ALPHA-LIPOIC ACID AND MESUAGENIN C-INDUCED CO-REGULATION OF NF-κB-CYTOKINES AND CHEMOKINES VIA PI3K-AKT/GSK-3B AND ERK1/2 IN *IN VITRO* NEURONAL MODELS

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KUALA LUMPUR

2016

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE UNIVERSITY OF MALAYA
KUALA LUMPUR

2016

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

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Registration/Matric No: SHC120040

Name of Degree: Doctor of Philosophy

Title of Thesis ("this Work"):

Alpha-lipoic acid and mesuagenin c-induced co-regulation of NF-κB, cytokines and chemokines via PI3K-AKT/GSK-3β and ERK1/2 in *in vitro* neuronal models**.**

Field of Study: Biochemistry

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ABSTRACT

(R-)(+)-α-lipoic acid and mesuagenin c were shown to protect the NG108-15 cells against H_2O_2-induced cell death by mitigating the caspase-dependent mitochondrial-mediated pathway. (R)-(+)-α-lipoic acid activated both mTORC1 and mTORC2 components whereas mesuagenin c only activated mTORC2 component which led to activation of PI3K-Akt pathway. This was followed with the reduction of both Bax/Bcl2 and Bax/Bcl-xL ratios and inhibition of cleaved caspase-3. Both compounds suppressed the NF-κB p65 nuclear translocation by inactivating the GSK-3β which reduced IL-6 and TNF-α by increasing the production of IL-10. Following H_2O_2 exposure, the level of CCL21 was significantly increased and pretreatment with both compounds decreased the CCL21 level in NG108-15 cells. Since both compounds modulated the co-regulation of NF-κB, cytokines and chemokine, therefore their anti-neuroinflammatory properties and mechanisms against LPS-stimulated BV-2 cells co-cultured with NG108-15 cells were investigated. Pretreatment with both compounds increased the BV-2 cells viability and inhibited both inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). This was followed with attenuation of nitric oxide, intracellular reactive oxygen species (ROS) and prostaglandin E_2 level. Cytokines protein array revealed that both compounds displayed different efficacy in suppressing the production of anti- and pro-inflammatory chemokines and cytokines. Furthermore, both compounds attenuated the production of Galectin-3 which is required for resident microglia activation. Pretreatment with (R)-(+)-α-lipoic acid and mesuagenin c activated the PI3K-Akt which inactivated GSK-3β(Ser9) and subsequently suppressed NF-κB p65 translocation. Moreover, the addition of lithium chloride and API-2 significantly reversed LPS-induced pro-inflammatory cytokines and chemokines production which accentuated the importance of PI3K-Akt/GSK-3β pathway in modulating NF-κB, cytokines and chemokines co-regulation. Following CCL21 knockdown, both transfected and wild-type NG108-15 co-cultured with BV-2 cells which were pretreated with both compounds displayed suppression of microglial inflammation. Moreover, The knockdown-CCL21, (R)-(+)-α-lipoic acid- and mesuagenin c-microglia conditioned media improved neuronal viability and mitigated neuronal cells death which suggested that downmodulation of CCL21 is crucial in suppressing microgliosis-induced neuronal cell death. Additionally, mesuagenin c induced neuritogenesis by activating of PI3K-Akt and ERK1/2 pathways which increased the neurofilament protein (-70, 150 and 200 kDa) expression. The neuritegenesis observation was abolished following the addition of API-2, UO126 and Wortmannin inhibitors. Interestingly, mesuagenin c modulated the production CCL21, Galectin-1 and other pro-inflammatory cytokines and chemokines as compared to control and inhibitors-treated cells. However, pretreatment with Pertussin Toxin significantly abolished neuritogenesis highlighting the importance of chemokines in neuritogenesis. Following knockdown of CCL21, mesuagenin c-induced neuritogenesis through production of cytokines was reduced as compared to recombinant CCL21-treated cells which suggested CCL21 potential novel role in neuritogenesis. Aberrant co-regulation of NF-κB, cytokines and chemokines is detrimental in neuronal system. Nevertheless, this paradoxical finding may suggest otherwise and merit further investigation of mesuagenin c and CCL21 roles which coincides with its both anti- and pro-inflammatory mechanisms. Taken together, these findings exemplify the natural products ability to modulate NF-κB, chemokines and cytokines reciprocal regulation via PI3K-Akt/GSK-3β and ERK1/2 pathways.

ABSTRAK

(R)-(+)-α-asid lipoik dan mesuagenin c dibuktikan melindungi sel NG108-15 daripada kematian sel teraruh H_2O_2 dengan merencat pengisyaratan pengantaraan mitokondria bersandarkan kaspase. (R)-(+)-α-asid lipoik mengaktifkan kedua-dua komponen mTORC1 dan mTORC2 manakala mesuagenin c hanya mengaktifkan komponen mTORC2 yang seterusnya mengaktifkan pengisyaratan PI3K-Akt. Ini diikuti dengan pengurangan nisbah Bax/Bcl-2 dan Bax/Bcl-xL dan perencatan kaspase-3 terbelah. Kedua-dua sebatian menyekat translokasi nukleus NF-κB p65 dengan menyahaktif GSK-3β yang seterusnya mengurangkan penghasilan IL-6 dan TNF-α menerusi peningkatan IL-10. Tahap CCL21 bertambah secara signifikan selepas didedahkan kepada H_2O_2 dan prarawatan dengan kedua-dua sebatian menurunkan tahap CCL21 dalam sel NG108-15 cells. Memandangkan kedua-dua sebatian memodulasikan pengawalaturan sama NF-κB, sitokina dan kemokina, maka ciri-ciri anti-keradangan saraf dan mekanisme terhadap sel BV-2 dirangsang LPS yang dikultur bersama sel NG108-15 seterusnya disiasat. Prarawatan kedua-dua sebatian meningkatkan viabiliti sel BV-2 dan merencatkan nitrik oksida sintase teraruhkan (iNOS) dan siklooksigenase-2 (COX-2). Ini diikuti dengan penurunan tahap nitrik oksida, intrasellular spesies oksigen reaktif (ROS) dan prostaglandin E_2. Protein 'Array' sitokina menunjukkan kecekapan berbeza kedua-dua sebatian dalam menghalang penghasilan sitokina dan kemokina anti- dan pro-keradangan. Tambahan pula, kedua-dua sebatian menyekat penghasilan Galectin-3 yang diperlukan dalam pengaktifan mikroglia residen. Prarawatan dengan (R)-(+)-α-asid lipoik dan mesuagenin c mengaktifkan isyarat PI3K-Akt yang merencat GSK-3β(Ser9) dan seterusnya menyekat translokasi NF-κB p65. Di samping itu, penambahan litium klorida dan API-2 membalikkan tindakan LPS merangsang penghasilan sitokina dan kemokina pro-keradangan yang menonjolkan kepentingan modulasi pengisyaratan PI3K-Akt/GSK-3β dalam mengawalatur sama NF-κB, sitokina dan kemokina. Kedua-dua sel NG108-15 transfeksi dan asal dikultur bersama sel BV-2 yang dirawat dengan kedua-dua sebatian menunjukkan penyekatan keradangan sel mikroglia setelah CCL21 dilenyapkan. Tambahan itu, mikroglia 'conditioned media' iaitu 'knockdown-CCL21', (R)-(+)-α-asid lipoik dan mesuagenin c menunjukkan peningkatan viabliti dan menghalang kematian sel-sel saraf yang menyarankan penurunan kawalatur CCL21 adalah penting dalam menghalang mikrogliosis merangsang kematian sel-sel saraf. Selain itu, mesuagenin C merangsang neuritogenesis melalui pengaktifan pengisyaratan PI3K-Akt dan ERK1/2 yang meningkatkan ekspresi protein neurofilamen (-70, 150 and 200 kDa). Penambahan perencat-perencat API-2, UO126 dan Wortmannin menghapuskan neuritogenesis. Lebih menarik lagi, rawatan dengan mesuagenin c memodulasikan penghasilan CCL21, Galectin-1, sitokina dan kemokina pro-keradangan apabila dibandingkan dengan sel kawalan tanpa rawatan dan sel dirawat perencat. Walau bagaimanapun, penambahan 'Pertussis Toxin' menghalang neuritogenesis dengan signifikan dan menonjolkan kepentingan kemokina dalam proses neuritogenesis. Setelah CCL21 dilenyapkan, proses mesuagenin c merangsang neuritogenesis menerusi penghasilan sitokina didapati berkurangan apabila dibandingkan dengan sel yang dirawat dengan CCL21 rekombinan. Ini seterusnya mencadangkan kebarangkalian potensi baru CCL21 dalam proses neuritogenesis. Kawalatur bersama NF-κB, sitokina dan kemokina yang aberan akan memudaratkan sistem saraf. Meskipun begitu, hasil kajian paradoksikal ini menyarankan sebaliknya dan ini memerlukan kajian lanjutan fungsi-fungsi mesuagenin c dan CCL21 yang berlaku serentak dengan mekanisme anti- dan pro-keradangannya. Secara kolektif, kajian ini menonjolkan keupayaan produk semulajadi dalam

memodulasikan pengawalaturan resiprokal NF-κB, sitokina dan kemokina menerusi pengisyaratan PI3K-Akt/GSK-3β dan ERK1/2.

ACKNOWLEDGEMENTS

'The starting point of all achievement is desire'

Napoleon Hill

Since young, I always desire to achieve big dreams in my life. Definitely, one of the dreams is to have awarded a Ph.D degree which will allow me to go for the big things in my life. In doing so, I am grateful to the Almighty God for his divine blessing and guidance throughout this journey. All of would be impossible without the guidance of my supervisor, Professor Dr Habsah Abdul Kadir. A brief meeting that we had five years ago (during my undergraduate year) have given me the encouragement and taught me that the measure of who we are is what we do with what we have. She is such an open-minded person who shares her views on academic, research and life and taught me that the value of satisfaction in life come from your hard work. I have encountered many obstacles and mind-boggling observations, but Prof. Dr Habsah was the person who truly placed her trust in me, lent her expertise and allowing me to explore various methods to overcome the hindrances. Such is her dedication and persona that have helped and inspired me along this journey. I may not have thanked her enough all this while and I am taking this opportunity to do so, Thank you, Prof Dr Habsah Abdul Kadir.

I would also like to express my gratitude to Professor Dr Khalijah Awang, for her kind advices, guidance and most importantly, her agreement and permission to work with Dr Chan Gomathi. A special thank to Dr Chan Gomathi, for her generous supply of the compound, and the collaborative work that we have undertaken that led to the isolation and biactivities of mesuagenin c.

I would like to thank this institution, University of Malaya for providing the funding (HIR-Chancellery, FRGS, UMRG and PPP grants) that have allowed me to conduct the research. Along with this, I am thankful for the kind helps and cooperation given by the Head, science officers and staffs of Institute Science of Biology as well as Faculty of Science overall. In addition to this, I would love to thank my colleagues, Chan Chim Kei, Hadi Supriady, Lo Jia Ye and Sharifah Salwa for the awesome experience of working together, sharing ideas and for tolerating with my intense working attitude.

I believe the good things in life come from what we care about and for that, I would like to thank both of my parents, Mr Kamarudin bin Kichut and Mrs Noor Azizah bt Ahmad for constantly providing the kind advices, the constant supports and being patient when I needed it the most. I cannot thank them enough for their trust and hopes in me throughout this journey. Not to forget, my siblings, Siti Nawar, Nur Khairunnisaq, Nurul Sazwani and Mohd Hisham, who always stand by my side, providing their supports and couraging words especially throughout this journey.

Joy is the simplest form of gratitude.

Karl Bath

Thank you.

TABLE OF CONTENTS

Abstract iv

Abstrak v

Acknowledgements vii

Table of Contents ix

List of Figures xvi

List of Tables xx

List of Abbreviations xxi

List of Symbols xxvii

List of Appendices xxviii

CHAPTER 1: INTRODUCTION 1

CHAPTER 2: LITERATURE REVIEW 5

2.1 Neuronal cell death in neurodegenerative diseases 5

2.2 Neuroinflammation as the key event of neurodegenerative diseases 6

2.2.1 Oxidative stress and inflammatory mediators in neuroinflammation 7

2.2.1.1 Reactive oxygen species 7

2.2.1.2 Nitric oxide (NO) and inducible nitric oxide synthase (iNOS) 9

2.2.1.3 Cyclooxygenase-2 (COX-2) and Prostaglandin E_2 (PGE_2) 10

2.2.2 Cytokines as the major modulator of neuroinflammation 12

2.2.2.1 Interleukin-4 (IL-4) 16

2.2.2.2 Interleukin 6 (IL-6) 17

2.2.2.3 Interleukin-10 (IL-10) 19

2.2.2.4 Tumor necrosis factor-alpha (TNF-α) 21

2.2.3 Chemokines in brain inflammation and function 22

2.2.3.1 CCL21 as neuron-glia signaling mediator in inflammation 27
2.2.4 The roles of galectins in central nervous system 29
2.2.4.1 Galectin-1 in neuronal differentiation and inflammation 29
2.2.4.2 Galectin-3 as the inducer of microglia activation 30
2.2.5 The modulation of innate immunity in the brain 32
2.2.6 Microglia as the guardian of innate immunity 34
2.2.7 Toll-like receptors (TLRs) in innate immunity 38
2.2.7.1 Toll-like receptor 4 regulation in neuroinflammation 40
2.3 Neuronal apoptosis in neurodegenerative diseases 44
2.3.1 Molecular machinery of neuronal apoptosis 45
2.4 Neuritogenesis in the central nervous system 48
2.5 The signaling pathways in neuroprotection and neuritogenesis 49
2.5.1 Mammalian target of rapamycin (mTOR) 49
2.5.2 Phosphatidylinositol 3-kinase (PI3K) - protein kinase B (AKT) 53
2.5.3 Glycogen synthase kinase-3 (GSK-3) in neuroinflammation 57
2.5.4 Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) – the major transcription factor in neuronal processes 60
2.6 Mitogen-activated protein kinases (MAPKs) signaling pathways 63
2.7 Therapeutic approaches in neurodegenerative diseases 67
2.7.1 Natural products as therapeutic agents 69
2.7.1.1 Alpha-Lipoic Acid 70
2.7.1.2 Mesuagenin c from *Mesua kunstleri* 72

CHAPTER 3: 77

3.1 Introduction 78
3.2 Literature review 80
3.3 Materials and method 82

3.3.1 Cell culture and materials 82
3.3.2 Cell viability assay 82
3.3.3 Nitric oxide (NO) assay 83
3.3.4 Measurement of intracellular ROS level 83
3.3.5 Determination of Prostaglandin E_2 (PGE_2) level 83
3.3.6 Western blot analysis 84
3.3.7 CCL21 knockdown in NG108-15 cells 84
3.3.8 Real-time quantitative polymerase chain reaction (qPCR) analysis 85
3.3.9 Cytokines and chemokines antibody array 86
3.3.10 Co-culture of BV-2 and NG108-15 cells 86
3.3.11 Flow cytometric cytokine bead array (CBA) 87
3.3.12 Data analysis 87
3.4 Results 87
3.4.1 (R)-(+)-α-lipoic acid and mesuagenin c protected BV-2 cells against LPS-induced toxicity 87
3.4.2 (R)-(+)-α-lipoic acid and mesuagenin c reduced intracellular ROS, NO and PGE_2 level by attenuating COX-2 and iNOS in BV-2 cells 89
3.4.3 (R)-(+)-α-lipoic acid- and mesuagenin c-induced rapid PI3K-Akt activation and GSK-3β inactivation is essential in suppressing LPS-induced NO and PGE_2 in BV-2 cells 93
3.4.4 (R)-(+)-α-lipoic acid and mesuagenin c suppressed NF-κB p65 nuclear translocation through Akt activation and GSK-3β inactivation 97
3.4.5 (R)-(+)-α-lipoic acid and mesuagenin c suppressed galectin-3, pro-inflammatory cytokines and chemokines production by augmenting IL-4 and IL-10 through PI3K-Akt/GSK-3β pathway 98

3.4.6 CCL21 knockdown enhanced (R)-(+)-α-lipoic acid and mesuagenin c protective effects against LPS and inflammatory responses in neuron-glia co-culture system through Akt activation....103

3.5 Discussion....107

3.6 Conclusion....117

CHAPTER 4: 118

4.1 Introduction....119

4.2 Literature review....120

4.3 Materials and method....121

4.3.1 Cell culture and materials....121

4.3.2 Cell viability assay....122

4.3.3 Nuclear morphological analysis....122

4.3.4 Total intracellular glutathione (GSH) content....123

4.3.5 Mitochondrial membrane potential ($\Delta\psi m$)....123

4.3.6 Cytomteric detection of phosphatidylserine externalization....124

4.3.7 Measurement of intracellular ROS level....124

4.3.8 Cytometric immunofluorescence staining of Bax and Bcl-2 protein....125

4.3.9 Assessment of Caspase-3/7 & -9 activities....125

4.3.10 Western blot analysis....126

4.3.11 NF-κB p65 translocation assay....127

4.3.12 Cytokines measurement....127

4.3.13 Data analysis....127

4.4 Results....128

4.4.1 Mesuagenin c protected NG108-15 against H_2O_2-induced cell death....128

4.4.2 Mesuagenin c suppressed H_2O_2-induced nuclear morphologic changes in NG108-15 cells....128

4.4.3 Mesuagenin c mitigated the externalization of phosphatidylserine 130
4.4.4 Mesuagenin c increased the intracellular GSH level 130
4.4.5 Mesuagenin c attenuated $\Delta\psi m$ dissipation in NG108-15 cells 133
4.4.6 Mesuagenin c modulated Bax and Bcl-2 protein expression 133
4.4.7 Mesuagenin c inhibited activation of caspase-3/7 and -9 136
4.4.8 Mesuagenin c induced activation of PI3K-Akt via mTORC2 138
4.4.9 Mesuagenin c modulated cytokines production by inactivating GSK-3β and NF-κB p65 translocation 138
4.5 Discussion 144
4.6 Conclusion 150

CHAPTER 5: 151

5.1 Introduction 152
5.2 Literature review 153
5.3 Materials and method 155
5.3.1 Cell culture and material 155
5.3.2 Cell viability assay 155
5.3.3 Nuclear morphological analysis 156
5.3.4 Determination of glutathione/glutathione disulfide (GSH/GSSG) ratio 156
5.3.5 Mitochondrial membrane potential ($\Delta\psi m$) 156
5.3.6 Measurement of intracellular ROS level 157
5.3.7 Western blot analysis 157
5.3.8 NF-κB p65 translocation assay 158
5.3.9 Cytokines measurement 158
5.3.10 Data analysis 158
5.4 Results 159
5.4.1 (R)-(+)-α-lipoic acid protected against H_2O_2-induced cell death 159

5.4.2 (R)-(+)-α-lipoic acid suppressed nuclear morphological changes 159
5.4.3 (R)-(+)-α-lipoic acid attenuated intracellular ROS by augmenting the GSH/GSSG ratio 162
5.4.4 (R)-(+)-α-lipoic acid inhibited dissipation of $\Delta\psi m$ 164
5.4.5 (R)-(+)-α-lipoic acid modulated apoptotic proteins and caspase-3 expression 164
5.4.6 Pretreatment with (R)-(+)-α-lipoic acid induced PI3K-Akt activation via mTORC1 and mTORC2 168
5.4.7 Inactivation of GSK-3β by (R)-(+)-α-lipoic acid inhibited NF-κB p65 translocation and modulated cytokines production 171
5.5 Discussion 174
5.6 Conclusion 182

CHAPTER 6: 183

6.1 Introduction 184
6.2 Literature Review 185
6.3 Materials and Method 186
6.3.1 Cell culture and materials 186
6.3.2 Cell viability assay 187
6.3.3 Neuritogenesis assessment of mesuagenin c in NG108-15 Cells 188
6.3.4 Immunofluorescence staining and microscopy analysis 188
6.3.5 Western blot analysis 189
6.3.6 CCL21 knockdown in NG108-15 cells 189
6.3.7 Real-time quantitative polymerase chain reaction (qPCR) analysis 190
6.3.8 Cytokines and chemokines antibody array 191
6.3.9 Data analysis 191
6.4 Results…. 192

6.4.1 Toxicity and cell proliferative effects of mesuagenin c 192
6.4.2 Mesuagenin c induced neuritogenesis in NG108-15 cells 194
6.4.3 Mesuagenin c enhanced the expression of neurofilament protein 198
6.4.4 Mesuagenin c induced neuritogenesis through the rapid activation of Akt and ERK1/2 in NG108-15 cells 198
6.4.5 Mesuagenin c induced full activation of Akt via mTORC2 199
6.4.6 Activation PI3K-Akt, ERK1/2 and chemokines by mesuagenin c induced activation of NF-κB p65 204
6.4.7 Mesuagenin c modulated galectin-1, pro-inflammatory cytokines, chemokines and CCL21 through PI3K-Akt and ERK1/2 signaling pathways 207
6.5 Discussion 212
6.6 Conclusion 219

CHAPTER 7: CONCLUSION 220

References 223
Appendix A- Papers Presented and Awards 273
Appendix B- List of Publication 276
Appendix C- SPECTROSCOPIC DATA OF MESUAGENIN C 287

LIST OF FIGURES

Figure 2.1	Figure 2.1 The production of ROS, NO, peroxynitrite (ONOO-) and PGE2 through iNOS and COX-2 in the activated astrocytes and microglia	14
Figure 2.2	The aberrant regulation of pro-inflammatory cytokines, ROS and RNS exacerbate the activation of microglia that leads to neuronal cell death.	15
Figure 2.3	The regulation of chemokines and receptors in chronic neuroinflammation observed in neurodegenerative diseases.	26
Figure 2.4	The role of microglia in inducing the innate immune response.	37
Figure 2.5	The activation of TLR4 through MyD88-dependent and MyD88-independent (TRIF cascade).	43
Figure 2.6	The execution of apoptosis through mitochondrial (intrinsic) and death receptor (extrinsic) pathways.	47
Figure 2.7	The cellular signaling following mTOR activation by various signals.	52
Figure 2.8	The activation of PI3K and its downstream signaling through the activation of GPCRs and RTK.	56
Figure 2.9	The signaling pathways (PI3K-Akt, mTOR and wingless (Wnt)–frizzled (Fz)) that activates or inactivates GSK-3β.	59
Figure 2.10	The role of NF-κB within the cellular context of the nervous system.	62
Figure 2.11	Mechanism of ERK activation through the tyrosine kinases receptor (TRKs) or G protein-coupled receptors (GPCRs).	66
Figure 2.12	Structure of (A) (R)-(+)-α-lipoic acid (B) (S)-(-)- α-lipoic acid.	73
Figure 2.13	The bark of M. kunstleri (b) Chemical structure of mesuagenin c.	76

Figure 3.1	The protective effects of (R)-(+)-α-lipoic acid and mesuagenin c against LPS-induced toxicity in BV-2 cells.	88
Figure 3.2	The effects of (R)-(+)-α-lipoic acid and mesuagenin c on LPS-induced the production of NO, PGE2 and intracellular ROS.	91
Figure 3.3	Western blot analysis of (A) (R)-(+)-α-lipoic acid (100 μM) and (B) Mesuagenin c (20 μM) on the activation of Akt(Ser473), total Akt and GSK-3β(Ser9) inactivation.	94
Figure 3.4	The suppression of NF-κB p65 nuclear translocation through activation of Akt and inactivation of GSK-3β (Ser9) by (R)-(+)-α-lipoic acid and mesuagenin c.	99
Figure 3.5	Figure 3.5 The modulatory effects of (R)-(+)-α-lipoic acid and mesuagenin c on Galectin-3, inflammatory cytokines and chemokines production through PI3K-Akt/GSK-3β pathway.	102
Figure 3.6	The suppression of LPS-induced toxicity and neuron-glia inflammation by (R)-(+)-α-lipoic acid and mesuagenin c vida modulation of IL-4, IL-6, IL-10 nd TNF-α following triple knockdown of CCL21 (48 – 120 h).	105
Figure 3.7	(R)-(+)-α-lipoic acid and mesuagenin c suppress the inflammation by modulating NF-κB, cytokines, chemokines and Gal-3 via PI3K-Akt/GSK-3β in LPS-stimulated BV-2 co-cultured with NG108-15 cells.	117
Figure 4.1	Dose-dependent increase in cell viability by pretreatment with mesuagenin c in H_2O_2-induced cell death prior to $H2O2$ exposure for 10 h.	129
Figure 4.2	Mesuagenin c prevented H_2O_2-induced morphological changes in NG108-15 cells.	129
Figure 4.3	The effect of mesuagenin c on the externalization of PS in NG108-15.	131
Figure 4.4	The effect of mesuagenin c on total intracellular GSH level.	132
Figure 4.5	Mesuagenin c mitigated alterations in the Δψm of NG108-15 cells.	134

