Physiological Society 2012 to Present
- Academy of Pharmaceutical Sciences of Great Britain 2015 to Present

AWARDS AND ACHIEVEMENTS

- Huddersfield Association of Women Graduates (AWG) Postgraduate Award 2014
- Postgraduate Student representative- School of Applied Science School Board, University of Huddersfield 2013/2014
- Research student representative – Graduate Council University of Huddersfield 2014

LEISURE INTERESTS

- I enjoy travelling, taking walks, and spending time with friends. I equally do cake and sugar crafts in my part time. These I love doing as they help me unwind, relax and improve my creativity.

REFERENCES

Available on request