Figure 4.6	Mesuagenin c modulated Bax and Bcl-2 protein expression in H_2O_2-treated NG108-15 cells.	135
Figure 4.7	Mesuagenin c prevented H_2O_2-induced activation of caspase-3 and caspase-9 in H_2O_2-treated NG108-15 cells.	137
Figure 4.8	Western blot analysis of mesuagenin c on Akt(Th308, Ser473), mTOR and rictor expression in NG108-15 cells.	139
Figure 4.9	Western blot analysis of mesuagenin c on pGSK-3β (Ser9) and total GSK-3β and NF-κB p65 translocation.	142
Figure 4.10	Schematic figure of the neuroprotective mechanisms of mesuagenin c in NG108-15 cells through actiavation of PI3K-Akt/GSK-3β and suppression of NF-κB-cytokines.	150
Figure 5.1	Neuroprotective effect of (R)-(+)-α-lipoic acid by MTT cell viability assay.	160
Figure 5.2	(R)-(+)-α-lipoic acid reduced the H_2O_2-induced morphological changes in NG108-15 cells.	161
Figure 5.3	The effect of (R)-(+)-α-lipoic acid on GSH/GSSG and ROS levels.	163
Figure 5.4	Flow cytometric analysis of Δψm in NG108-15 cells following pretreatment with (R)-(+)-α-lipoic acid.	166
Figure 5.5	Western blot analysis of (R)-(+)-α-lipoic acid (LA) on Bax, Bcl-2, Bcl-xL, procaspase-3 and cleaved caspase-3 expression in NG108-15 cells.	167
Figure 5.6	Western blot analysis of (R)-(+)-α-Lipoic acid (LA) on pAktTh308, pAktSer473, total Akt in the absence or presence of H_2O_2 after 2 h.	169
Figure 5.7	Western blot analysis of (R)-(+)-α-lipoic acid (LA) on GSK-3β(Ser9) and NF-κB p65 translocation. Pretreatment with (R)-(+)-α-lipoic acid (LA; 50 µM) and ethyl 3,4-dihydroxycinnamate (10 µM) modulated the cytokines production following H_2O_2 exposure.	172
Figure 5.8	Schematic figure of the neuroprotective mechanisms of (R)-(+)-α-Lipoic acid in NG108-15 cells through actiavation of PI3K-Akt/GSK-3β and NF-κB-cytokines suppression.	182

Figure 6.1	The cytotoxicity evaluation mesuagenin c (0.1 – 100 µM) in NG108-15 cells for 48 h.	193
Figure 6.2	Neuritogenesis evaluation of mesuagenin c at different dose and time in NG108-15 cells.	196
Figure 6.3	Figure 6.3 Immunofluroscence staining and western blot analysis of neurofilament heavy (200 kDa) of mesuagenin c-treated cells (1 µM) from 1 – 48 h. 200	200
Figure 6.4	Mesuagenin c (1 µM) induced rapid activation of Akt and ERK1/2 in NG108-15 cells.	201
Figure 6.5	Mesuagenin c (1 µM) induced the activation of mTORC2.	203
Figure 6.6	Activation of PI3K-Akt by mesuagenin c (MC;1 µM) induced the NF-κB p65 nuclear translocation in NG108-15 cells. Mesuagenin c (1 µM) induced the expression of CCL21 and chemokines during neuritogenesis in NG108-15 cells.	205
Figure 6.7	Modulatory effects of mesuagenin c (MC) on Gal-1, inflammatory cytokines and chemokines production. The phase-contrast neutite outgrowth following CCL21 knockdown, addition of recombinant CCL21, PTx or mesuagenin c.	209
Figure 6.8	Schematic illustration of neuritogenic mechanisms of mesuagenin c in NG108-15 cells.	219
Figure 7.1	Activation of the PI3K-Akt/GSK-β and ERK1/2 pathways that modulate the regulation of NF-κB, cytokines, chemokines and Galectins.	222

LIST OF TABLES

Table 3.1 The primer sequence for CCL21 and HMBS.....85

Table 6.1 The primer sequence for CCL21 and HMBS.....190

LIST OF ABBREVIATIONS

•OH	Hydroxyl radical
Aβ	Amyloid Beta
AChE	Acetylcholinesterase
AIF	Apoptosis-inducing factor
AJs	Adherens junctions
Akt	Protein kinase B
APAF-1	Apoptotic protease-activating factor-1
AP-1	Activator protein -1
APS	Ammonium persulfate
APCs	Antigen presenting cells
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
BAFF	B cell-activating factor
Bak	Bcl-2 Homologous Antagonist/Killer
Bax	Bcl-2-associated X protein
B cells	B lymphocytes
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma extra large
BDNF	Brain-derived neurotrophic factor
BH	Bcl-2 homology
Bid	BH3-interacting domain
BBB	Blood brain barrier
CARD	Caspase activation and recruiting domain
CD4+ T-cell	Cluster of differentiation 4 T helper cells

CNS	Central nervous system
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CRDs	Carbohydrate-recognition domains
CREB	Cyclic amp-responsive element-binding protein
CX3CL1	Fractalkine
DAMPs	Damage-associated molecular patterns
DED	Death effector domain
DEPTOR	DEP domain containing mTOR-interacting protein
DISC	Death-inducing signal complex
DR	Death receptor
DC	Dendritic cells
DMEM	Dulbelcco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
ETC	Electron transport chain
FADD	Fas-associated death domain protein
FasL	Fas ligand
FasR	Fas receptor
FBS	Fetal bovine serum
FoxO	Transcription factors forkhead
GDNF	Glial-derived neurotrophic factor
GPCR	G-protein coupled receptors

GSH	Glutathione
GSK-3β	Glycogen synthase kinase 3β
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HClO	Hypochlorous acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidise
HSP	Small heat shock protein
ICAD	Caspase-activated DNase
IFN-γ	Interferon gamma
IGF	Insulin growth factor
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRS	Insulin receptor substrate
JAK	Janus kinase
JNK/SAPK	c-jun n-terminal kinases/ stress activated protein kinases
LBP	LPS binding protein
LPS	Lipopolysaccharides
LTD	Long-term depression
LTP	Long-term potentiation
MIP1α	Macrophage inflammatory protein 1α
MIP-1β	Macrophage inflammatory protein-1β
Mal/TIRAP	MyD88-adapter-like

MAPK	Mitogen-activated protein kinases
MHC-II	Major histocompatibility complex II
mLST8	Mammalian lethal with sec-13 protein 8
MPTP	Mitochondria permeability transition pores
mRNA	Messenger ribonucleic acid
mSin1	Mammalian stress-activated map kinase-interacting protein 1
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MCP-1	Monocyte chemotactic protein 1
MyD88	Myeloid differentiation primary response protein88
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAIDS	Non-steroidal anti-inflammatory drugs
O2-	Superoxide anion
ONOO-	Peroxynitrite
ONOOH	Peroxynitrous acid
p38 MAPK	p-38 mitogen activated protein kinases
PAMPs	Pathogen-associated molecular patterns

PARP	Poly-ADP-ribose polymerase
PBS	Phosphate buffered saline
PDK-1	Phosphoinositide-dependent kinase-1
PI	Propidium iodide
PI3K	Phosphatidylinositide-3-kinase
PIP2	Phosphatidylinositol (3,4)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PGs	Prostaglandins
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PRAS40	Proline-rich Akt/PKB substrate 40 kDa
PRRs	Pattern recognition receptors
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
Raptor	Regulatory associated protein of mTOR
Rictor	Rapamycin-insensitive companion of mTOR
RIP	Receptor-interacting protein
RNAi	RNA interference
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSK	Protein S6 kinase
SDF-1	Stromal cell-derived factor 1
siRNA	Small interfering RNAs
STAT1	Signal transducers and activators of transcription 1
STAT2	Signal transducers and activator of transcription 2
STAT3	Signal transducers and activator of transcription 3

STAT5	Signal transducers and activator of transcription 5
SVC	Subventricular zone
TAK1	Transforming growth factor beta-activated kinase 1
TBI	Trauma brain injury
T cells	T lymphocytes
TEMED	Tetramethylethylenediamine
Th1	T helper type 1
Th2	T helper type 2
Th17	T helper type 17
TIR	Toll/interleukin-1 (IL-1) receptor domain
TLRs	Toll-like receptors
TLR4	Toll-like receptor-4
TNF-α	tumor necrosis factor- alpha
TNFR1	TNF receptor 1
TNFR2	TNF receptor 2
TRADD	TNF receptor-associated death domain adaptor protein
TRAIL-R1	TNF-related apoptosis inducing ligand receptor 1
TRAIL-R2	TNF-related apoptosis inducing ligand receptor 2
Tram	Trif-related adaptor molecule
Trif	TIR-related adaptor protein inducing interferon
Rtk	Receptor tyrosine kinase
TSC1	Tuberous sclerosis complex 2 or tuberin
TSC2	Tuberous sclerosis complex 1 or tuberin

LIST OF SYMBOLS

α	Alpha
β	Beta
°C	Degree celcius
C	Carbon
δ	Delta
ε	Epsillon
γ	Gamma
g	Gram
h	Hour
H	Hydrogen
kDa	Kilodalton
kg	kilogram
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
µM	Micromolar
µg	Microgram
µL	Microlitre
$\Delta\psi m$	Mitochondrial membrane potential
Na_2CO_3	Sodium bicarbonate
p	Pico
S.E.	Standard error

LIST OF APPENDICES

Appendix A: List of Publications 261

Appendix B: Papers presented and awards 264

CHAPTER 1: INTRODUCTION

Neurodegenerative diseases is an umbrella term for a sphere of conditions which primarily affect the neurons in the human brain which includes broad changes of biochemical pathways that include protein misfolding, oligomerization and aggregation, proteolysis, post-translationalism, activation of stress, inflammation, pro-apoptotic responses and others (Jucker & Walker, 2013; Outeiro et al., 2008; Smith, Das, Ray, & Banik, 2012). Generally, it is known that neurodegenerative diseases are eviscerating conditions that are incurable and will result in progressive or advanced degeneration and/or death of nerve cells. Additionally, dementia which is characterized by the decline in mental ability (memory, learning and other cognitive skills) is shown to be accountable for the greatest burden of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Irwin, Lee, & Trojanowski, 2013; McKhann et al., 2011; Zlokovic, 2011).

In 2013, Alzheimer's Disease International reported that the number of people affected by dementia is estimated to be 135 millions by 2050 with 71% of the population is from the low or middle income countries (Prince et al., 2013). Recently, the latest report projected a total case of 46.8 million of people to be affected by dementia worldwide in 2015 (Pratchett, 2015). The recent statistics also estimated approximately 9.9 million new cases to be reported annually or more accurately one in every 3.2 seconds. Moreover, the total global cost of dementia is valued to be at $818 billion from the economic and social views that include various medical costs, direct social and informal care cost (Pratchett, 2015). Despite numerous advancements in neuroscience research, the etiology of these chronic neurodegenerative diseases remains partly understood. By far, dysregulation of neuronal cell death through aberration inflammation and apoptosis has been shown to be implicated in the pathogenesis of

neurodegenerative diseases (Okouchi, Ekshyyan, Maracine, & Aw, 2007). Stimuli such as trauma and bacterial toxins can initiate the immediate activation of innate immune system in the central nervous system (CNS), particularly the brain (Quan & Banks, 2007). These inflammatory responses include the activation of microglia that are capable of releasing numerous anti- and pro-inflammatory cytokines, chemokines and other inflammatory mediators (Suzumura, 2013) leading to aberrant inflammation and progressive neuronal damage observed markedly in Alzheimer's disease and Parkinson's disease (Lehnardt, 2010a; McGeer & McGeer, 2004). Numerous evidences have demonstrated the involvement of dysregulated inflammatory responses between glial cells and neurons in mediating neuronal cell death and degeneration (Minghetti, 2005).

The current available therapies for neurodegenerative diseases only alleviate the symptoms with moderate level of therapeutic effects (Moore et al., 2010). Moreover, their efficacy in preventing the underlying degeneration of neurons is rather compromised (Vlad, Miller, Kowall, & Felson, 2008). Therefore, the work of this thesis investigate the mitigation of neuronal cell death though suppression of inflammation and apoptosis between the neurons and glial cells via PI3K-Akt/GSK-3β that modulates NF-κB, cytokines and chemokines. The use of cognitive enhancers that promote the reconstruction of complex neuronal circuits is important in attenuating the symptoms of associated cognitive decline particularly in Alzheimer's disease (Kim et al., 2007; Mileusnic, Lancashire, & Rose, 2004; Wang et al., 2011b). Furthermore, various compounds with neuritogenic activity have been demonstrated to improve the memory process in Alzheimer.s disease model (Tohda, Matsumoto, Zou, Meselhy, & Komatsu, 2004; Tohda, Nakamura, Komatsu, & HATTORI, 1999). Even though inflammation can exacerbate neuronal damage, it can also profoundly alter the structure and function of the nervous system (Olofsson, Rosas-Ballina, Levine, & Tracey, 2012). In addition,

pro-inflammatory cytokines such as IFN-γ (Ishii et al., 2013) , IL-4 (Gölz et al., 2006), IL-6 (Yang, Wen, Ou, Cui, & Fan, 2012), IL-17 (Chisholm, Cervi, Nagpal, & Lomax, 2012) and TNF-α (Muñoz-Fernández et al., 1994) have been demonstrated to influence nervous system plasticity by initiating neuritogenesis.

In view of this, the neuroprotective effects of (R)-(+)-α-lipoic acid and mesuagenin c through mitigation of inflammation were first investigated. The anti-inflammatory property of (R)-(+)-α-lipoic acid and mesuagenin c in BV-2 cells was shown to be associated with their ability to regulate NF-κB, cytokines and chemokines via PI3K-Akt/GSK-3β pathway. The neuron-glia inflammatory network though PI3K-Akt/GSK-3β was determined with special attention to chemotactic chemokine, CCL21. Following this, their neuroprotective activity and mechanisms against H_2O_2-induced cell death in NG108-15 cells through PI3K-Akt/GSK-3β were investigated. (R)-(+)-α-lipoic acid and mesuagenin c were demonstrated to protect the NG108-15 against H_2O_2 through the activation of PI3K-Akt/GSK-3β via mTORC1 and/or mTORC2. Additionally, mesuagenin c was shown to mediate neuritogenesis in NG108-15 cells by activating the PI3K-Akt and ERK1/2 pathways that induced NF-κB, inflammatory cytokines and chemokines regulation.

The neuroprotective and neuritogenic properties of natural products that promote neuroprotection, neuroregeneration and ehancement of cognitive function can lead to the development of nutraceuticals for the intervention of neurodegenerative diseases. The combination of neuropotective and neuritogenic activities by natural products together with the deeper understanding on the molecular mechanisms would allow for them to be developed as therapeutics that aim different target sites in every stage of neurodegenerative diseases. Therefore, this thesis aimed to investigate the anti-

inflammatory, neuroprotective and neuritogenic effects of (R)-(+)-α-lipoic acid and mesuagenin c in neuronal models.

The specific objectives of this study were:

1. To determine the anti-neuroinflammatory activities and molecular mechanisms of (R)-(+)-α-lipoic acid and mesuagenin c against LPS-stimulated BV-2 microglial cells.
2. To investigate the modulation of neuron-glia communication through regulation of NF-κB, cytokines and chemokines via PI3K-Akt/GSK-3β pathway by (R)-(+)-α-lipoic acid and mesuagenin c in neuronal models.
3. To determine and establish the involvement of CCL21 in neuroinflammation and neuritogenesis models by using siRNA CCL21 knockdown study.
4. To determine CCL21 regulation by (R)-(+)-α-lipoic acid and mesuagenin c.
5. To evaluate the neuroprotective effects of (R)-(+)-α-lipoic acid and mesuagenin c against H_2O_2-induced neuronal cell death in NG108-15 cells.
6. To elucidate the molecular mechanisms underlying (R)-(+)-α-lipoic acid and mesuagenin c neuroprotective effects against H_2O_2-induced neuronal cell death in NG108-15 cells.
7. To evaluate the neuritogenic activity of mesuagenin c and molecular mechanisms involved in NG108-15 cells.
8. To investigate the involvement and regulation of NF-κB, cytokines and chemokines via PI3K-Akt/GSK-3β pathway by (R)-(+)-α-lipoic acid and mesuagenin c in neuroprotection and neuritogenic models.

CHAPTER 2: LITERATURE REVIEW

2.1 Neuronal cell death in neurodegenerative diseases

The neuronal cell death is an essential component of programmed cell death that regulates the brain homeostasis and normal function (Pettmann & Henderson, 1998). Programmed cell death is a tightly regulated physiological process that helps to remove cells discretely without disturbing the development of neighboring or remaining cells (Bredesen, Rao, & Mehlen, 2006). This physiological cell death are also utilized in most part of the body for the proper organ development that allows the appropriate control of cell number and as a defense mechanism that clear the damaged or infected cells (Bredesen et al., 2006). Programmed cell death can be triggered by various stimuli such as acute ischemia, environmental stress and inflammatory signals that eventually leads to neuronal apoptosis, neuroinflammation and necrosis (Brady & Morfini, 2010; Ermak & Davies, 2002). Nonetheless, the dysregulation in neuronal cell death processes is reported to be responsible for the degeneration of neurons that leads to the development of neurodegenerative diseases (Bredesen et al., 2006). For instance, Alzheimer's disease is associated with the loss of cognitive ability due to degeneration of neurons in the basal forebrain and hippocampal (Querfurth & LaFerla, 2010).

The brain is equipped with a sophisticated inflammatory network that continuously monitors its surrounding in order to detect and eliminate threats and insults that can dampen its functions (Quan & Banks, 2007). The human brain was once considered to be an immune-privileged organ in which the immune responses did not take place (Pachter, de Vries, & Fabry, 2003). Nevertheless, the emerging understanding of the immune responses in the CNS has provided the evidences that inflammation does occur in the brain and regulated by the resident glia cells (Minghetti, 2005; Suzumura, 2013). Generally, the glials are the immune cells that actively regulate the innate and adaptive immunity while participating in the CNS surveillance (Lehnardt,

2010a). Various empirical evidences have demonstrated that almost all neurological disorders are regulated in some parts, by exclusive inflammatory components (Doty, Guillot-Sestier, & Town, 2014; González, Elgueta, Montoya, & Pacheco, 2014). For example, the overproduction of pro-inflammatory factors such as chemokines, cytokines, reactive oxygen species (ROS), reactive nitrogen species (RNS) and inflammatory enzyme (COX-2, iNOS) induce the neuronal damage and degeneration in the Alzheimer's disease (Rubio-Perez & Morillas-Ruiz, 2012). Moreover, several studies have shown that pro-inflammatory mediators enhance the processing of amyloid precursor protein and tau protein phosphorylation leading to their deposition along with neurofibrillary that leads to cell death cycle in Alzheimer's disease (Heneka, O'Banion, Terwel, & Kummer, 2010; McAlpine et al., 2009; Quintanilla, Orellana, Gonzalez-Billault, & Maccioni, 2004). Therefore, the proper understanding of the intricate inflammatory processes that leads to neuronal cell death should be precisely distinguished and understood in developing potential therapeutics that can delay and/or prevent the onset of neurodegenerative diseases.

2.2 Neuroinflammation as the key event of neurodegenerative diseases

Neuroinflammation is recognized to be an important feature of various neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, narcolepsy and autism (Brambilla et al., 2005; Carson et al., 2009). Neuroinflammation is a complex integration of the immune responses of all cells present within the CNS, including neurons, microglia and the infiltrating leukocytes (Bradl & Hohlfeld, 2003; Carson et al., 2009). Brain inflammation has been shown to contribute to the pathology of neurodegenerative diseases, meningitis and brain trauma (Zipp & Aktas, 2006; Engelhardt, 2010; Lee, 2013). Even though neuroinflammation is believed to induce neuronal damage, the local immune responses in the brain also offer beneficial effects on the traumatized tissue. (Taupin, 2008; Giatti et al., 2012).

Neuro-protective effects of inflammation include the clearance of cellular debris, secretion of neurotrophic factors, cytokines, and activation of proteases. In this framework, neuroinflammation is viewed as an intricate local immune response that serves to deal with any threat towards neuronal environment with minimal side effects (Hirsch & Hunot, 2009). Such threat can occur within the disease, physical trauma, ischemia/hypoxia, or with cellular damage due to multiple initiating stimuli, including exposure to neurotoxicants (Harry & Kraft, 2009). The inflammation which is mediated by activated microglia in response to inflammatory signals that induce a cascade of events such as ROS formation and secretion of cytotoxic inflammatory mediators (Helmy, De Simoni, Guilfoyle, Carpenter, & Hutchinson, 2011). When the activating stimulus ceases, the microglia establishes a complex signaling cascades to down-modulate the inflammatory immune response. However, if the stimuli failed to cease and the inflammation prolongs, microglia will be excessively activated and induce aberrant inflammatory responses that lead to chronic neuroinflammation (Glass, Saijo, Winner, Marchetto, & Gage, 2010). During this chronic inflammation, the aberrant microglial activation can result in the accumulation of toxic factors that promote neurodegeneration (Gao & Hong, 2008).

2.2.1 Oxidative stress and inflammatory mediators in neuroinflammation

2.2.1.1 Reactive oxygen species

Oxidative stress associated with mitochondrial dysfunction is one of the major factors in the degeneration of neurons (McGeer & McGeer, 2004). Oxidative stress is a condition that takes place following the imbalance between the production of free radicals such as ROS (Zhao et al., 2011) and cellular antioxidants defense system in favor of the pro-oxidants leading to cell damage (De Felice et al., 2007). Oxidative stress is normally followed with mitochondrial dysfunction, inflammatory responses, aberrant activation of microglia, lipid peroxidation and protein degradation (Yamamoto

et al., 2007). By far, one of the risk factors for neurodegenerative diseases is aging through the accumulation of mitochondrial DNA (mtDNA) mutations associated with net production of ROS and RNS.

In the CNS, ROS facilitates some beneficial normal cell functions such as cell differentiation, proliferation and survival (Le Belle et al., 2011). The most current ROS are superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH) (Halliwell et al., 1992; Maiese, 2009). Mammalian cells have developed a variety of antioxidant enzymes to counteract the effects of ROS (Baker et al., 1992). Nonetheless, following the diminution of intracellular enzymatic antioxidants level, the excessive production of diffusible ROS such as H_2O_2 into cytosol can induce oxidative stress. Although ROS provides beneficial cell functions, ROS can attack cellular membranes, DNA and various proteins inducing damages which can modulate various signaling pathways causing cellular toxicity and cell death.

In addition, ROS can initiate pro-inflammatory pathways and further perpetuates the deleterious environment towards the vulnerable neuronal populations (Figure 2.2) (Block and Hong, 2007). For example, the superoxide can also quickly react with RNS molecule such as nitric oxide (NO) to produce a more cytotoxic radical, the peroxynitrite anions ($ONOO^-$) that can react with carbon dioxide to cause extensive damages on protein through the formation of nitrotyrosine and lipid oxidation (Emerit et al., 2004). Various studies has reported that ROS is an important messenger in innate and adaptive immune cells (Kamiński, Röth, Krammer, & Gülow, 2013; Sena et al., 2013; West, Shadel, & Ghosh, 2011b). For example, the increased production of ROS in immunce cells can lead to aberrant inflammation that promotes tissue damage (Mittal, Siddiqui, Tran, Reddy, & Malik, 2014). Moreover, research in the last decade has demonstrated that LPS induces TLR inflammatory signaling by generating high

level of ROS via NADPH oxidase and mitochondria(West et al., 2011a). In the context of inflammation, a slight increase in ROS production can induce either beneficial or detrimental effects in cells. For instance, mice that lack the uncoupling protein 2 (UCP2) demonstrated a low increase level of ROS that improved normal immunity against bacterial pathogens (Arsenijevic et al., 2000). In contrast, the elevation of ROS significantly increased the production of pro-inflammatory cytokines in NRF2-deficient mice which aggravated the inflammatory responses towards pathogens (Rangasamy et al., 2005; Thimmulappa et al., 2006).

2.2.1.2 Nitric oxide (NO) and inducible nitric oxide synthase (iNOS)

Nitric oxide (NO) is a free radical gas produced in mammalian cells via the metabolism of amino acid L-arginine by the enzyme nitric oxide synthase (NOS) (Crane et al., 1998). There are three isoforms of NOS which are genetically different; (1) endothelial nitric oxide synthase (eNOS) that induces the endothelial smooth muscle relaxation, (2) inducible nitric oxide synthase (iNOS) which is inducibly expressed in astrocytes and microglia following exposure to inflammatory stimuli and (3) neuronal nitric oxide synthase (nNOS). During inflammation, the expression of iNOS is strongly induced and its activation requires the increased cellular uptake of L-Arginine into brain cells by cationic amino acid transporters. As an example the generation of ROS can lead to the increase expression of iNOS that enhances NO production in glial and endothelial cells (Harry & Kraft, 2009; Terazawa et al., 2013). NO is one of the key cytotoxic molecules that can instigate a series of deleterious effects. Following its production, NO will form the short-lived but highly reactive RNS, peroxynitrite ($ONOO^-$) by reacting with superoxide anion (Sies, 1997) and can be protonated to form peroxynitrous acid (ONOOH) which can generate •OH and nitrate (NO_2^-).

Moreover, high expression of iNOS was detected from post mortem of brain tissue samples of patients diagnosed with Parkinson's disease (Hunot et al., 1996), multiple sclerosis (Bo et al., 1994) and Alzheimer's disease (Vodovotz et al., 1996; Wallace, Geddes, Farquhar, & Masson, 1997). The production of NO by iNOS has also been shown to be regulated following NF-κB activation (Nomura, 2001), signal transducers and activators of transcription (STAT) as well as activator protein-1(AP-1) (Saha & Pahan, 2006). Despite being detrimental to the CNS, NO has been documented to play pivotal roles in many physiological functions under normal conditions such as host immune defense, vascular regulation, cerebral blood flow and neurotransmission (Misko, Schilling, Salvemini, Moore, & Currie, 1993). NO also plays critical roles as mediators of signaling and as effector molecule in various biological systems (Bogdan, 2001; Jaffrey & Snyder, 1995). Nevertheless, the excessive production of RNS (NO and $ONOO^-$) induces aberrant inflammation by causing oxidative and nitrosative/nitrative stress, lipid peroxidation and disruption of the cell membrane integrity (Figure 2.2) (Bertrand, 1985; Calabrese, Bates, & Stella, 2000; Dalle-Donne et al., 2005; Sharma, Al-Omran, & Parvathy, 2007). Nitrosative/nitrative stress occurs following the high level of NO or $ONOO^-$ that influence the nitration of tyrosine residues to produce nitrotyrosine in various proteins (Broniowska & Hogg, 2012; Calcerrada, Peluffo, & Radi, 2011; Lancaster, 2006). These processes can lead to the disruption of mitochondrial ETC complexes that alters the mitochondrial functions which induces mitochondria leakage with enhanced ROS production (Lancaster, 2006; Moon, 2013). Furthermore, high level of nitrotyrosine has been observed in the midbrains of Parkinson's patients (Giasson et al., 2000; Hunot et al., 1996; Malinski, 2007).

2.2.1.3 Cyclooxygenase-2 (COX-2) and Prostaglandin E_2 (PGE_2)

Cyclooxygenase (COX) is an enzyme that catalyzes the synthesis of prostaglandins (PGs) through the conversion of arachidonate (arachidonic acid) to

prostaglandin H2 (Williams, Mann, & DuBois, 1999). COX mainly exist in two isoforms, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) and both are encoded by different genes with different inflammatory properties (Aïd & Bosetti, 2011; Lyman et al., 2013). COX-1 is constitutively expressed in most cells and is important in regulating normal physiological responses, such as gastric epithelial cytoprotection, integrity of platelet function and renal homeostasis (Dubois et al., 1998). Conversely, COX-2 expression is induced by various stimuli such as growth factors, pathogens and inflammatory cytokines. (Samad et al., 2001). COX-2 is also expressed in CNS cells, particularly in glial and neuronal cells and shown to be involved in neuroinflammation, synaptogenesis and process of memory (Liang et al., 2007). COX-2 can mediate detrimental inflammatory responses in the CNS by inducing dioxygenation that allows initially catalyzes arachidonic acid to prostaglandin G2. This prostaglandin G2 will undergo peroxidase reaction and converted to prostaglandin H2 to form prostaglandin E_2 (PGE_2) (Aïd & Bosetti, 2011). PGE_2 is a major eicosanoid that is highly produced in various CNS inflammatory diseases (McGeer and McGeer, 1995).

The COX-2 gene promoter normally contains multiple regulatory elements that are regulated by different transcription factors which includes AP-1, CREB and NF-κB depending on cells type (Tanabe & Tohnai, 2002). The dysregulation of these transcription factors can mediate the overexpression of various pro-inflammatory genes. For instance, deregulated AP-1 enhanced the expression of pro-inflammatory genes such as *PTGS2, NOS2* and *TNF* that lead to the production of COX-2, iNOS and TNF-α in activated microglia (Bae et al., 2006; Kang et al., 2004). During neuroinflammation, the prolonged NF-κB activation mediates iNOS-induced NO production (Bhat et al., 2002; Davis et al., 2005), cytokine expression (Jana et al., 2002; Nakajima et al., 2006) as well as COX-2-induced PGE_2 production in glial cells (Figure 2.2). Furthermore, high expression of iNOS and COX-2 were found in the glial cells of substantia nigra

pars compacta of post-mortem Parkinson's patients (Knott *et al*., 2000) and in rodent brain following exposure to LPS treatment (Boje and Arora, 1992; Minghetti *et al*., 1999). In different inflammation-mediated diseases such as arthritis, colon cancer and cardiovascular disease, COX-2 serves as the important source of prostanoid formation (COX metabolites of arachidonic acid which includes prostaglandin (PG) D(2), PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2) (Dubois et al., 1998).

2.2.2 Cytokines as the major modulator of neuroinflammation

Cytokines are a group of small protein (8–30 kDa) that can be produced by various immune cells such as lymphocytes, monocytes and macrophages. In the human brain, cytokines are endogenously produced by astrocytes, microglia and neurons. Cytokines are normally produced in high level following inflammation, injury, infection and/or immunological alterations. Following this stimuli, cytokines are produced and involved in the tissue repair and restoration of homeostasis (Nathan, 2002; Woodroofe, 1995). In normal conditions, cytokines concentration is found in the picomolar to nanomolar range and act as autocrine or paracrine signaling molecules that modulate local cellular activities such as cell survival, growth and differentiation (Deverman & Patterson, 2009).

Cytokines are important in the development and regulation of homeostasis in the brain since they mediate signals between immune cells through the interplay between pro-inflammatory and anti-inflammatory cytokines which promote and suppress inflammatory responses (Suzumura, Takeuchi, Zhang, Kuno, & Mizuno, 2006). During chronic neuroinflammation, a number of pro-inflammatory cytokines are expressed by activated microglia and neurons (Figure 2.2). Moreover, in cases of most neurodegenerative diseases elevated levels of several cytokines are observed (Rubio-Perez & Morillas-Ruiz, 2012; Smith et al., 2012; Steinman, 2013). For example, the

level of IL-1β, IL-6, TNF-α, granulocyte-macrophage colony-stimulating factor (GMSF), IFN-α and the type B of IL-8 receptor (IL-8RB) are highly expressed in the Alzheimer's disease brain tissue (Rubio-Perez & Morillas-Ruiz, 2012). Additionally, TNF-α was also reported to mediate astrogliosis and neuronal cell death that contributed to the development of schizophrenia (Ashdown et al., 2006; Meyer, Feldon, & Yee, 2009).

The production of pro-inflammatory cytokines also stimulates the production of anti-inflammatory cytokines in glial cells triggering a negative feedback that serves to reduce the production of pro-inflammatory cytokines and thus, subsiding the neuroinflammation (Ouyang, Rutz, Crellin, Valdez, & Hymowitz, 2011) (Figure 2.2). Even though the release of cytokines is essential in removing the threats towards the CNS milieu, the persistent production of cytokines can lead toward an imbalanced pro-inflammatory phenotype resulting in increased neuronal injury and cell death (Smith et al., 2012; Steinman, 2013). In this context, the aberrant activation of microglia is responsible for the production of various pro-inflammatory cytokines which can compromise the permeability of blood brain barrier (BBB) and while inducing its disruption. (Guyon, Massa, Rovère, & Nahon, 2008; Helmy et al., 2011). This event can lead to the infiltration of leukocytes and macrophages that can further enhance the inflammatory responses by producing more pro-inflammatory cytokines (González et al., 2014). Therefore, tunderstanding the role of cytokines and their regulation during inflammation are vital since a number of them possess dual roles in neurodegenerative diseases.

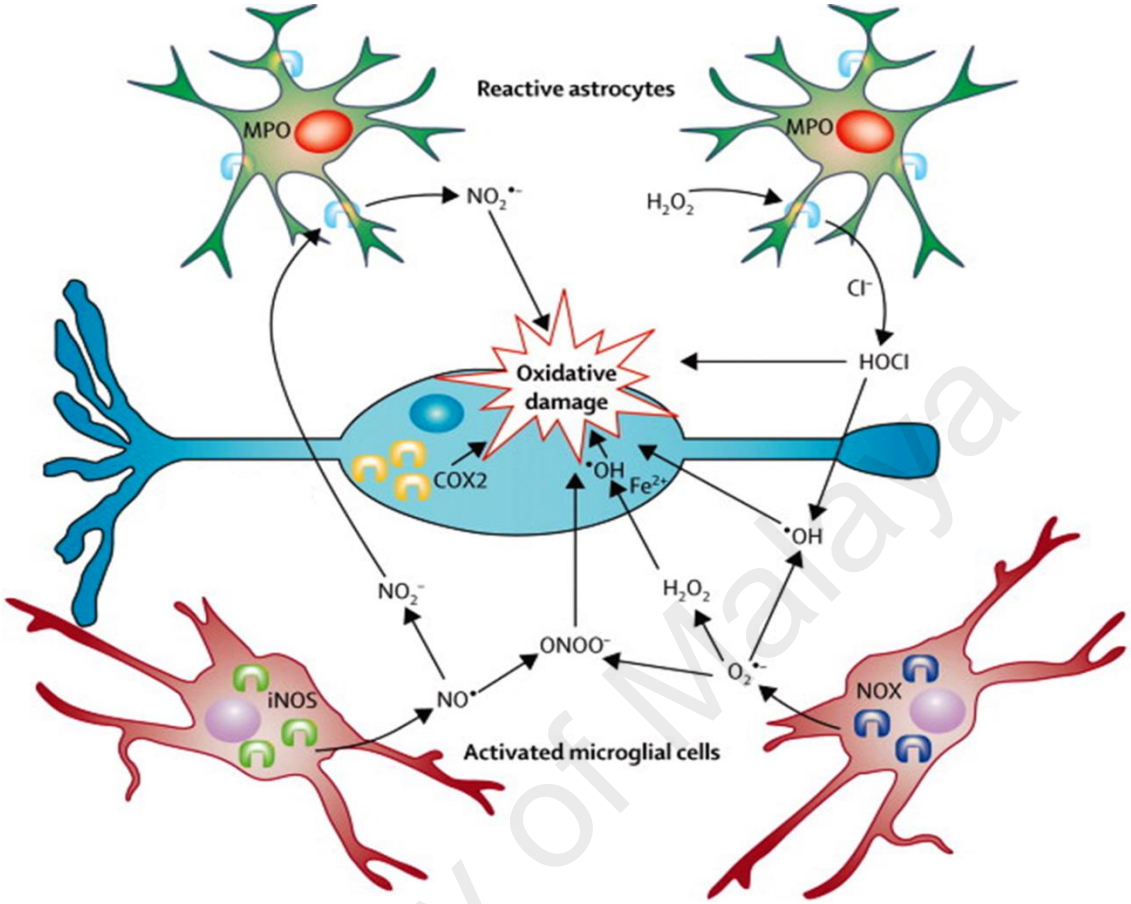


Figure 2.1 The production of ROS, NO, peroxynitrite ($ONOO^-$) and PGE_2 through iNOS and COX-2 in the activated astrocytes and microglia that leads to the oxidative stress and damages in the dopaminergic neurons as observed in Parkinson's disease. Adapted from Hirsch, E. C., & Hunot, S. (2009).

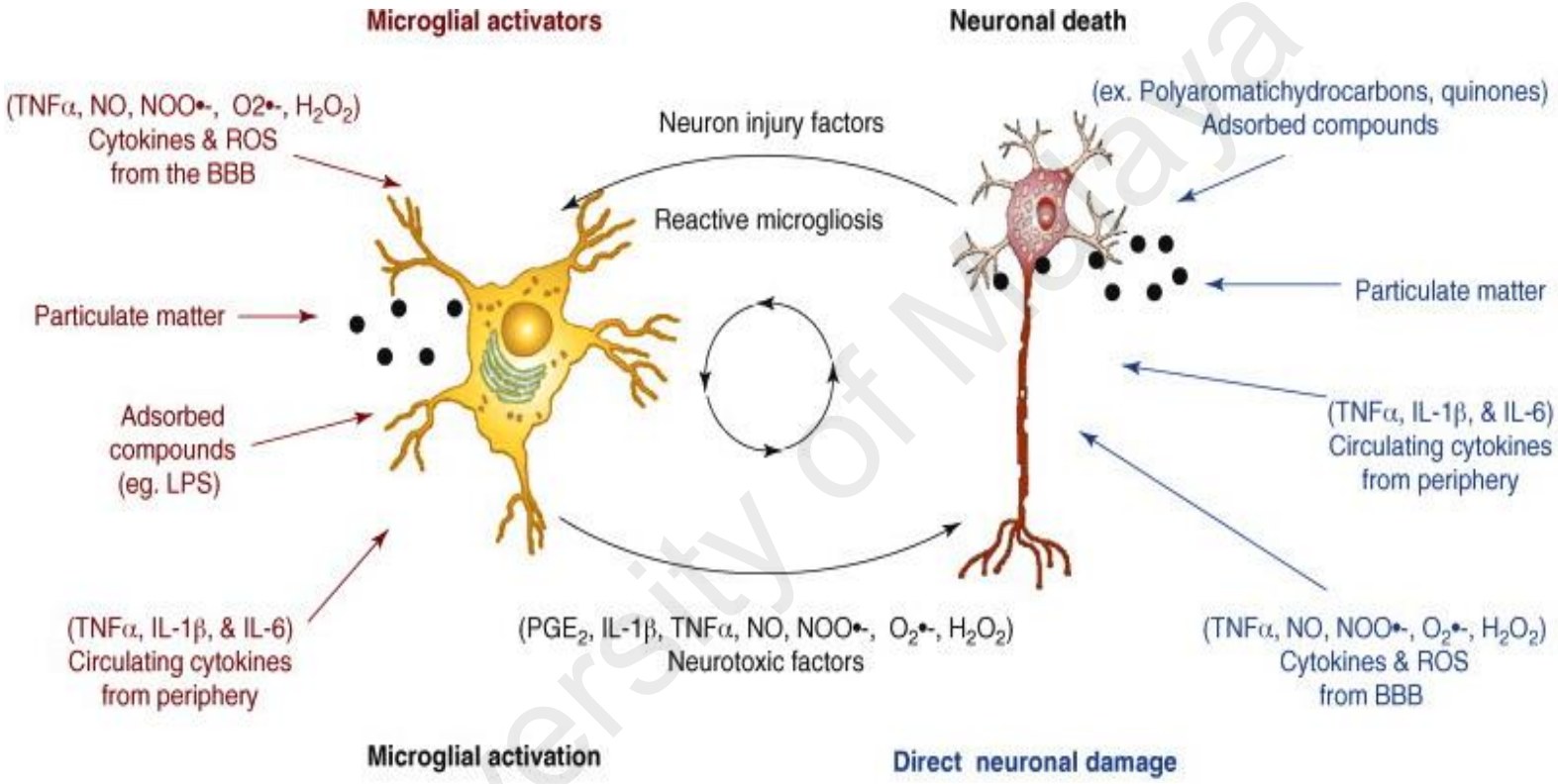


Figure 2.2 The aberrant regulation of pro-inflammatory cytokines, ROS and RNS exacerbate the activation of microglia that leads to neuronal cell death. Adapted from Block, M. L., & Calderón-Garcidueñas, L. (2009).

2.2.2.1 Interleukin-4 (IL-4)

Interleukin 4 (IL-4) is a pleiotropic cytokine that act as a powerful regulator of immunity secreted primarily by eosinophils, basophils, mast cells and Th2 cells. IL-4 was formerly identified as a B cell differentiation factor (BCDF) and as a B cell stimulatory factor (BSF1). Over the years, IL-4 was shown to be an important regulator in leukocyte survival under both physiological and pathological conditions. IL-4 was shown to modulate the Th2 cell-mediated immunity, IgE class switching in B cells as well as homeostasis and tissue repair through the alternative macrophage activation. (Akbari et al., 2003; Ferrick et al., 1995; van Panhuys et al., 2011).

IL-4 was first reported to possesss anti-inflammatory property since its administration downregulated the production of inflammatory cytokines and antagonized IFN-γ–driven MHCII expression while increasing IGF-1 levels in macrophages (Gadani, Cronk, Norris, & Kipnis, 2012). Moreover, T cell-derived IL-4 was shown to be a critical participant in brain cognitive functions where the IL-4 deficient mice demonstrated impaired performance in spatial learning task (Derecki et al., 2010; Kipnis, Gadani, & Derecki, 2012; Ziv et al., 2006). However, this observation was reversed following the transplantation of IL-4-competent bone marrow. In Alzheimer's disease, the expression of IL-4 has been shown to reduce the neuroinflammation induced by amyloid-β *in vivo* and *in vitro* (Lyons, Griffin, Costelloe, Clarke, & Lynch, 2007). Recently, IL-4 was reported to protect the injured CNS neurons through Akt and MAPK signaling pathways (Walsh et al., 2015). However, IL-4 is not entirely an anti-inflammatory cytokine since it induces macrophages priming that is followed by the induction of pro-inflammatory factors resulting in an advanced inflammatory response (Gordon, Helming, & Estrada, 2014; Martinez et al., 2013). IL-4 can also induce a robust M2a phenotype in microglial cells *in vitro* and *in vivo* models which correlates to increased microgliosis (Latta et al., 2015). In addition, *in vivo*

models, the treatment with chronic high dosage or transgenic overexpressed IL-4 resulted in the increased IFN-γ expression, histiocytosis, erythrophagocytosis, extramedullary hematopoiesis and weight loss (Milner et al., 2010).

2.2.2.2 Interleukin 6 (IL-6)

Interleukin-6 (IL-6) is a 184 amino acid glycosylated protein (26 kDa) cytokine that can be synthesized and secreted by many cell types including glial cells, monocytes, neurons, T-cells and endothelial cells. IL-6 is a typical four-helix bundle cytokine and is the original member of the neuropoietins (Tanaka, Narazaki, & Kishimoto, 2014). IL-6 is also known as B-cell differentiation factor, T-cell differentiation factor and hepatocyte stimulating factor. IL-6-type cytokines have been documented to play important roles in the communication between cells of multicellular organisms (Tanaka et al., 2014). In general, IL-6-type cytokines are involved in the complex regulation of processes such as cell differentiation, cancer and immune response (Ataie-Kachoie, Pourgholami, & Morris, 2013; Tanaka et al., 2014).

IL-6 can mediate its cellular functions through classical and trans-signaling pathways. In the classical signaling, IL-6 binds and activates a membrane-bound receptor protein complex comprised of a non-signaling membrane-associated α subunit receptor (IL-6Rα, 80 kDa) and two signal-transducing component gp130 or also known as CD130 or IL-6 signal transducer. The activation of IL-6R-gp130 complex can induce the transcriptional activity of STAT1 and STAT3 through Janus kinases (JAKs) (Heinrich, Behrmann, Muller-Newen, Schaper, & Graeve, 1998). In addition, IL-6R-gp130 complex can activate MAPKs signaling cascade and induce IL-6 survival effects through PI3K/Akt signaling. The trans-signaling pathway involves the association of cell surface gp130 to a soluble form of IL-6R (sIL-6R) (Chalaris et al., 2007). The production of sIL-6R can be initiated though the proteolytic cleavage of IL-6R. Hence,

in human, sIL-6R can also be produced by translation of an alternatively spliced mRNA. Generally, only a few cells express membrane bound IL-6R whereas the gp130 is almost ubiquitously expressed in most cells on the cell surface. Therefore, only cells that have gp130 can respond to the IL-6-sIL-6R complex in the trans-signaling (Rose-John, 2012).

The anti-inflammatory property of IL-6 is normally associated to the classical signaling pathway whereas the trans-signaling is responsible of the pro-inflammatory responses (McLoughlin et al., 2005; Rose-John, 2012). Therefore, IL-6 has been viewed as a pleiotropic cytokine in the CNS. In support of this, IL-6 was reported to be increased in neurological conditions, such as depression and Alzheimer's disease (Galimberti et al., 2008). In addition, increased serum levels of IL-6 and of other cytokines in brain atrophy during normal aging (Jefferson et al., 2007). IL-6 has been shown to promote neuronal injury (Hang, Shi, Li, Li, & Wu, 2005; Ziebell & Morganti-Kossmann, 2010) and inflammation in various *in vitro* and *in vivo* models (Turner, Nedjai, Hurst, & Pennington, 2014). On the contrary, IL-6-dependent activation of its classical signaling was associated to anti-inflammatory and immunosuppressive activities that may reduce inflammatory conditions (Petersen & Pedersen, 2006; Tilg, Dinarello, & Mier, 1997). IL-6 can also act as developmental neurotrophic factor that improves neuronal survival following excitotoxic and ischemic insults (Gadient & Otten, 1997; Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011). Moreover, IL-6 has also been demonstrated to promote neuronal plasticity by eliciting the growth of axons and synaptogenesis, LTP and memory (Schafer, Mestres, Marz, & Rose-John, 1999; Shuto et al., 2001; Yang et al., 2012). In summary, IL-6 with its dual neurodegenerative and neuroprotective biological functions may serve as an excellent cytokine target in the intervention of various neurodegenerative diseases (Scheller et al., 2011). Moreover, the paradoxical properties of IL-6 are distinguished by the type of

signaling activated; anti-inflammatory activities are mediated by *classic signaling* whereas pro-inflammatory responses are rather regulated by *trans-signaling*. Therefore, the IL-6 therapeutic potential can be distuingished by targeting and modulating the activation of these signaling pathways that will result in different therapeutic effects. Additionally, the development of IL-6-based therapeutic has also considered the long-term global blockade of this cytokine. Although various therapeutics posses the ablity to block both classic- and trans-signaling, nevertheless, specific therapeutics that block only the trans-signaling is currenttly developed (Kopf, Bachmann, & Marsland, 2010).

2.2.2.3 Interleukin-10 (IL-10)

Interleukin-10 (IL-10) is a well-known homodimeric anti-inflammatory cytokine and was originally named as cytokine synthesis inhibitory factor (Sabat, 2010). To date, IL-10 is the main member of the IL-10 superfamily which also includes IL-19, IL-20, IL-22, IL-24, IL- 26 and type III IFN-γ subfamily (Sabat, 2010). IL-10 can by secreted by various cell types including macrophages, microglia, neurons and T cell subsets (Saraiva & O'Garra, 2010). IL-10 is a powerful inhibitor of inflammation since it is capable to reduce the Th1 cell activity and the subsequent pro-inflammatory cytokines production. IL-10 induces its anti-inflammatory activity by binding to its receptor, (designated IL-10R1/2), which recruits and activates Jak1 and Tyk2 (Couper, Blount, & Riley, 2008; Saraiva & O'Garra, 2010). The expression of IL-10 also inhibits MHC class II and costimulatory molecule B7-1/B7-2 expression on monocytes and macrophages which is responsible to repress the production of pro-inflammatory cytokines and chemokines (Couper et al., 2008; Ouyang et al., 2011). In addition, during mycobacterial infection, the autocrine signaling of IL-10 was shown to inhibit the chemokine production which prevented their trafficking and led the Th1

differentiation of naive T cells. IL-10 can also act directly on $CD4^+$ T cells and inhibited the proliferation and production of IL-2, IL-5, IFN-γ and TNF-α (Couper et al., 2008).

The constitutive expression of IL-10 is a vital anti-inflammatory mechanism that allows well-controlled normal physiological processes in the peripheral nervous system and CNS (Strle et al., 2001; Taylor et al., 2006). The expression of IL-10 can inhibit the production of various pro-inflammmatory cytokines such as IL-1β, IL-6, IL-12 and TNF-α (Hovsepian, Penas, Siffo, Mirkin, & Goren, 2013; Ouyang et al., 2011). In addition, the expression of IL-10 also promotes the production of other anti-inflammatory factors such as the IL-1β receptor antagonist and soluble TNF-α receptors p55 and p75 (Ouyang et al., 2011; Sanjabi, Zenewicz, Kamanaka, & Flavell, 2009). IL-10 was reported to suppress the production of pro-inflammatory cytokines in reactive glia and macrophage following the exposure to LPS (Green & Nolan, 2012; Qian, Hong, & Flood, 2006). Furthermore, IL-10 also downregulates the expression of MHC class II molecules (both constitutive and IFN-γ-induced), as well as that of co-stimulatory molecule, CD86, and adhesion molecule, CD58 (Koppelman, Neefjes, de Vries, & de Waal Malefyt, 1997; Mellman & Steinman, 2001). IL-10 was reported to protect the cerebellar granule cells (Bachis et al., 2001) and retinal ganglion cells (Boyd et al., 2003), protecting them from chemically-induced apoptosis through the activation of its receptor, IL-10R. Moreover, the anti-inflammatory property of IL-10 is important in maintaining the CNS homeostasis (Strle et al., 2001). Furthermore, the impaired production of IL-10 contributes to the development of neuroinflammatory disease models such as experimental autoimmune encephalomyelitis, traumatic injury, Alzheimer's disease and Parkinson's disease (Cua et al., 2001; Brewer et al., 1999; Bethea et al., 1999; Frenkel et al., 2005; Qian et al., 2006).

2.2.2.4 Tumor necrosis factor-alpha (TNF-α)

Tumor necrosis factor-alpha (TNF-α) is another type of pleiotropic inflammatory cytokine. TNF-α was first isolated during the identification of tumor necrosis factors that is responsible for necrosis of the sarcoma Meth A (Carswell et al., 1975). Generally, TNF-α can exist either as a 26 kDa membrane-bound protein or a 17 kDa soluble protein form generated by metalloprotease known as TNF-α converting enzyme. Originally thought to be circulating factor that induces necrosis of tumor cells, TNF-α has been identified and studied as one of the key players in the inflammatory responses. In the CNS, TNF-α is mainly produced by microglia, although numerous reports have demonstrated that astrocytes and neurons are also capable of producing TNF-α (Breder et al., 1994; Lee, Liu, Dickson, Brosnan, & Berman, 1993).

The signaling of TNF-α can occur through the activation of two distinct receptors TNFR1 (*TNFRSF1a*/p55) and TNFR2 (*TNFRSF1b*/p75). TNFR1 is expressed almost ubiquitously among cell types while TNFR2 present in a lower level in specific neuronal subtypes and glial cells in the brain (Choi et al., 2005; Tartaglia & Goeddel, 1992). The binding of TNF-α to TNFR1 or TNFR2 can elicit various signal transduction pathways that are responsible for a plethora of cellular responses such as cell death, survival, differentiation, proliferation, inflammation and migration (Kastl et al., 2014; Sade-Feldman et al., 2013; Sedger & McDermott, 2014). The activation of TNFR1 is important in the promotion of neuronal cell death through activation of pro-apoptotic or survival proteins via the activation of NF-κB (Gururaja et al., 2007). Moreover, the neuronal TNFR2 signaling pathway can result in the amplification of the NF-κB-mediated transcription of target genes via the activation of PI3K/Akt and stimulation of NF-κB inducing kinase which in turn promotes IKK activity and thus, NF-κB activation (Wajant, Pfizenmaier, & Scheurich, 2003).

TNF-α plays important role in various inflammatory-mediated diseases such as septic shock, cancer, diabetes, rheumatoid arthritis, trauma, meningitis and ischemia-reperfusion injury (Bradley, 2008). The overproduction of TNF-α by aberrant NF-κB activation is a common observation in most *in vivo* and *in vitro* neuroinflammation model following the exposure to LPS. Moreover, the overproduction of TNF-α by activated microglia was associated with the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (McCoy & Tansey, 2008; Perry, Collins, Wiener, Acton, & Go, 2001), Parkinson's disease (Boka et al., 1994) and multiple sclerosis (Magnano, Robinson, & Genovese, 2004). In support of this, high level of TNF-α was detected in the cerebrospinal fluid and serum of Alzheimer's and ALS patients (Terenghi, Allaria, & Nobile-Orazio, 2006). In addition, TNF-α, with other pro-inflammatory cytokines (IFN -γ and IL-6) were shown to induce the *in vitro* differentiation of the neuroblastoma cell which indicates its pleiotropic property (Muñoz-Fernández et al., 1994). Taken together, the production of TNF-α by microglia and neurons in the CNS is not only important in the regulation of inflammation, but equally essential in the modulation of neuronal differentiation depending on the nature of the stimuli that affects the CNS milieu.

2.2.3 Chemokines in brain inflammation and function

Chemokines are a large family of cytokine that exert their biological effects by acting on the superfamily of G-protein-coupled receptors (Cardona, Li, Liu, Savarin, & Ransohoff, 2008). Chemokines or chemotactic cytokines belong to a superfamily of small secreted protein (8–14 kDa) that were initially identified according to their ability to regulate leukocyte trafficking (Ransohoff, 2009). Chemokines exert their biological effects by binding and activating GPCRs that mediate intracellular signals through heterotrimeric GTP binding protein (Bacon et al., 2001; Chemokine, 2002; Koelink et al., 2012). The chemokine receptors include ten CC receptors (from CCR1 to CCR10),

seven CXC receptors (CXCR1–7), one XCR1 and one CX3CR1 (Chemokine, 2002; Ransohoff, 2009). Chemokine receptors activate various signaling pathways, such as the MAPKs, the phospholipase C and PI3K pathways (Koelink et al., 2012; Lee & Kim, 2007) leading to various biological responses.

Chemokines which function as inflammatory factors in the brain are expressed in neurons and glia cells (Réaux-Le Goazigo, Van Steenwinckel, Rostène, & Parsadaniantz, 2013; Rostène, Kitabgi, & Parsadaniantz, 2007). In addition to their chemotactic activity, chemokines have been implicated in the modulation of cell adhesion (Burns et al., 2006), phagocytosis (Shimaoka et al., 2003), cytokine secretion (Gouwy, Struyf, Proost, & Van Damme, 2005), cell proliferation (Bajetto et al., 2006), apoptosis (Kaul & Lipton, 1999; Vlahakis et al., 2002), angiogenesis (Strieter et al., 2006) and viral pathogenesis (Biber et al., 2006; Galea et al., 2007; Rebenko-Moll et al., 2006). Chemokines can also be functionally classified into inflammatory and homeostatic chemokines (Ransohoff, 2009). Inflammatory chemokines, such as CCL5 (RANTES) is expressed in inflamed tissues in response to pro-inflammatory cytokines or contact with pathogens (Huang et al., 2009b; Zaremba, Ilkowski, & Losy, 2006). CCL5 and other chemotactic chemokines are responsible for the recruitment of effector cells such as monocytes, granulocytes and effector T cells to sites of inflammation (Turner et al., 2014). Homeostatic chemokines, such as SDF-1/CXCL12, tend to be constitutively expressed in discrete environments within tissues and are characterized by their involvement in trafficking of cells that belong to the adaptive immunity (Cartier, Hartley, Dubois-Dauphin, & Krause, 2005). For instance, in physiological conditions, the constitutive expression of CXCL1, CXCL8 and CXCL12 is important to regulate neurotransmitter release and ion channel activity at both presynaptic and postsynaptic sites (Ragozzino et al., 2006; Rostène et al., 2007).

In a normal physiological state, the brain is protected from immune cell intrusion by the BBB, limiting leukocyte trafficking from the blood to the brain parenchyma (Palmer, 2010; Shlosberg, Benifla, Kaufer, & Friedman, 2010). However, factors such as severe infection and traumatic brain injury can lead to overexpression of cytokines and chemokines causing chronic inflammation which disrupts the integrity of the BBB leading to immune cell infiltration (Figure 2.3) (Shaftel et al., 2007; Wispelwey, Lesse, Hansen, & Scheld, 1988; Zlokovic, 2008). Additionally, chemokines and their receptors are found to be highly expressed following the pathogenesis of neurodegenerative diseases and brain injury (Koelink et al., 2012; Minami, Katayama, & Satoh, 2006). For example, a number of chemokines (CCL2–5, CCL7, CCL8, CXCL1, CXCL10 and CXCL12) are found to be elevated in the brain of multiple sclerosis patients (Bartosik-Psujek & Stelmasiak, 2005; Krumbholz et al., 2006; Sørensen et al., 1999). In addition, various chemokines and their receptors (CCL2, CCL3, CCL4, CCL5, CXCL10 / CXCR3, CCR3, CCR5) are highly expressed in brain tissues from Alzheimer's disease patients (Azizi, Khannazer, & Mirshafiey, 2014; Liao, Guan, & Ravid, 2011; Weeraratna et al., 2007).

Despite their involvement in the pathogenesis of neurodegenerative diseases, the role of the chemokines in the brain is not only limited to the above pathological situations. In addition to macrophages and glial cells, neurons are able to express chemokines and multiple chemokine receptors which may function as neuromodulators/ neurotransmitters in the homeostatic brain (Conductier, Blondeau, Guyon, Nahon, & Rovère, 2010; Miller et al., 2008). Moreover, the chronic CXCL10 administration was shown to alter the level of activated ERK1/2, CREB and NF-κB in primary hippocampal cells that induced neurite outgrowth and neuroplasticity (Bajova, Nelson, & Gruol, 2008; Cho, Nelson, Bajova, & Gruol, 2009). Therefore, it is important to distinguish chemokines involvement under normal condition and various pathological

states. The release of cytokines and chemokines in the brain mainly mediates local immune responses that help to eliminate threats as well as to repair neuronal damage and restore homeostasis. However, the dysregulation of inflammatory responses can produce excessive pro-inflammatory cytokines and chemokines that disturb the homeostatic balance leading to advance neuronal damage.

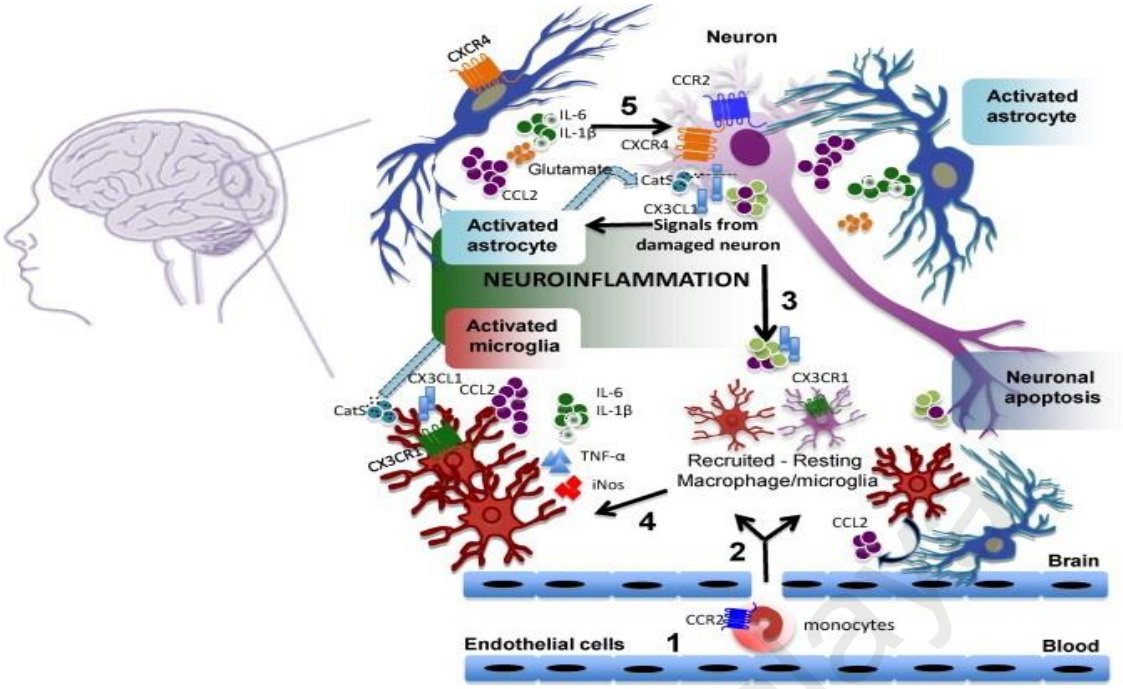


Figure 2.3 The regulation of chemokines and receptors in chronic neuroinflammation observed in neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and after traumatic injury and pathogen infection. Adapted from Réaux-Le Goazigo, A., Van Steenwinckel, J., Rostène, W., & Parsadaniantz, S. M. (2013).

2.2.3.1 CCL21 as neuron-glia signaling mediator in inflammation

CCL21 is rapidly expressed in endangered neurons (Biber, Sauter, Brouwer, Copray, & Boddeke, 2001) particularly after chronic exposure to glutamate-induced excitotoxicity in cortical neurons (Biber et al., 2001; de Jong et al., 2005). The rapid expression of CCL21 in endangered neurons after injury indicates a brain injury specific role of CCL21. Moreover, it was demonstrated that the overexpression of CCL21 in the brain induced a massive brain inflammation in transgenic mice within three days after the expression onset (Chen et al., 2002a), whereas CCL21 expression in the skin was not accompanied by any inflammatory responses (Chen et al., 2002b).

Under stress conditions, neurons specifically express the microglia-activating CCL21 (Biber et al., 2001; Biber et al., 2011; Dijkstra, Hulshof, Van Der Valk, Boddeke, & Biber, 2004; Rappert et al., 2004; Redondo-Muñoz, Terol, García-Marco, & García-Pardo, 2008) which can contribute to neurodegeneration. Moreover, neuronal CCL21 was found in vesicle-like structures that were distributed throughout neurons (de Jong et al., 2005) and suggested to be a neuronal signal to induce microglia activation at distant sites from a primary lesion (de Jong et al., 2005). The upregulation of CCL21 following neuronal injury was shown to induce the distant activation of microglia through chemokine receptor CXCR3 (Biber et al., 2001). A follow up study using *CXCR3*$^{-/-}$ mice strongly supported this finding where CCL21 was shown to trigger a chloride current and chemotaxis in murine microglia (Dijkstra et al., 2004; Rappert et al., 2004). Based on these findings, neuronal CCL21 is viewed as a microglia-activating chemokine which is solely found in endangered neurons in the brain. Therefore, CCL21 may serve as a candidate for neuron-microglia signaling and its rapid expression of in endangered neurons advocates the specific role of CCL21 as pro-inflammatory chemokine in the brain. Additionally, the CCL21/CCR7 axis was reported to upregulate matrix metalloproteinase-9 (MMP-9) expression via extracellular signal-regulated

kinase-1/2 signaling that induced the B-cell chronic lymphocytic leukemia cell invasion and migration (Redondo-Muñoz et al., 2008).

In a different study, CCL21 was demonstrated to induce the sufficient regulation of dendritic cells homeostasis and function *in vivo* such as migration, maturation and immune responses (Britschgi, Favre, & Luther, 2010). Previous *in vivo* findings proposed that CCL21 plays a cardinal role in the development of autoimmune diseases (Christopherson, Kent, & Hromas, 2004; Columba-Cabezas, Serafini, Ambrosini, & Aloisi, 2003), atherosclerosis (Damås et al., 2007) and rheumathoid arthritis (Manzo et al., 2005; Pickens et al., 2012). Moroever, mice that were deficient in *CCL19/CCL21* or their receptor *CCR7* were shown to be resistant against autoimmune encephalomyelitis where the numbers of Th1 and Th17 cells were found to be reduced (Kuwabara et al., 2009). Apart from all these reports, CCL21 was also reported to up-regulate the microglial P2X4 receptor expression in *vitro* and *in vivo* models that contributed to the development of tactile allodynia (Biber et al., 2011). Cumulatively, the recents studies on CCL21 mainly describe its immunomodulatory property that contributes to delevopment of various pathological conditions. However, to date, there is no scientific evidence that describe its involvement in neuronal differentiation, particularly neuritogenesis. Therefore, a greater understanding on CCL21 regulation and its regulatory effects in neuronal differentiation would provide a better view on its immunomodulatory roles in the development of CNS.

2.2.4 The roles of galectins in central nervous system

Classified under the lectin superfamily, galectins are groups of β-galactoside–binding proteins with carbohydrate-recognition domains with possess multifarious cellular processes (Yang, Rabinovich, & Liu, 2008b). Galectins are regulator of various biological processes which include cell growth, apoptosis, innate and adaptive immune responses (Henderson & Sethi, 2009; Hughes, 2001; Yang et al., 2008b). A total of 15 galectins with each one having one or two carbohydrate-recognition domains of ~130 amino acids has been identified in mammals. The classification of galectins is mainly based on their structures and the presence of carbohydrate-recognition domains which are the prototype, chimera type, and tandem repeat type (Yang et al., 2008b). More importantly, various galectins have specific characteristics in their distributions, expression patterns, and binding abilities which then explain their unique functions as regulators in different cellular processes.

2.2.4.1 Galectin-1 in neuronal differentiation and inflammation

Galectin-1 is a soluble carbohydrate binding protein that acts in both the extracellular and intracellular space which binds specifically to β-galactosides. Galectin-1 is the first reported protein identified in a family of β-galactoside binding animal lectins to be involved various cellular functions (Camby, Le Mercier, Lefranc, & Kiss, 2006). The intracellular functions of galectin-1 are associated with its lectin-independent interactions with other proteins whereas most of its extracellular functions require its lectin activity (Camby et al., 2006; Yang et al., 2008b). Galectin-1 is involved in the development and regeneration of the nervous system (Sakaguchi, Imaizumi, & Okano, 2007; Sakaguchi et al., 2006; Sango, Horie, Yanagisawa, Watabe, & Kadoya, 2012) and the pathogenesis of neurological disorders, such as amyotrophic lateral sclerosis (Chang-Hong et al., 2005), Parkinson's disease (Qu et al., 2010; Werner, Heyny-von Haussen, Mall, & Wolf, 2008), brain ischemia (Kurushima et al.,

2005; Qu et al., 2010) and immune-mediated peripheral neuropathies (Peng, 2012). For instance, a single injection of galectin-1 into lateral ventricles stimulates neurogenesis and improves neurological function following focal cerebral ischemia. Furthermore, the infusion of galectin-1 antibodies or knockout of the galectin-1 gene that decreased the number of endogenous neural stem cells (Yamane et al., 2010) while its delivery stimulated neurogenesis in the subventricular zone after ischemia (Ishibashi et al., 2007).

Besides its neurogenic property, previous reports have successfully demonstrated that galectin-1 also possesses anti-inflammatory property. Galectin-1 is capable of inhibiting the proliferation of astrocytes and attenuating astrogliosis by downregulating the expression of iNOS and IL-1β following cerebral ischemia (Qu et al., 2011). Moreover, galectin-1 also stimulates astrocyte differentiation that enhances BNDF production indicating its neuroprotective roles during neuroinflammation (Qu et al., 2011; Sasaki, Hirabayashi, Manya, Kasai, & Endo, 2004). The neuroprotective property of galectin-1 was further demonstrated by its ability to suppress microgliosis and the subsequent pro-inflammatory responses associated with CNS demyelinating diseases (Starossom et al., 2012).

2.2.4.2 Galectin-3 as the inducer of microglia activation

Galectin-3 is a unique chimera-type member of the galectin family that can be found in cytoplasm, nucleus, cell surface and in the extracellular fluid surroundings of several cell types (Lepur et al., 2012; Sato & Nieminen, 2002). It was also reported that both extracellular and intracellular galectin-3 are involved in a variety of cellular functions such as cell adhesion, migration, chemotaxis, phagocytosis and apoptosis (Dumic, Dabelic, & Flögel, 2006; Nangia-Makker, Balan, & Raz, 2008; Wesley, Vemuganti, Ayvaci, & Dempsey, 2013). The extracellular galectin-3 has been reported

to exhibit various autocrine and paracrine effects that mediate cell adhesion and chemoattractant for glial cells (Pasquini et al., 2011).

In the last years, growing evidences have highlighted galectin-3 as a potent immune regulator (Breuilh et al., 2007; Rabinovich & Toscano, 2009; Shin, 2013) that modulates the activation of various immune-associated cells, monocytes, dendritic cells, macrophages and glial cells (Henderson & Sethi, 2009; Sato & Nieminen, 2002). For instance, galectin-3 is secreted into the extracellular space upon exposure to various inflammatory stimuli such as lipopolysaccharide (LPS) (Dhirapong, Lleo, Leung, Gershwin, & Liu, 2009; Li et al., 2008) and interferon-γ (IFN-γ) (Jeon et al., 2010). Recently, galectin-3 expression was augmented in microglial cells upon exposure to various inflammatory stimuli indicating its involvement in the CNS inflammation (Lalancette-Hébert et al., 2012; Rotshenker, 2009; Wesley et al., 2013). The up-regulation of galectin-3 induces chronic inflammatory responses by mediating injury-induced microglial activation and proliferation in the unilateral transient focal cerebral ischemia model in mice (Lalancette-Hébert et al., 2012). In addition, galectin-3-deficient mice exhibited abnormal neutrophil recruitment with a significant decreased in macrophage survival exemplifies its pro-inflammatory role during inflammation (Colnot et al., 1998). Moreover, the production of pro-inflammatory mediators in microglial cells were significantly augmented through the activation of tyrosine phosphorylation of Janus kinase 2 (JNK2) and signal transducers and activator of transcription 1 (STAT1), STAT3 and STAT5 (Filer et al., 2009; Jeon et al., 2010; Maeda et al., 2003) following exposure to galectin-3. Even though various evidences have implicated the pro-inflammatory role of galectin-3, the molecular mechanisms that regulate its production remain to be elucidated. Therefore, the elucidation of the molecular mechanisms that modulate Galectin-3 in microglia would be beneficial in the intervention of inflammation-mediated neurological disorders.

2.2.5 The modulation of innate immunity in the brain

The activation of innate immunity in the CNS particularly in the brain is important in providing the first line of defense against neuronal insults (Heneka, Kummer, & Latz, 2014). The innate immune responses in CNS are mediated by microglia and astrocytes that can exert multiple functions such as neuroprotective and neurorestorative towards the damaged or infected tissues (Ransohoff & Brown, 2012). However, microglia and astrocytes can engage in significant cross-talk with CNS-infiltrating T cells and other components of the innate immune system that further exacerbate the inflammatory responses (Lampron, ElAli, & Rivest, 2013; Ransohoff & Brown, 2012). The overproduction of cytokines and chemokines by microglia that compromise the integrity of BBB can promote the trafficking of leukocytes and macrophages from the periphery which exacerbate the inflammatory responses (Dimitrijevic, Stamatovic, Keep, & Andjelkovic, 2006; Minami et al., 2006).

The BBB is a tightly controlled membrane formed by specialized endothelial cells that are closely attached to each other via tight junctions (TJs) and adherens junctions (AJs) (Hermann and Elali, 2012; Hawkins and Davis, 2005). BBB plays a central role in innate immunity regulation as it restricts and prevents the entry of blood-borne molecules and peripheral cells into the brain (Wilson et al., 2010; Pardridge, 2003). By virtue of this, the BBB is a barrier that separates the brain from the peripheral immune cells as well as various infectious agents (Pachter et al., 2003). Several reports have demonstrated that some of immune cells are capable of infiltrating the BBB (Carson et al., 2006) but this process is limited under normal physiology. Nevertheless, neuronal injury and chronic neuroinflammation can induce disruption in the BBB permeability causing the peripheral immune cells to infiltrate the brain and further exacerbates the local inflammatory responses (Wilson et al., 2010).

In innate immunity, microglia act as the chief player within the brain that constantly monitor the brain microenvironment through the pattern recognition receptors (PRRs) such as Toll-like receptors (TLRS) and Nod-like receptors (NLRs) (Heneka et al., 2014). The activation of PRRs can result in the activation of transcription factors that provide the endpoint of the innate immune responses by producing cytokines, chemokines and free radicals on microglia that can initiate the phagocytosis of pathogen (Lampron et al., 2013; Turner et al., 2014). Moreover, neurons also serve as the main source of inflammatory stimuli and therefore, contribute to the activation of innate immune responses (De Haas, Van Weering, De Jong, Boddeke, & Biber, 2007; de Jong et al., 2005; Suzumura, 2013).

Additionally, astrocytes are another type of brain immune cells that can induce innate inflammatory responses within the brain. Similar to microglia, astrocytes can act as scaffold cells that guide the neurons during the brain development as well as orienting newly formed brain capillaries (Jacobs and Doering, 2010; Bozoyan et al., 2012). Generally, astrocytes are capable of producing various bioactive molecules such as VEGF, TGFβ, bFGF, TNFα, IL-1β, IL-3, IL-6, Ang-1, B cell-activating factor (BAFF) and glial-derived neurotrophic factor (GDNF) (Igarashi et al., 1999; Chung and Benveniste, 1990; Farina et al., 2007; Abbott et al., 2006). During aberrant inflammation, the overactivation of microglia can lead to the excessive activation of astrocytes which serves as a hallmark of CNS inflammation. Nonetheless, it is known that astrocytes also offer neuroprotective functions by regulating the level of inflammation and initiate tissue repair following danger elimination (Bsibsi et al., 2006; Cabezas, El-Bachá, González, & Barreto, 2012).

2.2.6 Microglia as the guardian of innate immunity

In peripheral tissues, macrophages are generally the first line of defense against any injury and infection. In the brain, macrophages are substituted by other types of immune cells known as astrocytes and microglia (González et al., 2014; Suzumura, 2013). Among these immune cells, microglia constitute 5 - 20% of the brain cell population and are the key cellular entities in inflammation-mediated neurodegenerative diseases (González et al., 2014; Tambuyzer, Ponsaerts, & Nouwen, 2009). The activation microglia regulates the synthesis of various inflammatory cytokines, chemokines and other inflammatory mediators that are either cytotoxic or protective to the brain system (Tambuyzer et al., 2009).

During the brain development, microglia exert various physiological functions that contribute at regulating number of neuronal cells (Ashwell, 1990; Egensperger et al., 1996; Marin-Teva et al., 2004; Streit, 2001). Nevertheless, the understanding on how microglia regulate the physiological function mainly depends on the CNS microenvironment. In a normal condition, they are maintained in a resting state with a ramified morphology characterized by a small cell body with fine cellular projections(branches) and low expressions of activation-associated surface antigens. In this state, microglia are highly dynamic cells that are actively moving and monitoring the brain against any threat (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011; Kreutzberg, 1996). Unlike other cells in the brain, microglia are extremely sensitive towards any kind of stimuli. Following nerve injury or the exposure to infectious bacteria, microglia will be rapidly activated within a few of minutes, migrating toward the lesion site and change their shape to adopt a rounder phenotype that will facilitate the phagocytosis of debris and mediate inflammatory reactions (Boche, Perry, & Nicoll, 2013).

Microglia are capable of promoting synaptogenesis through the synthesis of neurotrophic factors during the brain development. (Aarum et al., 2003; Elkabes et al., 1996; Walton et al., 2006) Moreover, microglia support the regulation of synaptic transmission and remodeling of the brain (Fourgeaud and Boulanger, 2007; Jonakait et al., 1996, 2000; Stevens et al., 2007). Nonetheless, aberrant microglial activation has been associated with various injuries and pathogenesis of neurodegenerative diseases (Boille´e et al., 2006; Long-Smith et al., 2009; Mount et al., 2007; Schwab and McGeer, 2008). These neurotropic and neuroinflammatory properties of microglia are mediated through the innate and adaptive immunity that trigger the release of inflammatory mediators (Hanisch, 2002b; Kielian, 2004). These processes are mediated by the activation of pattern recognition receptors (PRRs) that allows microglia to detect both pathogen- and host-derived ligands within the CNS (Figure 2.4) (Jack et al., 2005; Olson & Miller, 2004).

Neurons, astrocytes and oligodendrocytes can produce ligands that bind to the receptors in microglia and induce signaling that promote or inhibit microglial activation (Kettenmann et al., 2011). Neuronal damage followed by degeneration can result in the reduction of neuronal ligands that bind to the inhibitory receptors on the microglia and thus, triggering the microglia priming process and activation (Norden & Godbout, 2013; Perry & Holmes, 2014). The activated microglia secrete various pro-inflammatory mediators that can propagate the inflammatory activity of astrocytes and result in excessive production of pro-inflammatory factors (Hanisch, 2002a; van Rossum & Hanisch, 2004). These pro-inflammatory mediators (cytokines and chemokines) can induce the remote activation and migration of microglia to the injured sites thus resulting in exacerbated inflammatory responses. This result in the disruption of BBB and leukocytes trafficking that induces the infiltration of other activated immune cells (Dheen, Kaur, & Ling, 2007; Walter & Neumann, 2009).

The aberrant activation of microglia in the brain can lead to the development of chronic neuroinflammation-mediated neurodegenerative diseases (Akiyama et al., 2000b; McGeer & McGeer, 2010). Nonetheless, microglia are also highly plastic cells and have been demonstrated to induce neuroplasticity in neuronal models (Cullheim & Thams, 2007; London, Cohen, & Schwartz, 2013). Moreover, microglia are responsible for the modeling of neurons and synapses in the brain (Tremblay & Majewska, 2011; Xavier, Menezes, Goldman, & Nedergaard, 2014). For example, anti-inflammatory cytokines such as IL-4 or IL-13, can induce the polarization of microglia activation toward M2 phenotype to promote the scavenging of dead cells, thus favouring wound healing (Ponomarev et al., 2007; Butovsky et al., 2006; Kawanokuchi et al., 2008). Additionally, microglia produce neurotrophic factor such as BDNF, NGF and GDNF which suppress neuronal apoptosis (Batchelor et al., 2002; Elkabes, DiCicco-Bloom, & Black, 1996; Honda, Nakajima, Nakamura, Imai, & Kohsaka, 1999; Presta et al., 1995). Since their discovery, various immunotherapies that target the microglial activity have been developed to modulate their protective and regenerative properties in suppressing chronic neuroinflammation (González et al., 2014)

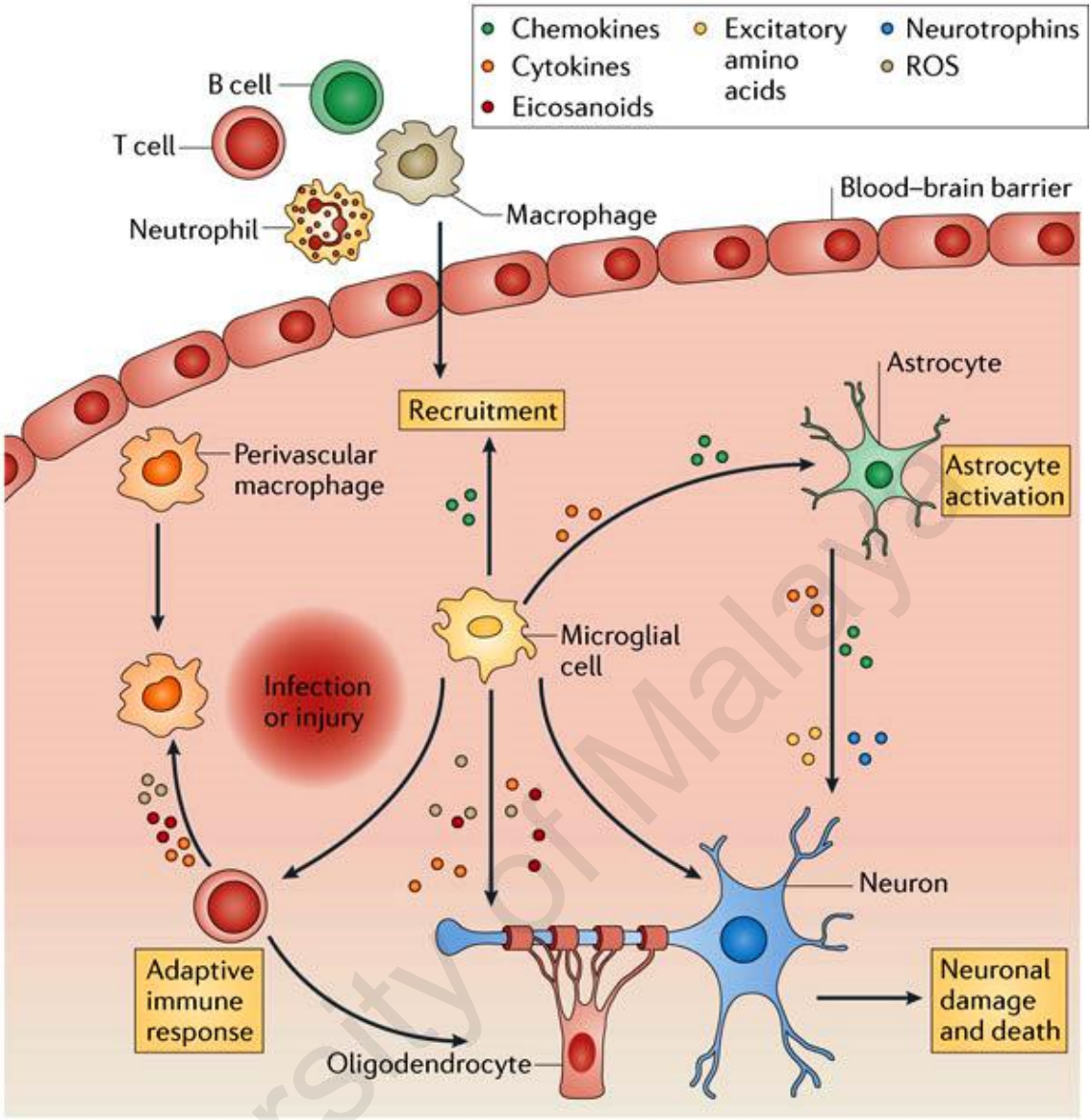


Figure 2.4 The role of microglia in inducing the innate immune response that leads to alterations in the CNS microenvironment at almost every level of control, from gene expression and cellular differentiation to changes in cellular composition through the recruitment of blood-derived cells (Adapted from Walsh, J. G., Muruve, D. A., & Power, C. (2014).

2.2.7 Toll-like receptors (TLRs) in innate immunity

Innate immune cells are strategically distributed throughout the organism to play an important role in immune response by detecting microorganisms or pathogens .The detection and recognition of pathogens are triggered by the PRRs that recognize broad groups of microbial structures known as pathogen-associated molecular patterns (PAMPs) (Akira, Uematsu, & Takeuchi, 2006). PAMPs are also generically known as microbial-associated molecular patterns (MAMPs). PRRs can be grouped into two main classes which are transmembrane (TLRs and C-type lectin receptors) and cytoplasmic proteins (NOD-like receptors, retinoic acid-inducible gene-I-like receptors, pyrin and HIN domain-containing family members) (Kawai & Akira, 2010; Ransohoff & Brown, 2012). Almost all of the PRRs modulate the immune responses by inducing the activation of highly conserved transcriptional factors, such as NF-κB and AP-1. Recently, a new class or PRRs known as inflammosomes was identified and reported to induce a non-transcriptional program that is responsible for the activation of inflammatory caspases (caspase-1) required for the maturation of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18 (Hanamsagar, Hanke, & Kielian, 2012).

Almost two decades ago, *Drosophila* Toll was the first receptor to be identified and showed to participate in innate immune responses against fungal infections (Akira, Takeda, & Kaisho, 2001; Rock, Hardiman, Timans, Kastelein, & Bazan, 1998). Following this discovery, various homologues of Toll known as Toll-like receptors (TLRs) have been identified to recognize PAMPs and elicit innate immune responses (Jack et al., 2005). TLRs are a group of transmembrane pattern-recognition receptors that induce the initial signals in response to the recognition of diverse PAMPs (Kawai & Akira, 2009; Kumar, Kawai, & Akira, 2011). To date, thirteen mammalian TLR paralogues have been described (TLR1-10 in humans, TLR1-9 and TLR11-13 in mice) and most of them are capable of recognizing distinct PAMPs derived from various

microbial pathogens (Akira & Hemmi, 2003; Kumar et al., 2011). The process of TLR-mediated recognition of MAMPs and DAMPs can occur at the intracellular or the plasma membrane. For example, the primary PAMPs recognition of TLR1, TLR2, TLR4, TLR5 and TLR6 occurs at the plasma membrane (Beutler, 2009; Kumar et al., 2011). This interaction allows these TLRs to bind the specific lipid or protein structures that are either expressed on the surface of pathogens or released in the extracellular space. On the other hand, intracellular PAMPs recognition receptors are TLR3, TLR7, TLR8, TLR9, TLR11, TLR12 and TLR13 that are localized in intracellular vesicular compartments which recognize nucleic acids or microbial components (Beutler, 2009; Meylan, Tschopp, & Karin, 2006). These differences in the PAMPs recognition pattern could explain the different TLR activation induced by ligands from microbial pathogens (Kay, Scotland, & Whiteford, 2014). For instance, lipoproteins activate TLR2 while bacterial lipopolysaccharide (LPS) and flagellin induce the activation of TLR4 and TLR5, respectively (Kawai & Akira, 2011).

TLRs can activate signaling pathways that provide specific immunological responses tailored to the PAMP (Beutler, 2009). One of the major pathways that can be triggered by TLRs is the activation of NF-κB (Kawai & Akira, 2007). In addition, the activation of TLRs also activates the Jun amino-terminal kinase (JNK) and p38 MAP kinases that induce transcription and regulate the stability of mRNAs (Brown, Wang, Hajishengallis, & Martin, 2011). Even though TLRs are known to activate the NF-κB and MAP kinases signaling pathways, the final gene expression profile from the individual TLRs are different (Brown et al., 2011; Liu, Shepherd, & Nelin, 2007). The specific immune response initiated by individual TLRs is mainly dependent on the recruitment of a single or a specific combination of Toll-like interleukin-1β receptor (TIR) domain-containing adaptor protein (such as myeloid differentiation primary response protein88 (MyD88), MyD88-adapter-like (Mal or TIRAP), TIR-related

adaptor protein inducing interferon (Trif) and Trif-related adaptor molecule (Tram) (Kawai & Akira, 2006; Kawai & Akira, 2010). The MyD88 activation pathway is commonly utilized by all TLRs except TLR3 (Fitzgerald et al., 2001; Warner & Núñez, 2013). MyD88 has a Toll-IL-1 receptor (TIR) domain that is similar to TLRs that participates in the ligand-induced assembly of a TIR-TIR platform by the dimerization of two TLRs (except TLR2 and TLR4) (Lin, Lo, & Wu, 2010). TLR3 can activate an alternative pathway known as MyD88 independent TLR activation to induce the activation of NF-κB and IRF3 and the induction of type I IFN and inflammatory cytokines production (Bagchi et al., 2007; Yamamoto et al., 2003).

2.2.7.1 Toll-like receptor 4 regulation in neuroinflammation

Toll-like receptor 4 (TLR-4) was discovered as the receptor that responds to lipopolysaccharide (LPS) or endotoxin from the outer membrane of Gram-negative bacteria (Poltorak et al., 1998). Even though LPS can induce the transcription of TLR2 in the brain, this receptor is not capable of modulating the immune response to LPS (Naert, Laflamme, & Rivest, 2009). On the opposite, TLR4 is constitutively expressed in microglia (Lehnardt et al., 2003) and following its activation by LPS, pro-inflammatory mediators are synthesis through NF-κB signaling pathway (Akira & Takeda, 2004). The constitutive expression of TLR4 allows it to act as a sensor in initiating the cerebral innate immune response following systemic bacterial infections and when becomes abberant, may have detrimental to neuronal structures (Miller, Ernst, & Bader, 2005). Moreover, mounting evidences have demonstrated that TLR4 is also involved in the development and progression of neurodegenerative diseases (Balistreri et al., 2008; Crowley et al., 2015; Noelker et al., 2013).

The binding of LPS to TLR4 is assisted by the LPS binding protein (LBP) which transfers LPS to the receptor complex that is composed of CD14, MD-2 (or LY96) and

TLR4 (Lu, Yeh, & Ohashi, 2008; Nagai et al., 2002). Upon LPS binding, the TLR4–MD-2 complex may generate aggregates to form a homodimeric state complex (Buchanan, Hutchinson, Watkins, & Yin, 2010; Fitzgerald et al., 2003; Nagai et al., 2002). Following this, TLR4 through TIR domain will transmit intracellular signals that recruit adaptor molecules and kinases triggering the activation of downstream signaling cascade to produce of pro-inflammatory cytokines and chemokines (Fitzgerald et al., 2003; Lu et al., 2008; Martin, Rehani, Jope, & Michalek, 2005) (Figure 2.5). TLR3 and TLR4 are the two unique TLRs that can induce signal transduction through the MyD88-independent pathway (Fitzgerald et al., 2003; Kawai & Akira, 2006). More importantly, TLR4 is the only TLR that can induce the signal transduction through both MyD88-dependent and MyD88 independent pahways (Yamamoto et al., 2003; Zhai et al., 2004). The activation of TLR4 through MyD88-dependent pathway can lead to the rapid expression of iNOS and a wide range of pro-inflammatory cytokines, chemokines and their receptors (Gorina, Font-Nieves, Márquez-Kisinousky, Santalucia, & Planas, 2011; Lehnardt, 2010b).

The overactivation of TLR4 has been long thought to be the causative factor of Alzheimer's disease (Balistreri et al., 2008; Song et al., 2011; Walter et al., 2007). Moreover, numerous reports have demonstrated that TLR4 contributes to neuronal death and disruption of the BBB by its ability to activate the NF-κB signaling activation (Alfonso-Loeches, Ureña-Peralta, Morillo-Bargues, Gómez-Pinedo, & Guerri, 2015; Chen et al., 2009). This NF-κB activation led to the expression of various pro-inflammatory genes that encode cytokines, chemokines and enzymes such as COX-2 and MMP-9, which are responsible for the development of chronic neuroinflammation (Yao et al., 2013; Zhao, Zhou, Xu, & Zhang, 2014). Even though the expression of TLR4 was associated with chronic neuroinflammation and brain injury, the role of TLR4, it not restricted to the development of neurodegenerative diseases only.

Interestingly, the expression of TLR4 was linked to the increased uptake of Aβ peptide that promotes its clearance before its accumulation as extracellular amyloid fibrils (Akiyama et al., 2000a; Tahara et al., 2006). Moreover, the production of several heat-shock proteins (HSPs) that activate TLR4 during inflammation is also linked to the increased uptake and clearance of Aβ (Kakimura et al., 2002; Triantafilou & Triantafilou, 2004) Nevertheless, HSPs are also reported to promote neurodegeneration and neuropathic pain through the TLR4 signaling (Hutchinson et al., 2009; Lehnardt et al., 2008). These ambigous inflammatory properties of TLR4 underscore the importance of regulating the molecular signaling essential in normal brain function.

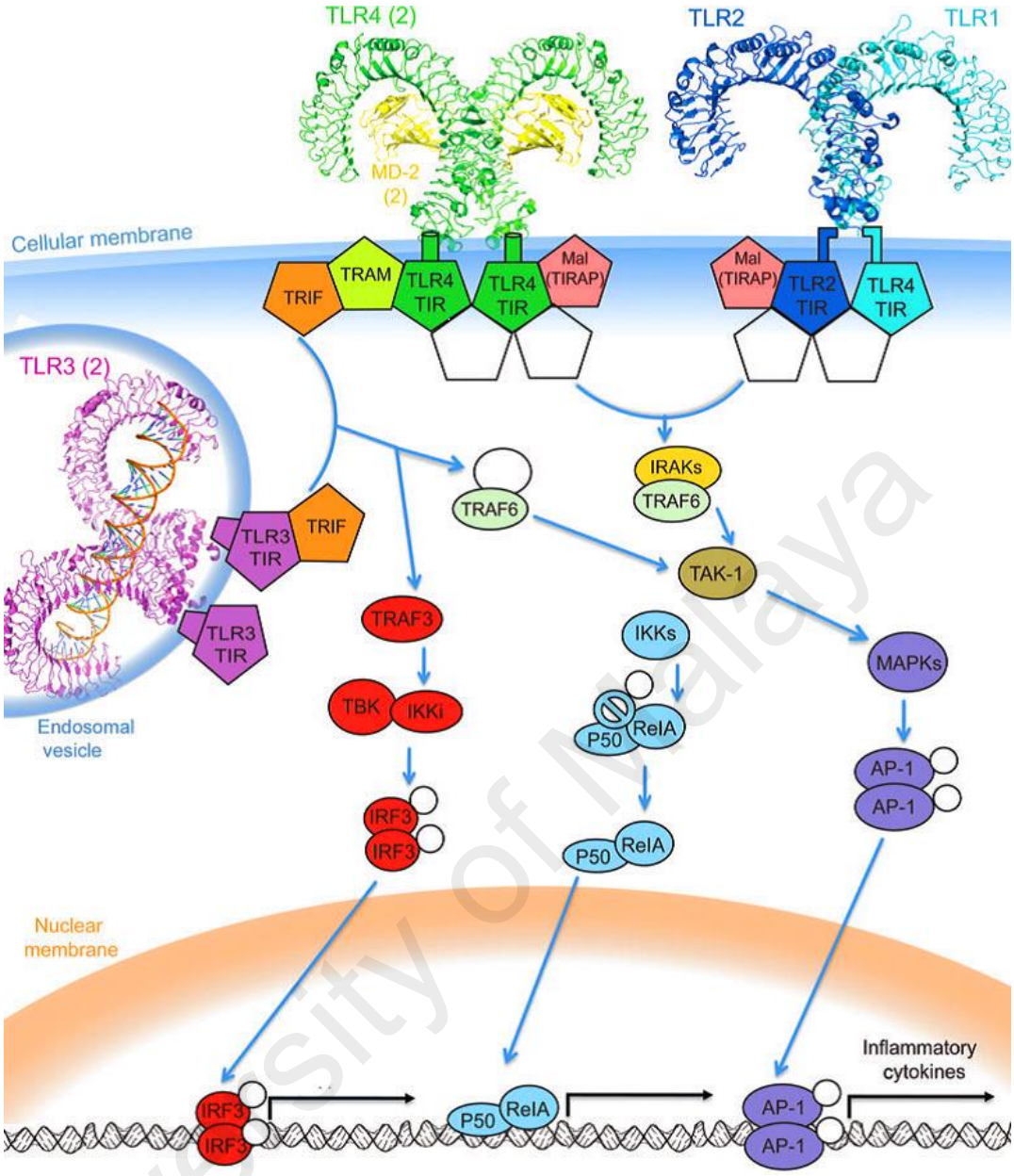


Figure 2.5 The activation of TLR4 through MyD88-dependent and MyD88-independent (TRIF cascade). The MyD88-dependent signaling is common to most TLRs except TLR3 whereas the TRIF cascade signaling is common for both TLR3 and TLR4. TLR4 is the only TLR that exclusively activate both MyD88-dependent and MyD88-independent pathways. Adapted from Buchanan, M. M., Hutchinson, M., Watkins, L. R., & Yin, H. (2010).

2.3 Neuronal apoptosis in neurodegenerative diseases

In a normal cycle of tissue proliferation, the aged and older cells are set to decease in order to give way for the generation of new cells. Such setting of programmed cell death or apoptosis is a highly organized and orchestrated form of cell death that is extremely vital in tissue homeostasis. Apoptosis can take place as a means of defense mechanism in immune reactions when cells are harmed by toxic and deleterious substances (Liu, Zhao, Ji, & Yu, 2014; Norbury & Hickson, 2001; Xu et al., 2015). During development of the central and peripheral nervous systems, neurons undergo apoptosis which coincides with synaptogenesis and neurogenesis (Aimone, Deng, & Gage, 2014; Gohlke, Griffith, & Faustman, 2004). Initial overproduction of neurons which is followed by death of some is thought to be the adaptive process that provides enough neurons to form nerve cell circuits that are precisely matched to their functional specifications (Buss, Sun, & Oppenheim, 2006; Southwell et al., 2012).

Apoptosis is an active process which can be characterized by the changes in morphological features such as cell shrinkage, chromatin condensation, loss of nuclear membrane integrity, plasma membrane blebbing and eventually the breaking off of cellular fragments giving rise to the formation of apoptotic bodies (Bredesen et al., 2006; Newton et al., 2014). Apoptosis holds a cardinal role in the maturation of the nervous system as well as neural architecture through the interplay between both anti- and pro-apoptotic protein (Czabotar, Lessene, Strasser, & Adams, 2014; Yuan & Yankner, 2000). Basically, neurons either will render an adaptive response or they would initiate apoptosis when they are exposed to stress signals or apoptotic stimuli such as withdrawal of neurotrophic factor, ischemic stroke, misfolded proteins, mitochondrial-complex inhibition, excessive calcium entry and excitotoxicity (Emdadul Haque et al., 2003; Mattson, 2000; Okouchi et al., 2007). For example, in adult neurodegenerative disorders, the formation of misfolded proteins such as β-amyloid

aggregates induces oxidative stress and excessive influx of intracellular Ca^{2+} which eventually lead to apoptosis (Fukushima et al., 2011).

2.3.1 Molecular machinery of neuronal apoptosis

Cellular apoptosis can be divided into extrinsic and intrinsic apoptosis pathways (Figure 2.6). The extrinsic pathway involves the activation of a death receptor upon binding of its ligand, recruitment of specific proteins at the death domain and downstream signaling through a cascade of protein–protein interactions (Beurel & Jope, 2006; Putcha et al., 2002; Yon, Daniel-Johnson, Carter, & Jevtovic-Todorovic, 2005). The best-characterized death receptors which function in apoptosis are Fas (CD95 or APO-1), TNF receptor 1(TNFR1), TNF-related apoptosis inducing ligand receptor 1 (TRAIL-R1) (death receptor 4, DR4), and TRAIL receptor 2 (TRAIL-R2) (death receptor 5, DR5) (Eimon & Ashkenazi, 2010; Lee, Seo, Jeong, Lee, & Song, 2012; Li, Liu, Qiao, & Zhang, 2009). The first step in the activation of receptor-mediated apoptosis is initiated by ligand binding to the death receptor that causes receptor trimerization and recruitment of adaptor proteins at the cytosolic death domain (DD) of the receptor. Fas- and TRAIL-associated death domain (FADD/TRADD) proteins will further bind to the pro-caspases-8 and/or -10 and form the death-inducing signaling complex (DISC) complex where these initiator caspase is activated (Lee et al., 2012; Wang & El-Deiry, 2003). In contrast, the intrinsic pathway involves the mitochondria and the release of pro-apoptotic factors into the cytosol with subsequent activation of executioner caspases. Generally, the intrinsic apoptotic pathway is regulated by the B-cell lymphoma 2 (Bcl-2) family of proteins which are further grouped into anti-apoptotic proteins and pro-apoptotic proteins, the adaptor protein Apaf-1 (apoptotic protease-activating factor 1) and the cysteine-aspartyl-specific proteases family (caspase) (Hu et al., 2014; Kerr, Winterford, & Harmon, 1994; Ola, Nawaz, & Ahsan, 2011).

The Bcl-2 family protein critically regulates the mitochondrial-mediated apoptosis by functioning either as promoters or inhibitors of the cell death process (Kuwana & Newmeyer, 2003). The first group of Bcl-2 proteins is anti-apoptotic proteins which include A1/Bfl1, Bcl-2, Bcl-w, Bcl-xL, Boo/Diva, Mcl-1, NR-13 and Nrf3 (Fu & Fan, 2002; Milosevic et al., 2003; Gross et al., 1999). The anti-apoptotic proteins function by binding and inhibit the pro-apoptotic proteins of the Bcl-2. Group II of Bcl-2 proteins are all pro-apoptotic proteins which include Bad, Bak, Bax, Bcl-rambo, Bcl-xS, Bid, Bik and Bim (Milosevic et al., 2003). Both Bax and Bak which are originally localized in the cytoplasm can translocate to the mitochondrial outer membrane resulting in conformational changes, oligomerization and insertion into the mitochondrial outer membrane that elevates the mitochondria permeability transition pores. This is followed by the release of cytochrome *c* from mitochondria which bind to an adaptor molecule, apoptotic protease-activating factor-1 (Apaf-1) forming apoptosome where procaspase-9 is recruited and activated in the presence of ATP or dATP. Caspase-9 further cleaves and activates the effector caspases, pro-caspase -3 and/or -7 that processes substrates like caspase-activated DNase or poly (ADP-ribose) polymerase-1 leading to DNA fragmentation (Ola et al., 2011). The anti-apoptotic protein can inhibit apoptosis by binding to pro-apoptotic protein that prevent the release of mitochondrial pro-apoptotic factors, such as cytochrome *c*, apoptosis inducing factor (AIF) and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) (Ola et al., 2011).

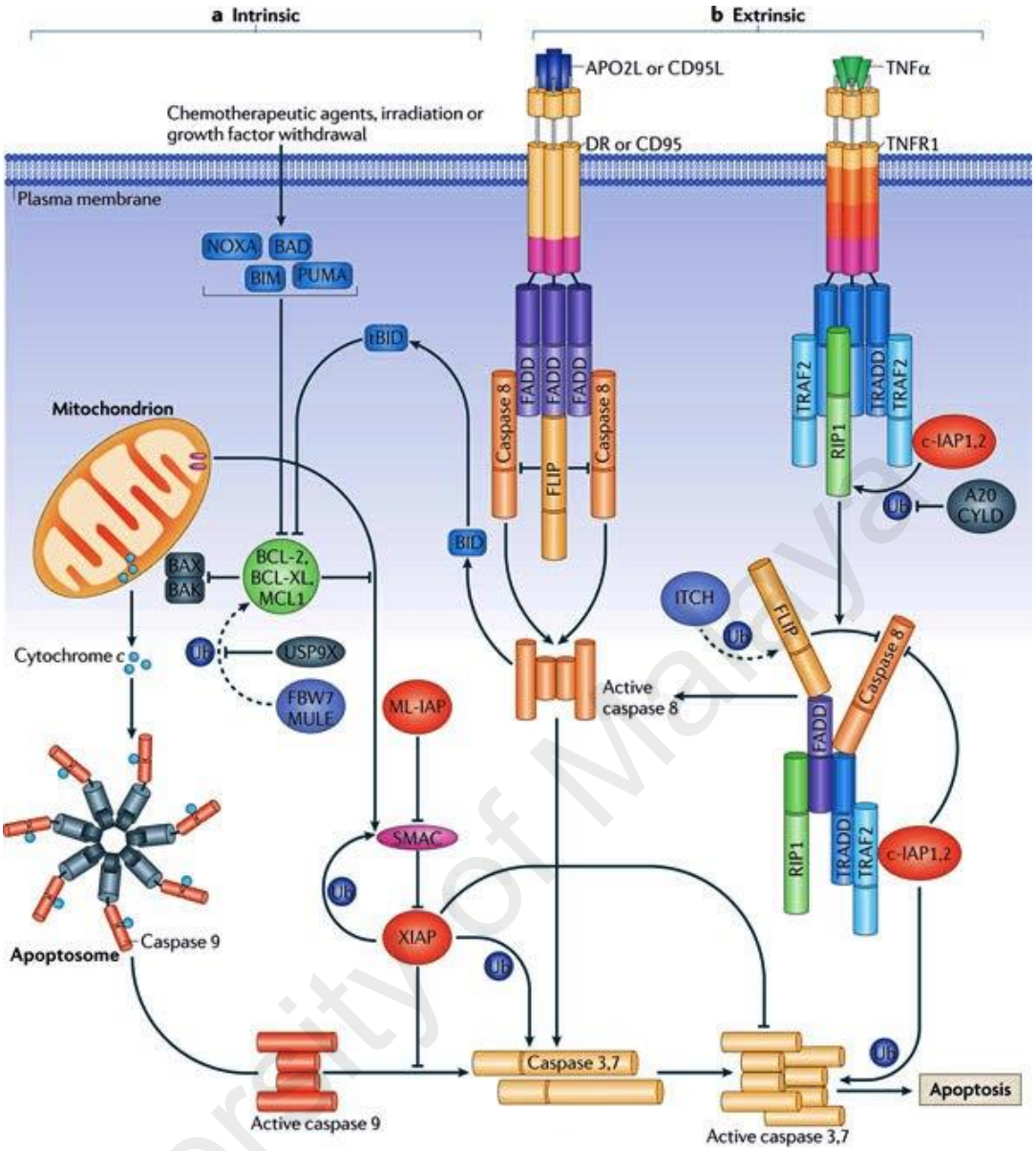


Figure 2.6 The execution of apoptosis through mitochondrial (intrinsic) and death receptor (extrinsic) pathways that result in the activation of caspases. (a) The intrinsic pathway cab be triggered by chemotherapeutic agents, irradiation or growth factor withdrawal whereas (b) the death receptor pathway is induced following the engagement of death ligands (CD95L/ FASL, APO2L/TRAIL or TNFα) engage to their respective receptors to trigger the formation DISC and activation of caspases. Adapted from Vucic, D., Dixit, V. M., & Wertz, I. E. (2011).

2.4 Neuritogenesis in the central nervous system

Neurons are highly specialized and electrically excitable cells with tree-like shape structure that form the building blocks of the CNS. The neurons in the brain are generally amitotic and as such, they are irreplaceable when damaged or die. Nevertheless, the advancement in neuroscience has led to the discovery of neural stem cells that are able to reproduce and differentiate into highly specialized neuronal cells (Lim et al., 2008; Yamane et al., 2010). Moreover, this finding is supported with the ability of neural progenitors in the dentate gyrus at the hippocampus and the subventricular zone that can differentiate into functional neurons and glia cells (Liu et al., 2008; Sakaguchi et al., 2006).

The human memory function is vulnerable to various conditions such as injury, stroke, head trauma, hypoxia, cardiac surgery, malnutrition, attention deficit disorder, depression, anxiety, and aging (Van Spornsen and Hoogenraad, 2010). Furthermore, the loss of memory and ability to learn is often associated with aging due to its frequent occurrence in later part of human lifespan. Neurogenesis, the birth of new neurons from stem cells, occurs rapidly during development of the nervous system and to a much more limited extent in some regions of the adult nervous system (Kempermann, 2008; Kempermann, Gast, & Gage, 2002). Neuroplasticity is believed to be an important mechanism of neurogenesis under physiological conditions and in brain repair following injury (Kempermann, Jessberger, Steiner, & Kronenberg, 2004; Kitamura et al., 2009). Neuroplasticity is the process of learning and memory is defined as a range of adaptive changes that occur in the structure and function of neurons in the CNS as a feedback to physiological or pathological perturbations (Ge, Sailor, Ming, & Song, 2008). Neuroplasticity is an essential process that involves chemical, electrical, molecular and cellular responses which induces the neuronal reorganization within and/or between brain regions. Furthermore, the formation of memory and the process of

learning are strongly correlated with neuroplasticity with broad changes in the expression of key molecules, intracellular signaling pathways, synaptic strength and formation of neurites that form the functional neuronal network. The field of neuroprotection can be further complemented with cognitive function enhancement through neuroplasticity process such as neuritogenesis and synaptogenesis which can improve the brain cognitive ability. The neuritogenesis or the sprouting and growth of axons or dendrites is responsible in strengthening the synapses, promoting neuronal survival and in neuronal pathfinding during brain development (Min et al., 2006). Neuritogenesis is a crucial step for the proper organization of functional neuronal networks during neuronal differentiation. Failure in generating accurate synaptic connections will cause the developing neurons to undergo neuronal apoptosis whilst in a developed brain, post-mitotic neurons become dependent on neurotrophic factors and neurotransmitters for survival (Read & Gorman, 2009b). Neuritogenesis is also essential in developing therapies that can promote neuronal (axonal) regeneration following nerve injury. Therefore, a deeper understanding of neuroplasticity, particularly neuritogenesis and the molecular signaling involved is important in the development of innovative therapeutic approaches against neurodegenerative diseases as well as in enhancing the recovery (neurorestoration) from neural trauma (Van Spronsen and Hoogenraad, 2010).

2.5 The signaling pathways in neuroprotection and neuritogenesis

2.5.1 Mammalian target of rapamycin (mTOR)

The mammalian target of rapamycin (mTOR), a target molecule of rapamycin is an atypical serine/threonine protein kinase (289kDa) that is ubiquitously expressed and belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family (Betz & Hall, 2013). mTOR has been widely reported to possess significant roles in cell growth and proliferation, apoptosis,inflammation, autophagy and cytoskeletal organization (Dazert

& Hall, 2011; Laplante & Sabatini, 2012). Generally, mTOR can be found in two cellular complexes, termed mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Both mTORC1 and mTORC2 can be differentiated based on their unique structures and substrates and sensitivity towards rapamycin (Feldman et al., 2009). The mTORC1 is mainly characterized by the presence of RAPTOR (regulatory-associated protein of mTOR). In addition, mTORC1 complex is formed by other proteins which are mammalian lethal with SEC-13 protein 8 (mLST8, also known as GbL), proline-rich Akt/PKB substrate 40 kDa (PRAS40) and DEP domain containing mTOR-interacting protein (DEPTOR) and and the TTI1/TEL2 complex (Feldman et al., 2009; Sancak et al., 2007; Yip, Murata, Walz, Sabatini, & Kang, 2010). In contrast, mTORC2 is defined by the presence of RICTOR (rapamycin-insensitive companion of mTOR) with the additional proteins which are mammalian stress-activated map kinase-interacting protein 1 (mSIN1) and protein observed with RICTOR 1 and 2 (PROTOR1/2) (Alessi, Pearce, & Garcia-Martinez, 2009; Feldman et al., 2009; Frias et al., 2006).

As shown in Figure 2.7, both mTOR complexes are important regulators of the intricate signaling pathways that can be activated by several stress conditions and growth factor signals. The mTORC1 complex activity can be inhibited following acute rapamycin exposure while prolonged rapamycin exposure can compromise the mTORC2 complex integrity to varying degrees leading to its suppression (Feldman et al., 2009). Nevertheless, the uncontrolled mTORC1 cellular activity has been reported to be associated with a number of pathological conditions which includes metabolic disorders (Dazert & Hall, 2011; Tsang, Qi, Liu, & Zheng, 2007), cancer and tumor (Shaw & Cantley, 2006; Zoncu, Efeyan, & Sabatini, 2011) and neurological disorders (Hoeffer & Klann, 2010; O'Neill, 2013). Moreover, the phosphorylation of mTOR pathway that is associated with PI3K-Akt signaling can either induce neuronal survival (Li et al., 2010a; Shang, Chong, Wang, & Maiese, 2012) or aberrant activation of

microglia (Russo, Lisi, Tringali, & Navarra, 2009). The involvement of mTOR in inflammation is due to its ability to promote the NF-κB activation that induces the expression of pro-inflammatory molecules such as cytokines, iNOS and COX-2 (Wang et al., 2011a). The activation of mTOR in the neurons contributes to the regulation of several processes in brain development such as synaptic plasticity, learning and memory (Jaworski & Sheng, 2006; Ma et al., 2010). Furthermore, mTOR protein synthesis and mTORC1 are required to induce long-term potentiation (LTP) and long-term depression (LTD) which contributes to the learning and memory process by inducing synaptic plasticity (Hoeffer & Klann, 2010; Stoica et al., 2011).

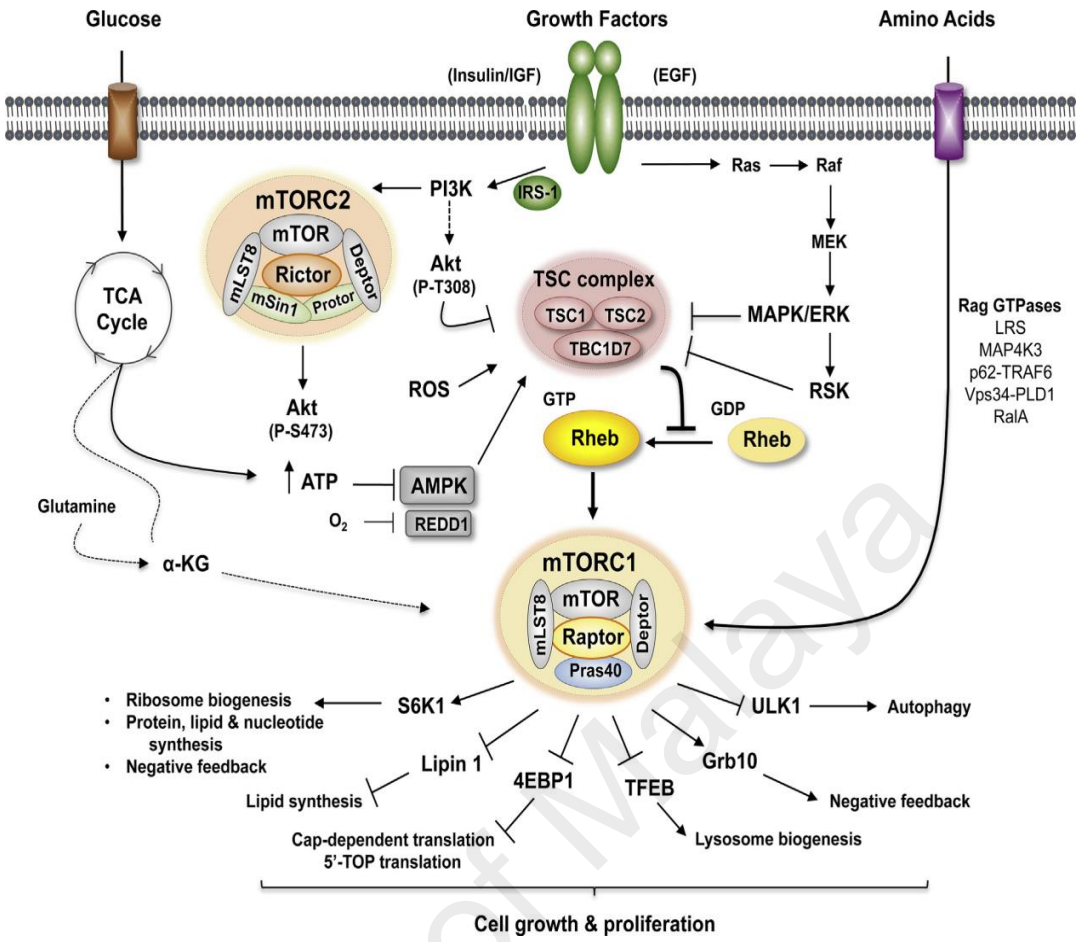


Figure 2.7 The cellular signaling following mTOR activation by various signals. Adapted from Huang, K., & Fingar, D. C., 2014.

2.5.2 Phosphatidylinositol 3-kinase (PI3K) - protein kinase B (Akt)

The PI3K-Akt is one of the major signaling pathways that is implicated in neuroprotection and neuritogenesis. PI3K is a well-known regulator of cell metabolism, growth and cell survival (Yap et al., 2008). Depending on the structure, distribution and mechanism of activation, PI3K can be grouped into three different classes (Franke, Kaplan, & Cantley, 1997; Yap et al., 2008). For instance, class I PI3Ks are grouped into class IA and class IB based on different associated protein adaptors. The class IA PI3Ks are usually activated by receptor tyrosine kinases (RTK) whereas class IB PI3Ks are activated by the GPCRs (Amzel et al., 2008; Walker, Perisic, Ried, Stephens, & Williams, 1999). Following its activation, PI3Ks will generate phospholipid second messengers that are crucial in the signaling cascade during intracellular signal transduction (Figure 2.8).

The serine/threonine protein kinase (Akt or protein kinase B) is the major downstream target following the activation of PI3Ks (Franke, 2008; Franke et al., 1997). In general, the human AKTs has three isoforms namely Akt1, Akt 2, and Akt3. Akt is normally inactive in cytosol and can be activated through stimulation of a variety of transmembrane receptors such GPCR and RTK. Once activated, PI3K phosphorylates the membrane phospholipid phosphatidylinositol (4,5)-diphosphate (PIP2), converting it to phosphatidylinositol (3,4,5)-triphosphate (PIP3). The accumulation of PIP3 promotes the translocation of Akt to the plasma membrane, where Akt binds to PIP3 via its pleckstrin homology (PH) domains, allowing phosphorylation of Thr308 residue of Akt1 by phosphoinositide-dependent kinase 1(PDK1) (Yap et al., 2008). The maximal activity of Akt requires the additional phosphorylation within the carboxyl-terminal regulatory domain (Ser 473 in Akt1) of Akt by PDK2 (Hanada, Feng, & Hemmings, 2004). Furthermore, Akt possesses the ability to inhibit the GTPase-activating protein (GAP) activity of the tuberous sclerosis complex 1 (TSC1) and TSC2

complex via its phosphorylation on TSC2 tuberin protein, leading to the accumulation and activation of the mTORC1 (Huang & Manning, 2009). Besides mTOR, Akt also mediates it effects by regulating the glycogen synthase kinase-3 beta (GSK-3β) that is important in cellular proliferation, programmed cell death, innate immunity as well as neurogenesis in the CNS (Emamian, 2012; Jo et al., 2011; Li et al., 2013).

Akt has been well documented to promote neuronal survival and its dysregulation has been associated with various pathological conditions (Jaworski, Spangler, Seeburg, Hoogenraad, & Sheng, 2005; Luo et al., 2015; Matsuda, Nakanishi, Wada, & Kitagishi, 2013). Akt promotes survival in neurons through phosphorylation of transcription factors forkhead/FOXO, GSK-3β, NF-κB or through phosphorylation of Bcl-2 family members (Brunet et al., 1999; Hanada et al., 2004; Malla et al., 2010). For example, the impairment of Akt signaling induceS neuropathological conditions such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS) (O'Neill, 2013; Rickle et al., 2004). Additionally, the changes in Akt signaling were also recorded in animal model of Huntington's disease and patients (Colin et al., 2005; Humbert et al., 2002). Moreover, a number of evidences have linked the pathogenesis of schizophrenia with the activation of GSK-3β associated with attenuated level of Akt1 in the peripheral lymphocytes and brains of schizophrenic patients (Emamian, 2012).

Akt is the key mediator of several aspects of neuritogenesis, including neurite elongation, branching and calibre (Read & Gorman, 2009b). Neurotrophins such as NGF and BDNF can influence neuritogenesis by binding to RTK receptor tyrosine kinases and activate the PI3K-Akt signal transduction (Jones, Tucker, Rahimtula, & Mearow, 2003; Mullen et al., 2012). The GSK-3β, mTOR (Zheng et al., 2011), CREB (Lai et al., 2011) and peripherin (Konishi et al., 2007) are among the proteins regulated by Akt in neuronal differentiation. For instance, the activation of P3K-Akt/mTOR and

Akt/GSK-3β by atorvastatin and latanoprost promote the neuritogenesis in differentiated RGC-5 cells and cortical neurons, respectively (Jin et al., 2012; Zheng et al., 2011). Additionally, activation of PI3K/Akt can activate CREB which is essential in learning, memory and synaptic plasticity plasticity (Benito & Barco, 2010) since it regulates the neural cell adhesion molecule (NCAM) stimulated axonal outgrowth of PC12-E2 cells (Jessen et al., 2001). Collectively, therapeutic strategy that aims at PI3K-Akt regulation by natural products and drugs should be of interest since modulation of it confers various beneficial cellular functions in the human brain.

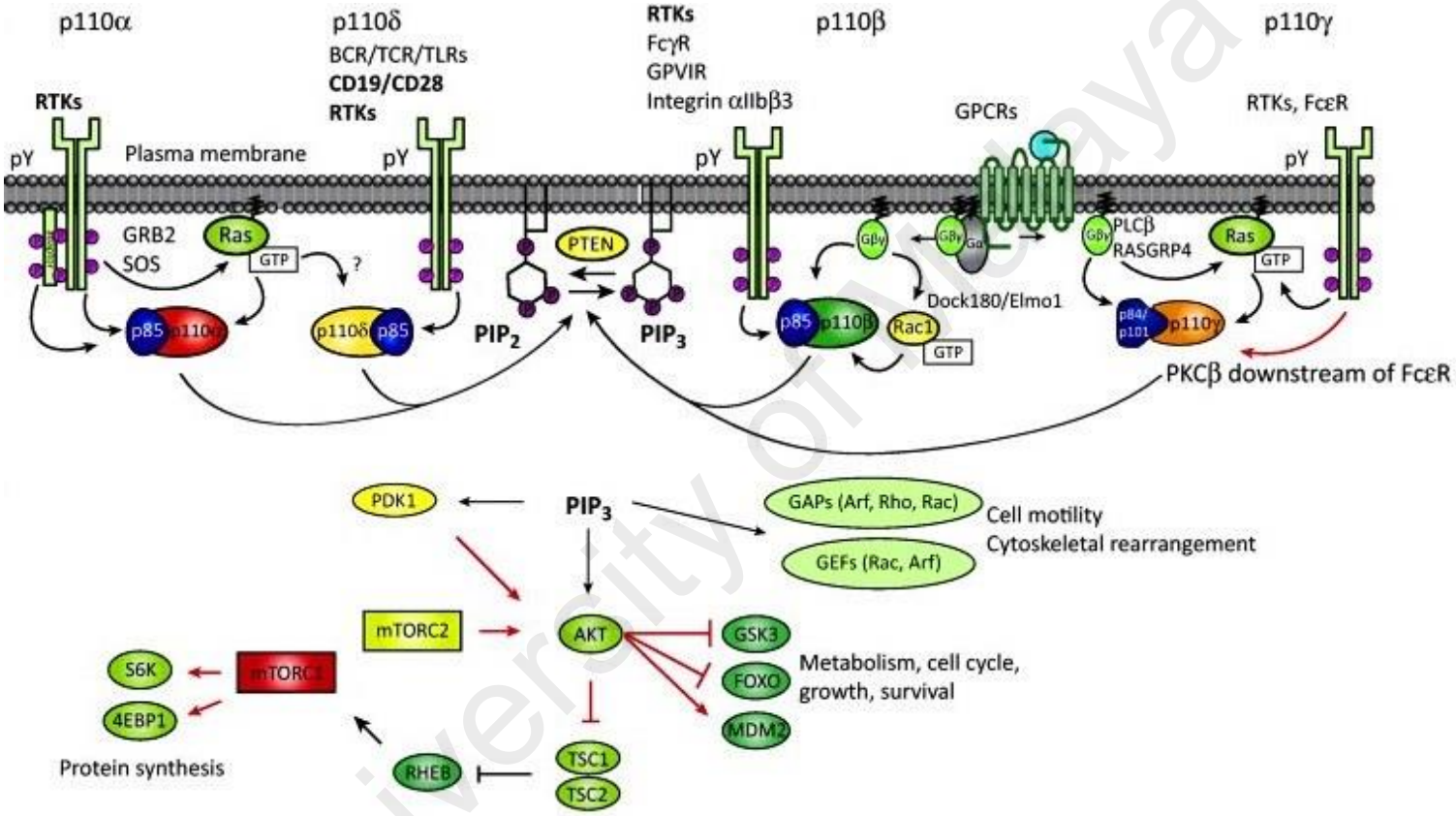


Figure 2.8 The activation of PI3K and its downstream signaling through the activation of GPCRs and RTK. PI3Ks catalyze the formation of PIP_3 which recruits Akt, PDK1 as well as RAS superfamily GEFs and GAPs. The phosphorylation of these kinases induces the phosphorylation of transcription factors such as GSK-3β, CREB and NF-κB that influence cellular functions. Adapted from Burke, J. E., & Williams, R. L. (2015)

2.5.3 Glycogen synthase kinase-3 (GSK-3) in neuroinflammation

Glycogen synthase kinase-3 (GSK-3) was initially discovered as a regulator in glycogen synthesis based on its ability to phosphorylate and inactivate glycogen synthase (Cohen et al., 1981). Recent findings on GSK-3 signaling and function have associated this particular kinase to other functions extending far beyond the regulation of glycogen metabolism (Beurel, Grieco, & Jope, 2015; Kaidanovich-Beilin & Woodgett, 2011). GSK-3 is a serine/threonine protein kinase that is divided into two isoforms, GSK-3α and GSK-3β. Several studies have reported that GSK-3 possesses the ability to phosphorylate and regulate more than 50 substrates in numerous cell signaling pathways (Kaidanovich-Beilin & Woodgett, 2011).

The activity of GSK-3 can be regulated by different mechanisms; (1) phosphorylation at an N-terminal serine, (2) phosphorylation of a tyrosine residue, (3) phosphorylation of a C-terminal serine residue and (4) disruption of the axin-β-catenin multiprotein complex (Duman & Voleti, 2012). The phosphorylation at Tyr216 or Tyr279 induces the activation of GSK-3β and GSK-3α, whereas phosphorylation at Ser9 or Ser21 inactivates GSK-3β and GSK-3α. The inactivation of GSK-3 is normally mediated by protein kinase A (PKA), Akt, protein kinase C (PKC) and ribosomal protein 6 kinase (S6K) that modulate transcription factors. The inactivation of GSK-3β by Akt regulates various transcription factors such as AP-1 (JUN family), CREB, STAT1-3 and NF-κB (Figure 2.9). More importantly, the aberrant activation of GSK-3 activity is strongly associated with the development of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, bipolar disorder and schizophrenia (Lei, Ayton, Bush, & Adlard, 2011).

During inflammation in the CNS, the activation of GSK-3β by TLRs in glial cells can suppress the production of major anti-inflammatory cytokine, (IL-10) which

enhances the production of pro-inflammatory mediators such as cytokines, chemokines and NO. GSK-3 is also expressed in other cells such as natural killer (NK) cells, macrophage-derived RAW264.7 cells and dendritic cells (DCs) where it regulates the Th1/Th2 balance by modulating the IL-12 expression (Beurel, Michalek, & Jope, 2010). For example, the suppression of GSK-3 in RAW264.7 cells significantly abolished the persistent STAT1 activation which reduced the expression of the pro-inflammatory mediators (Tsai et al., 2009). GSK-3β has emerged as one of the critical regulators of various cellular signaling pathways involved in inflammation, cell death, mobility and apoptosis (Beurel & Jope, 2006; Jope, Yuskaitis, & Beurel, 2007) (Figure 2.9). GSK-3β deficiency was associated to be involved in the progression of mood disorders such as bipolar disorder and neurodegenerative diseases such Alzheimer's disease (Lei et al., 2011). GSK-3β activity is important in inflammation mediated diseases since it regulates TLR-mediated cytokines release (IL-6, IL-10 and TNF-α) in monocytes and peripheral blood mononuclear cells. Furthermore, GSK-3β has been shown to induce both microglial activation and migration with enhanced production of inflammatory factors (Beurel et al., 2010). GSK-3β activation has been reported to inhibit CREB regulation which decreased nuclear translocation of CREB that concomitantly increased of pro-inflammatory cytokines and chemokines production (Beurel et al., 2010). Nevertheless, the inactivation GSK-3β increases CREB binding activity and reduced NF-κB p65 nuclear translocation which enhanced the expression of anti-inflammatory cytokines such as IL-10 (Beurel et al., 2010; Green & Nolan, 2012). Thus, the modulation of GSK-3 activity can confer protection against the deleterious effects of chronic neuroinflammation-mediated neurodegenerative diseases.

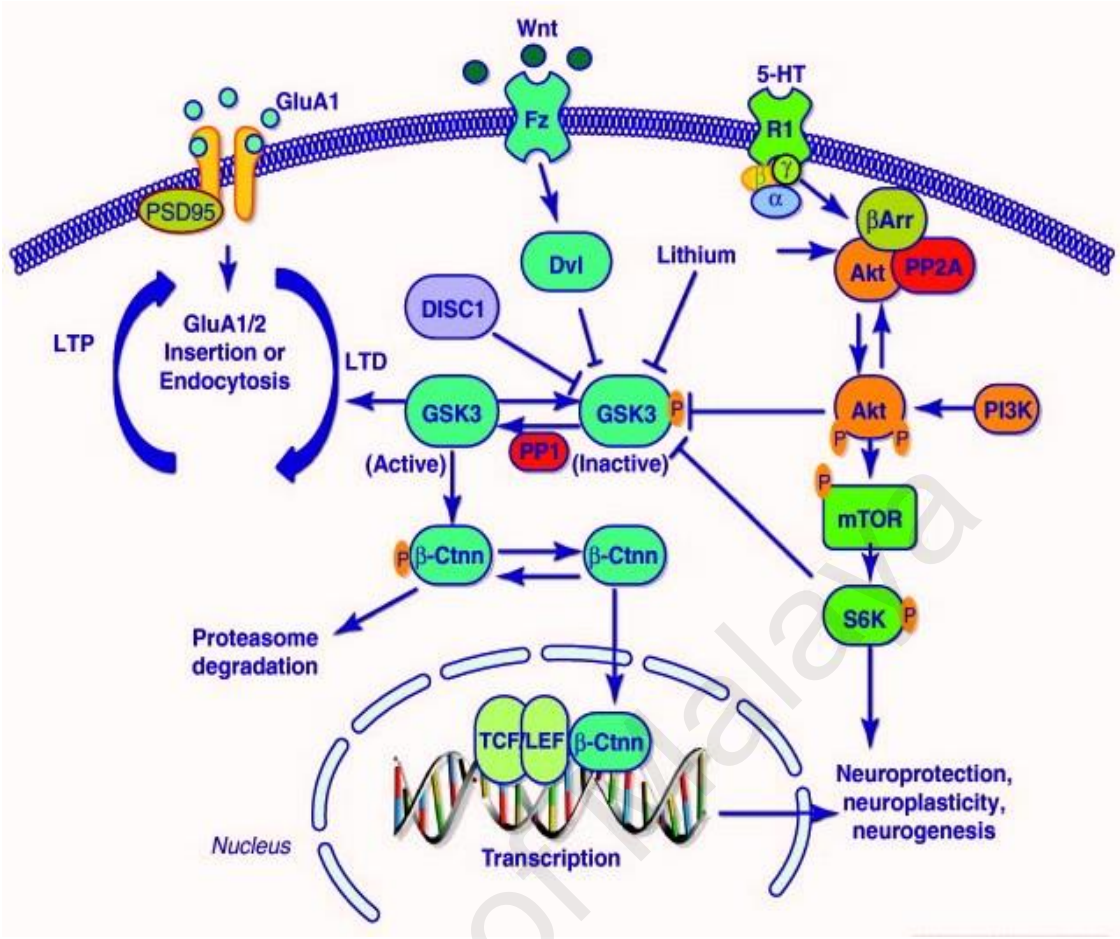


Figure 2.9 The signaling pathways (PI3K-Akt, mTOR and wingless (Wnt)–frizzled (Fz)) that activates or inactivates GSK-3β which modulates transcription factors that regulate cellular processes. Adapted from Duman, R. S., & Voleti, B. (2012).

2.5.4 Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) – the major transcription factor in neuronal processes

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) regulates various cell processes and the transcriptional programs regulated by NF-κB are important in the development and maintenance of the immune system (Hayden & Ghosh, 2011), skeletal muscle system (Jackman, Cornwell, Wu, & Kandarian, 2013) and epithelial homeostasis (Wullaert, Bonnet, & Pasparakis, 2011). Due to this immunomodulatory property, the dysregulation of NF-κB is generally involved in various human diseases such as autoimmune diseases, cancer, diabetes and neurodegenerative diseases (Baker, Hayden, & Ghosh, 2011).

The NF-kB family of transcription factors is composed of a dimer of five structurally related proteins, p50, p52, p65 (RelA), c-Rel, and RelB that form homo- and heterodimer that regulate gene expression by binding to specific DNA sequences within the promoters and enhancers of target genes (Ghosh, Wang, Huang, & Fusco, 2012). Without stimulation, NF-κB is sequestered in the cytoplasm in an inactive form via its association with one of three typical inhibitor proteins, IκB proteins (IκBα, IκBβ, or IκBε), the precursor proteins p100 and p105. These IκB proteins have the ability to mask the nuclear localization signal of NF-κB in the cytoplasm, thus suppressing NF-κB nuclear translocation (Hinz & Scheidereit, 2014). The phosphorylation of IκBs can alter the equilibrium between the cytosolic and nuclear signals that in turn favor the NF-κB nuclear translocation. IκBα is the most widely reported among IκBs and it is rapidly phosphorylated to undergo degradation following the activation of canonical NF-κB signaling pathway. The degradation of IκBα releases multiple NF-κB dimers, particularly, the p65:p50 heterodimer, allowing the p65 to translocate into nucleus (Hinz & Scheidereit, 2014). In contrast, non-canonical NF-κB pathway is regulated through proteasomal processing of p100 to p52 that releases p52 containing NF-κB

dimers that drive the transcriptional response (Sun, 2011). IκBα degradation (canonical) and p100 (non-canonical) processing have different regulation of NF-κB dimers, and thus, they regulate different sets of target genes (Shih, Tsui, Caldwell, & Hoffmann, 2011).

The multifaceted modulation of NF-κB signaling pathways are influenced by neurotrophic factors, neurotransmitters, cytokines and oxidative stress (Mincheva-Tasheva & Soler, 2013). NF-κB is proposed to be involved in the synaptic plasticity since it is present at the synaptic terminals and activated locally in response to synaptic transmission (Kaltschmidt et al., 2006; Mattson, 2005). NF-κB is a major regulator of neural processes particularly in controlling axon initiation, elongation, guidance, branching and dendritic arborization during development (Figure 2.10) (Gutierrez & Davies, 2011). NF-κB can be activated in neurons and glial cells following acute and chronic neurodegenerative conditions since it regulates the expression of immunoregulatory genes (Meffert and Baltimore, 2005; Memet, 2006). In addition, GSK-3β is capable of inducing rapid NF-κB transcriptional activity by directing the TNFα-/p65-dependent pathway and limiting NF-κB activation in BCL-3-dependent pathways. Generally, NF-κB mediates cellular immune responses to external stressors via the activation of TLRs that induces the expression of pro-inflammatory cytokines and chemokines in neurons and glial cells (Kawai & Akira, 2007). NF-κB in turn can be activated by the pro-inflammatory cytokines and chemokines causing its aberrant activation that leads to excessive microglia activation (Figure 2.10) (Kawai & Akira, 2007). While the activation of NF-κB in neurons promotes their survival and plasticity; its activation in glial cells may play a major role in inflammatory process that can damage and kill neurons (Baker et al., 2011; Crampton & O'Keeffe, 2013; Kaltschmidt & Kaltschmidt, 2009).

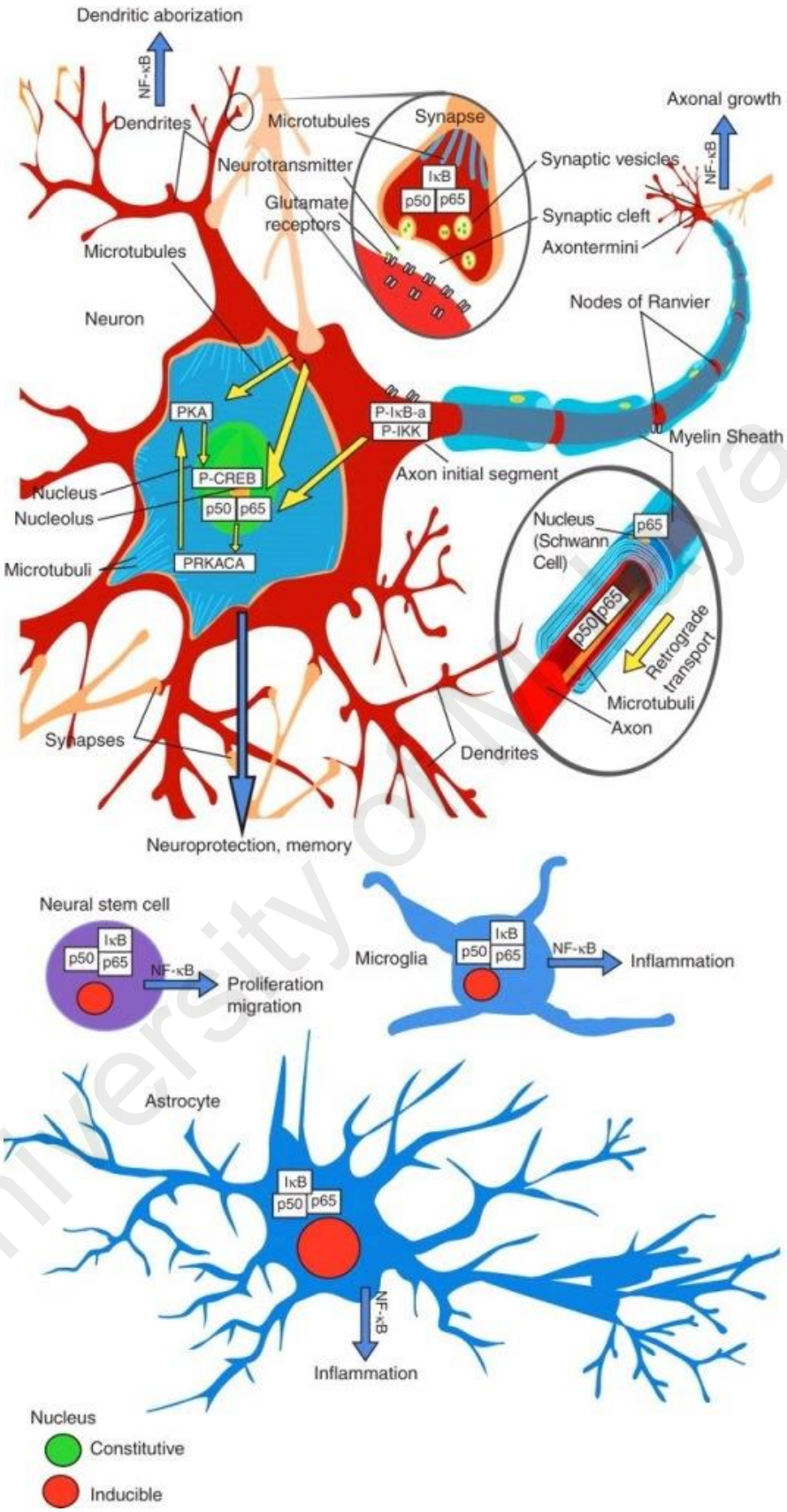


Figure 2.10 The role of NF-κB within the cellular context of the nervous system. Adapted from Kaltschmidt & Kaltschmidt (2009).

2.6 Mitogen-activated protein kinases (MAPKs) signaling pathways

Mitogen-activated protein kinases (MAPKs) signaling pathways are known to regulate cellular activities which include cell death, differentiation, proliferation, survival and transformation (Dhillon, Hagan, Rath, & Kolch, 2007; McCubrey, Lahair, & Franklin, 2006). In the last decade, at least four members of the MAPK family have been identified as the major signaling pathways of MAPKs, namely the extracellular-signal-regulated kinase 1/2 (ERK1/2), c-Jun-amino-terminal kinase (JNK) or stress activated protein kinases (JNK/SAPK), p38 family of kinases and extracellular signal-regulated kinase-5 (ERK5) (Son et al., 2011; Zhang & Liu, 2002). The JNK/SAPK which is primarily expressed in the heart, brain and testis is comprised of 3 isoforms (JNK1, JNK2 and JNK3) while the p38 family of kinases are consist of 4 isoforms (alpha, beta, gamma and delta) (Cowan & Storey, 2003). ERK5 is also known as the big MAP kinase 1 (BMK1) since it is twice the size of other MAPKs (Lee *et al*, 1995; Zhou *et al*, 1995) and plays a key role in cardiovascular development and neuronal differentiation. Moreover, all of the MAPKs have been documented to be involved in various aspects of neuronal differentiation by activating several transcription factors (West, Griffith, & Greenberg, 2002; Yang, Xia, Lu, Soong, & Feng, 2008a).

The extracellular signal regulated (Ras-Raf-MEK-ERK pathway) has been implicated in the regulation of cell growth and neuronal differentiation (Nishimoto & Nishida, 2006). In addition, the dysregulation of MAPK/ERK activation induces neuronal cell death (Subramaniam & Unsicker, 2010), neuroinflammation (Hovsepian et al., 2013; Supriady, Kamarudin, Chan, Goh, & Kadir, 2015) and tumorigenesis (Balmanno & Cook, 2009). ERK1 (p44 MAP kinase) and ERK2 (p42 MAP kinase) are closely related protein kinases of the MAP kinase family (Nishimoto & Nishida, 2006). ERK1/2 is regulated by dual phosphorylation at the Thr/Tyr residues that is carried out by MAP kinase kinase 1/2 (MKK1/2) (Roskoski, 2012).

The MAPK/ERK signaling pathway can be activated by a variety of stimuli such as growth factors, pro-inflammatory cytokines and hormones that interact with a multimolecular complex of receptors including TRKs and GPCRs (Roskoski, 2012). MAPK /ERK pathway is a three-staged kinase cascade comprising of; (1) MAP Kinase Kinase Kinase (MAPKKK/MAP3K/MEKK/MKKK), (2) MAP Kinase Kinase (MAPKK/MAP2K/MEK/MKK) and (3) Map Kinase (MAPK). The MAPKKK can induce the initial response by phosphorylating MAPKK which activates members of the MAPK family. The activation of TRKs and GPCRs allows the transmission of activating signals via recruitment of SOS through the adaptor protein growth-factor-receptor-bound-2 (Grb2) that stimulates Ras and converts guanosine diphosphate (GDP) to guanosine triphosphate (GTP). The conversion of GDP to GTP activates Ras and induces the interaction with various downstream effector protein which includes isoforms of the serine/threonine kinase, Raf. The activated Raf kinase phosphorylates MAPKK which subsequently activates MAPK (Figure 2.11).

Although ERK is associated with cell survival and proliferation, however, depending on the stimuli and cell types involved, the activation of ERK can mediate neuronal cell death. For instance, the activation of CREB and/or direct inhibition of Bad may mediate the pro-survival activity of ERK1/2 in trophic-deprived cerebellar granule neurons (Hetman & Gozdz, 2004). Conversely, a sustained ERK1/2 activation has been associated with neuronal death in various neuronal models (Jiang, Gu, Zhang, & Jing, 2000; Subramaniam & Unsicker, 2010). This finding was supported with the abnormal activation of the ERK1/2 in the pathogenesis of Alzheimer's disease (Pei et al., 2002; Shi et al., 2015).

In the CNS, ERK1/2 is crucial for neuronal differentiation, plasticity and neuronal survival (Alonso, Medina, & Pozzo-Miller, 2004; Vauzour, Vafeiadou, Rice-Evans, Williams, & Spencer, 2007). The activation of ERK1/2 is crucial for the development of NeuN-positive neurons, associated cytoskeletal and synaptic proteins (Li, Theus, & Wei, 2006). ERK1/2 activation has been linked to the development of peripheral neurons (Chen, Yu, & Strickland, 2007) and regeneration via neurotrophin induction (Markus, Patel, & Snider, 2002; Park, Luo, Hisheh, Harvey, & Cui, 2004). Moreover, neuritogenesis and LTP are also regulated by the ERK1/2 signaling pathway in response to a variety of extracellular stimuli (Markus, Zhong, & Snider, 2002; Seese, Wang, Yao, Lynch, & Gall, 2014; Waetzig & Herdegen, 2005). For example, exposure of Paju cells to IFN-γ induced neuritogenesis and rendered the cells to attain nearly pure populations of human neuron-like cells through the upregulation of p35 and Cdk5 mediated via ERK1/2 activation (Song et al., 2005). In addition, the administration of docosahexaenoic acid (DHA) induced neuritogenesis in SH-SY5Y neuroblastoma cells via the augmentation of ROS through ERK1/2 activation (Wu et al., 2009a).

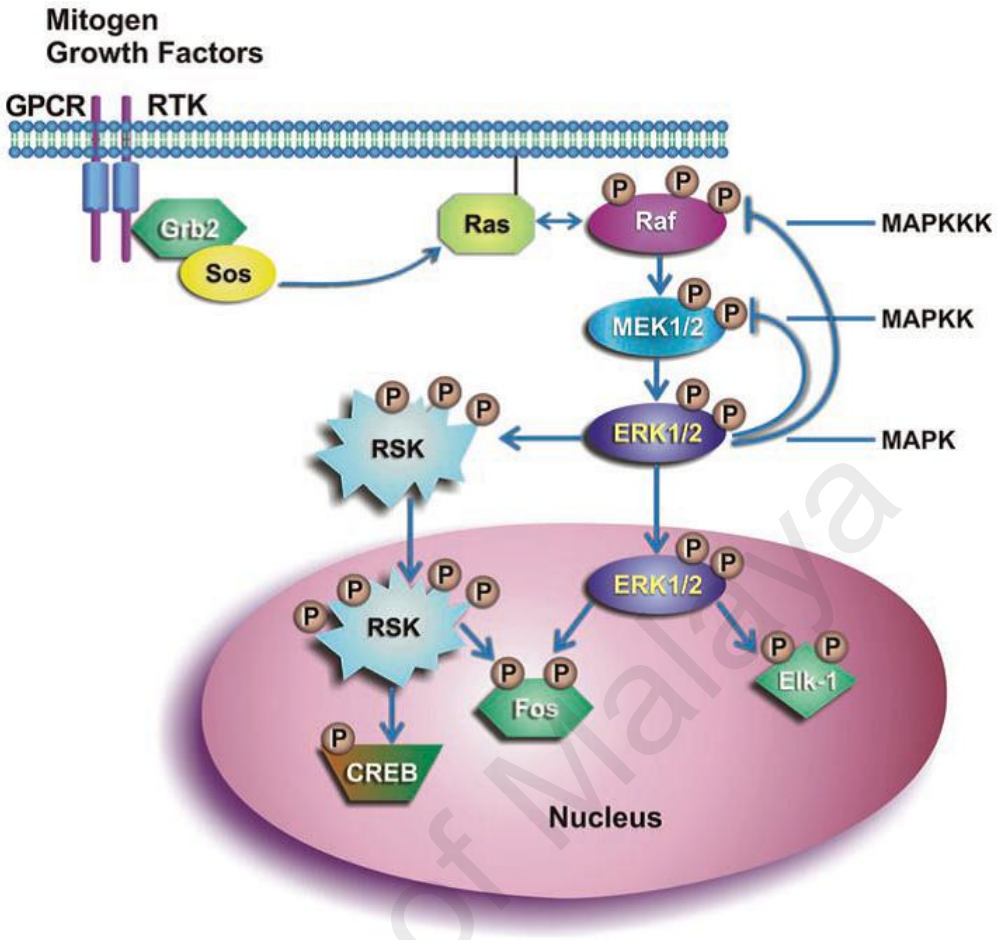


Figure 2.11 Mechanism of ERK activation through the tyrosine kinases receptor (TRKs) or G protein-coupled receptors (GPCRs). Adapted from Mebratu, Y., & Tesfaigzi, Y. (2009).

2.7 Therapeutic approaches in neurodegenerative diseases

The inhibition of chronic inflammation through suppression of pro-inflammatory mediators is thought to be beneficial in preventing excessive neuronal damage and therefore, could delay the onset of neurodegenerative diseases. In the late 90s, the suppression of COX-2 was demonstrated to delay the death of CA1 hippocampal neurons in global cerebral ischemia (Nagayama, Niwa, Nagayama, Ross, & Iadecola, 1999). COX-2 can be inhibited by several molecules and drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), flavonoids, polyphenol compounds and anti-inflammatory cytokines (IL-4 and IL-10) (Abdalla, Sanderson, & Fitzgerald, 2005). NSAIDs are competitive inhibitors of both COX-1 and COX-2 that inactivate the COX and inhibit the formation of prostanoids (Choi, Aid, & Bosetti, 2009; Yuan et al., 2009). Furthermore, numerous studies using different neurodegeneration and inflammation models reported that the suppression of iNOS together with COX-2 induced neuroprotective effects (Liang et al., 2007). Consistently, the use of selective COX-2 inhibitors such as celecoxib and rofecoxib also delayed the progession of some neurological diseases (Cudkowicz et al., 2006; Group, 2008).

Apart from direct inhibition of inflammatory mediators, potential therapeutics confer the ability to target specific and/or multiple protein in various signaling pathways (Magrone, Marzulli, & Jirillo, 2011). Moreover, the elucidation of the molecular mechanisms by which extracellular stimuli, misfolded protein and inflammatory insults induce the signaling pathways seems to be the more prominent and efficacious pharmacological intervention prior to neurodegeneration. Therefore, this approach is considered to provide better efficacy in preventing the aberrant inflammation and neuronal apoptosis by modifying the course of diseases. For instance, PI3K-AKT/GSK-3β pathway is one of the most targeted pathways that play a crucial role in neuroprotection, enhances cell survival by stimulating cell proliferation, inhibiting

apoptosis and induces neuronal differentiation (Matsuda et al., 2013; Nakaso, Ito, & Nakashima, 2008; Read & Gorman, 2009a). Moreover, drugs and natural products that induce the activation of PI3K-AKT modulate the inflammatory responses by regulating the expression of various genes via AKT downstream targets, such as FOXO (Brunet et al., 1999; Dong et al., 2014), GSK-3β (Emamian, 2012) and mTOR (Yap et al., 2008).

Since its discovery as an enzyme that regulates gene expression to promote inflammation, mood disorders and apoptosis, GSK-3 has been evaluated as a promising target in various neurological disorders such as Alzheimer's disease (Hooper, Killick, & Lovestone, 2008), bipolar disorder (Marmol, 2008) and schizophrenia (Emamian, 2012). Lithium is a monovalent cation drug that selectively inhibit GSK-3α and GSK-3β through the activation of the PI3K/AKT and MAPK signaling pathways in neurons (Zhang, Phiel, Spece, Gurvich, & Klein, 2003). The inhibition of GSK-3β provides neuroprotective effects as it induces the expression of anti-apoptotic, cell survival and anti-inflammatory genes in neurons and glia cells (Beurel & Jope, 2006; Beurel et al., 2010). At therapeutic concentration, lithium inhibits GSK-3β and reduces the phosphorylation of TAU proteins, the processing of APP to Aβ and enhances spatial memory in Alzheimer's disease (Alvarez et al., 2002). Moreover, some natural products such as *Achyranthes bidentata* polypeptides (Shen, Zhang, Gao, & Ding, 2011) and puerarin, the main isoflavone glycoside found in the Chinese herb (Wang et al., 2013), also suppressed neuronal apoptosis by activating the PI3K-AKT/GSK-3β pathway.

Despite numerous groundbreaking discoveries, to date, most current therapies only improve or delay the symptoms but are ineffective in preventing the progress of neurodegenerative diseases. This has led to the development of new therapeutics inventions which includes RNA interference (RNAi). RNAi therapies have been tested in several neurodegenerative diseases models as an attempt to evaluate its therapeutic

potentials. Moreover, numerous evidences have revealed that therapeutic manipulation of RNAi selectively suppressed disease linked genes and could offer a great potential for the treatment of neurodegenerative diseases (Basak, Patil, Alves, Larsen, & Møller, 2016; Johnson et al., 2015; Wittrup & Lieberman, 2015). The use of lentiviral for shRNA targeting endogenous β-site APP cleavage enzyme 1 (BACE1) reduced the Aβ production, amyloid plaques and neuronal death with significant improvement in learning and memory in transgenic mouse model (Miyagawa et al., 2016; Peng & Masliah, 2010; von Einem et al., 2015). RNAi directed against mutant human huntingtin successfully suppressed the expression of the transgene in the *stritum* and *cerebellum* in Huntington's disease mice with a significant pathological and behavioral improvement (Harper et al., 2005). Additionally, RNAi-based therapies have shown potential in the treatment of several other neurological conditions affecting the CNS. RNAi technologies have been applied to lessen and treat neuropathic pain (Doré-Savard et al., 2008), prevent neuronal cell death induced by brain ischemia (Sun et al., 2009), minimize the damage caused by prion proteins (Pfeifer et al., 2006; White et al., 2008) and induce apoptosis with increase chemotherapy susceptibility in brain tumors (Zhang et al., 2004). Collectively, the advancement in RNAi highlights its therapeutic potentials to be further developed in the intervention of various neurological disorders (Bhadra, 2016).

2.7.1 Natural products as therapeutic agents

Chemical substances or natural products derived from animals, plants and microbes have been traditionally used to treat various ailments. Over the last century, natural products served as the main source of pharmaceutical industry that drives the groundbreaking drug discovery. In the West, the use of natural products reached its peak in the period 1970–1980 and led to the rapid development of various drugs in the pharmaceutical landscape (Koehn & Carter, 2005). To date, natural products continue to

be one of the main sources in drug discovery owing to theirs ability to modulate multiple signalling pathways that either underly the pathogenesis or mitigate or delay the onset of various chronic diseases.

Globally, more than 200 billion dollars per year are spent for studying and treat AD, but despite this collective effort, AD remains without curative treatments (Wimo et al., 2013). Growing evidences have demonstrated that natural products may serve as promising targets for neurodegenerative diseases. For example, physostigmine, a short acting and potent reversible inhibitor of acetylcholinesterase (AChE) from *Physostigma venenosum* Balf. (Leguminosae) improved the cognitive functions in *in vivo* and in both normal and Alzheimer's disease patients (Houghton, Ren, & Howes, 2006). Curcumin has been shown to inhibit the formation of Aβ oligomers and reduced amyloidogenesis *in vivo* model of transgenic mice (Yang et al., 2005). In addition, curcumin attenuated the loss of dopaminergic neurons and protected rats from 6-OHDA induced Parkinson's disease symptoms (Zbarsky et al., 2005). Recently, evidences from *in vitro* and *in vivo* models supported the use of omega-3 in reducing the debilitating effects of inflammation while promoting tissue scarring within local tissues in various diseases such as rheumatoid arthritis, Alzheimer disease and irritable bowel syndrome (Calder, 2015). Therfore, the research of natural products is of great significance as it provides and leads to the development of newer therapeutics with lesser side effects in the intervention of neurodegenerative diseases.

2.7.1.1 Alpha-Lipoic Acid

Alpha-lipoic acid (α-lipoic acid) was discovered in 1951 as a molecule that assists in acyl-group transfer and as a coenzyme in the Krebs cycle (Biewenga, Haenen, & Bast, 1997). Alpha-lipoic acid is an organosulfur compound derived from octanoic acid ($C_8H_{14}S_2$, 206.33 g mol^{-1}) and can be found naturally in fruits, vegetables and

synthesized in animals and human (Babu, Kumar, & Singh, 2011). Other than acting as the key player in the mitochondrial energy production, it also functions as an antioxidant in both reduced and oxidized forms (Biewenga et al., 1997). Alpha-lipoic acid contains an asymmetrical carbon and thus has two possible optical isomers, namely, R-lipoic acid and S-lipoic acid (Figure 2.12). R-lipoic acid is a naturally occurring lipoic acid and functions as an essential cofactor in mitochondrial energy production and in the catabolism of alpha-keto acids and amino acids (Bustamante et al., 1998).

Numerous evidences have supported the intake of α-lipoic acid as nutraceutical in the prevention and treatment of chronic diseases. For instance, α-lipoic acid has been suggested as a potential anti-inflammatory agent for Alzheimer's disease (Maczurek et al., 2008), neuroprotective in various neuronal models (Fujita et al., 2008; Zaitone, Abo-Elmatty, & Shaalan, 2012) and modulator of various genes involved in cell survival, inflammation and oxidative stress (Bitar, Ayed, Abdel-Halim, Isenovic, & Al-Mulla, 2010; Salinthone, Yadav, Bourdette, & Carr, 2008). In addition, the activation of PI3K-Akt by α-lipoic acid was shown to protect the cerebral cortical neurons against Aβ peptide and H_2O_2–induced toxicity (Ratan & Baraban, 1995). Moreover, α-lipoic acid possesses the ability to cross and stabilizes the BBB integrity (Schreibelt et al., 2006). The combination of α-lipoic acid and N-acetylcysteine reversed the memory impairment and brain oxidative stress level in aged SAMP8 mice model (Farr et al., 2003). On the other hand, α-lipoic acid is widely used for the treatment of diabetes and its nerve related symptoms since it augments insulin sensitivity through the activation AMPK pathway in the skeletal muscle (Lee et al., 2005; Packer, Kraemer, & Rimbach, 2001). Recently, α-lipoic acid was demonstrated to mitigate the accumulation of 4-hydroxy-2-nonenal (4HNE) and protect the retinal neurons against oxidative stress by

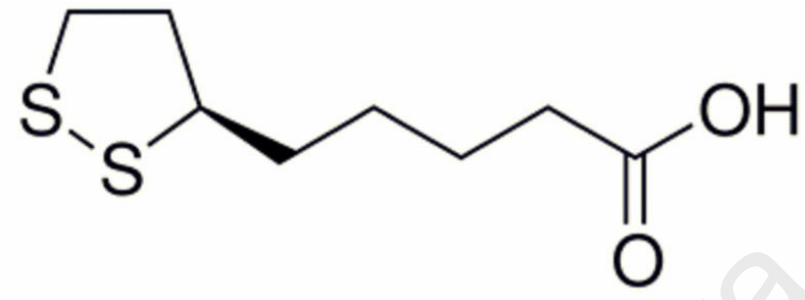
inducing HO-1 activity-dependent mechanism (Koriyama, Nakayama, Matsugo, & Kato, 2013a).

Although α-lipoic acid was reported to be neuroprotective and anti-inflammatory against various neuronal insults and suggested as potential therapeutic for Alzheimer's disease, most of the studies described its potential in a single neuronal model without integrating the neuron-glia model that can depict the regulation of inflammation that leads to neuronal death. Therefore, in this thesis, α-lipoic acid was chosen as a standard nutraceutical with the aims to integrate its diverse anti-inflammatory mechanism that could be associated with its neuroprotective effects in neuron-glia model.

2.7.1.2 Mesuagenin c from *Mesua kunstleri*

Mesua kunstleri is a forest timber plant and locally known as 'penaga'. It is listed under the family of Clusiaceae and the genus Mesua with more than 40 species along the region of Ceylon, India, Indo-China, Thailand, Malaysia and Queensland. Plants that fall under the genus *Mesua* have been used for various complementary medicinal purposes such as anti-allergic, rheumatism, anti-diarrhoetic and anti-bacterial as they are the fruitful sources of phytochemicals such as phloroglucinols, xanthones, neoflavonoids and coumarins (Awang et al., 2010; Verotta et al., 2004). These phytochemicals particularly coumarins, which are derivatives of cinnamic acid with the presence of a benzo-α-pyrene skeleton have been shown to exert multifarious biological activities such as neuromodulator, antidepressant, anti-HIV-1, anti-inflammatory, anti-tumor, antimicrobial, antiviral, antifungal and hepatoprotective effects (Li et al., 2003; Verotta et al., 2004; Wu, Wang, Xu, Farzaneh, & Xu, 2009b).

(A)



(B)

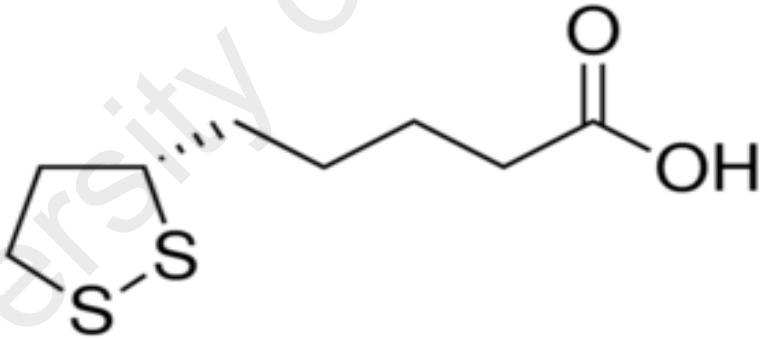


Figure 2.12 Structure of (A) (R)-(+)-α-lipoic acid (B) (S)-(-)- α-lipoic acid**.**

The bark of *M. kunstleri* is locally used to cure dyspepsis, a chronic, recurrent pain centered in the upper abdomen and renal diseases (Chan, Awang, Aadi, & Ng, 2008). The neuroprotective assay-guided fractionation and isolation of hexane extract has led to the identification five 4-phenylcoumarin compounds including mesuagenin c (Figure 2.13). Interestingly, 4-phenylcoumarins including mesuagenin c from *M. elegans* has been shown to possess acetylcholinesterase (AChE) inhibitory activity (Awang et al., 2010). Moreover, the isolated novel mesuagenin c has never been investigated and reported to be neuroprotective and anti-inflammatory against neurotoxicants despite having the ability to modulate AChE activity, relevant to Alzheimer's disease. Since, *M. kunstleri* can cure dyspepsis, this suggest that *M. kunstleri* might have bioactive compounds that alleviate the stomach pain through anti-inflammatory action. Therefore, following the neuroprotective-assay guided and isolation, mesuagenin c was subjected to various neuroprotective assays associated with its postulated anti-inflammatory property as an attempt to discover potential therapeutic for neurodegenerative disease. Moreover, various coumarins have been reported to protect several neurons against excitotoxicity-induced cell death. Osthol from *Cnidium monnieri* protected the NG108-15 by inhibiting the voltage-dependent L-type Ca^{2+} current (Wu, Lo, Chen, Li, & Chiang, 2002) whereas isopentenyl-oxycoumarin from *Citrus* species salvaged the neuronal mixed cortical cell culture model against NMDA-induced neurotoxicity (Epifano et al., 2008). In the primary neuron culture of rat cortical model, marmesin from *Angelica gigas* was reported to protect agent against glutamate-induced neurotoxicity (Kang & Kim, 2007).

The work in this thesis is the first study that evaluates and elucidates the neuroprotective, anti-inflammatory and neuritogenic mechsnisms of mesuagenin c in microglial and neuronal cells. Moreover, the study of novel mesuagenin c with multifarious properties could lead to the discovery of novel molecules that potentially

can act as new therapeutic target sites in combating neurodegenerative diseases. The scientific classification of *M. kunstleri* is as follow:

Kingdom : Plantae

Phylum : Tracheophyta

Class : Magnoliopsida

Order : Theales

Family : Guttiferae

Genus : Mesua

Species : *Mesua kunstleri*

Sp. Authority : King (kosterm)

(A)

(B)

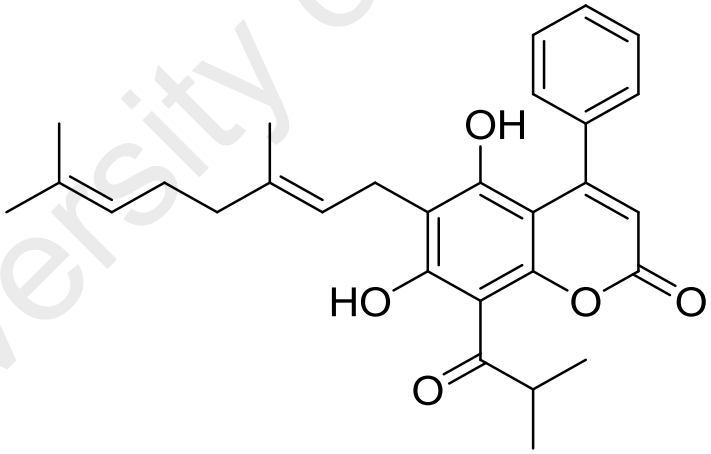


Figure 2.13 (a) The bark of *M. kunstleri* (b) Chemical structure of mesuagenin c.

CHAPTER 3:

The activation of PI3K-Akt/GSK-3β and CCL21 inhibition enhanced (R)-(+)-α-lipoic acid and mesuagenin c anti-inflammatory effects in BV-2 cells co-cultured with NG108-15 cells

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In preparation (submitted)

3.1 Introduction

Neuroinflammation is a defense mechanism that is aimed at protecting the central nervous system (CNS) against infectious insults and injury. Generally, inflammation ceases once the threat has been eliminated and homeostasis has been restored (Glass et al., 2010). For instance, activated microglia are responsible in scavenging dead cells from the CNS as well as to promote neuronal survival by secreting different neurotrophic factors (Glass et al., 2010; Tambuyzer et al., 2009). Nevertheless, in some instances, a perpetuation of insults can lead to aberrant microglial activation with prolonged release of pro-inflammatory and/or cytotoxic factors (Block, Zecca, & Hong, 2007; Suzumura, 2013) that can result in progressive neuronal damage observed in Alzheimer's disease, multiple sclerosis and Parkinson's disease (Block et al., 2007; Glass et al., 2010). Moreover, the subsequent damaged neurons are capable of releasing various soluble pro-inflammatory factors which in turn aggravate the microglial activation (Suzumura, 2013). Thus, the suppression of aberrant inflammatory responses through microglial activation and the neuron-glia interaction is important in developing therapeutic strategies against neurodegenerative diseases.

Lipoic acid is a powerful phytonutrient with diverse pharmacotherapeutic properties against various pathophysiological insults (Biewenga et al., 1997; Farr et al., 2003; Shay, Moreau, Smith, Smith, & Hagen, 2009). Lipoic acid has been proposed to be used as a potential anti-inflammatory drug since it is capable to interfere with the pathogenesis of Alzheimer's disease (Maczurek et al., 2008; Shay et al., 2009). Mesuagenin c, a novel 4-phenylcoumarin was previously reported to possess acetylcholinesterase (AChE) inhibitory activity and protected the NG108-15 cells against H_2O_2-induced mitochondria mediated caspase-dependent apoptosis (Awang et al., 2010; Chan et al., 2008; Chan et al., 2012). However, mesuagenin c has never been reported to possess anti-inflammatory activity against LPS-stimulated microglia cells.

In this chapter, both (R)-(+)-α-lipoic acid and mesuagenin c were demonstrated to modulate NF-κB signalling, cytokines, chemokines and Galectin-3 expression by activating PI3K-Akt and inactivating GSK-3β in LPS-stimulated BV-2 co-cultured with NG108-15 cells. Moreover, both compounds were shown to modulate the production of key chemotactic chemokines (CCL2, CCL3, CCL4 and CCL5) that minimize the aberrant activation of microglia. In this chapter, the glioma x neuroblastoma hybrid cells (NG108-15) is chosen as the neurpotection neuronal model. NG108-15 are somatic cell hybrid of glioma of rat (*Rattus norvegicus*) and neuroblastoma of mouse (*Mus musculus*) with a flat; round; 10 to 100 µmeters diameter. The cells were developed in 1971 by Bernd Hamprecht by the fusion of mouse N18TG2 neuroblastoma cells with rat C6-BU-1 glioma cells in the presence of inactivated Sendai virus. The cells were originally named 108CC15. The NG108-15 cell line is a relevant choice due to its characteristic that it exhibits angiotensin II receptor and it can be further differentiated into a cholinergic phenotype cell line. Several neuronal characteristics of differentiated NG108-15 cells have been examined, for example, the presence of a wide range of voltage-dependent membrane currents (Kasai, 1992), release of acetylcholine (ACh) in response to bradykinin stimulation (Higashida, 1988) and formation of cholinergic synapses with cultured myotubes (Nelson et al., 1981). Apart from that, NG108-15 has been extensively used as the neuroprotective neuronal model. Pramote, M., et al (2003)., shown the dual action of curcumin against H_2O_2-induced cell damaged in NG108-15 whereas Wong, Z.H., et al (2011) further proved the ability of catechin in minimizing the toxicity effect of H_2O_2 in NG108-15. More importantly, since NG108-15 cells are able to rapidly express CCL21 following exposure to insults, therefore, in this chapter, CCL21 gene in NG108-15 cells was knocked down as an attempt to further verify the neuron-glia inflammatory communication. This knockdown was postulated to enhance the attenuation of the inflammatory responses between neurons and microglial

and therefore, further enhance the anti-inflammatory effects of (R)-(+)-α-lipoic acid and mesuagenin c.

3.2 Literature review

The PI3K-Akt pathway has been reported to mediate inflammatory responses, neuronal survival and neuronal differentiation via its various downstream targets (De Oliveira et al., 2012; Kamarudin et al., 2014; Lee, Lim, Park, Park, & Koh, 2015). The activation of PI3K will result in the phosphorylation of its main downstream target, Akt, which regulates the expression of various inflammatory genes by inducing further catalytic changes of its downstream targets, such as such as glycogen synthase kinase-3 (GSK-3) and mammalian target of rapamycin (mTOR) (Sabatini & Sarbassov, 2011). In microglia, PI3K-Akt pathway can be activated by various stimuli including lipopolysaccharide (LPS)-dependent activation of the TLR4/CD14 complex (Fukao & Koyasu, 2003). Nevertheless, the roles of PI3K-Akt in microglia-induced inflammation still remain debatable as its activation is thought to be the predominant signaling pathway that is responsible for the production of pro-inflammatory factors. On another hand, several studies have reported that the inactivation of PI3K-Akt during inflammation can lead to the increased production of pro-inflammatory factors in microglia and hence, highlighted the importance of PI3K-Akt activation in suppressing inflammatory responses. For example, the inhibition of PI3K-Akt and its downstream targets, GSK-3β and mTOR, was shown to influence the production of prostanoid through the upregulation of COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1) in primary rat microglia (De Oliveira et al., 2012). GSK-3β is a multitasked kinase that plays imperative roles in various signaling pathways by regulating important metabolic and signaling proteins, structural proteins and transcription factors (Lei et al., 2011). The activation or inactivation of GSK-3β can result in either positive or negative modulation of various transcription factors that are critical in regulating the production

of inflammatory cytokines and chemokines such as cAMP-response element binding protein (CREB), signal-transducer and activator of transcription 1-3 (STAT1-3), and nuclear factor-kappaB (NF-κB) (Beurel et al., 2010; Grimes & Jope, 2001; Hoeflich et al., 2000)

The activation of NF-κB in glial and neuronal cells plays a significant role in the inflammatory processes since it activation induces the expression of pro-inflammatory cytokines and chemokines (Mincheva-Tasheva & Soler, 2013). NF-κB is in turn regulated by the pro-inflammatory cytokines and chemokines, thus creating a positive feedback mechanism which when becomes aberrant can result in excessive neuroinflammation. Cytokines and chemokines are proteins that originate from the immune system and endogenously produced in activated microglial cells and injured neurons suggesting their involvement in CNS defence mechanisms (Turner et al., 2014). In the brain, CCL21 is a microglia-activating chemokine, which is solely found in endangered neurons and therefore, serves as a candidate for neuron-microglia cros signaling. Under stress conditions, neurons specifically express CCL21 packed in vesicle-like structures and released as neuronal signal that induce microglia activation at distant sites from the primary lesion site (de Jong et al., 2005). Hence, it is thought that targeting chemotactic chemokines such as CCL21 in a complex neuron-glia system would be beneficial in minimizing the detrimental effects of inflammation. Other than cytokines and chemokines, NF-κB activation also induces the expression of Galectin-3 (GAL3) which has been reported to be essential for resident microglial activation and proliferation following ischemic injury (Lalancette-Hébert et al., 2012). Recently, GAL3 released from reactive microglia cells was shown to activate other surrounding immune cells in a paracrine manner by binding to and activating Toll-like receptor 4 (TLR4) (Burguillos et al., 2015).

3.3 Materials and method

3.3.1 Cell culture and materials

The immortalized mouse microglial BV-2 cells were developed in the laboratory of Dr Blasi (Perugia, Italy) (Blasi, Barluzzi, Bocchini, Mazzolla, & Bistoni, 1990) whereas NG108-15 (glioma x neuroblastoma hybrid cells) was obtained from the American Type Culture Collection (ATCC). Both cell lines were cultured in DMEM (Sigma Aldrich, St. Louis, MO, USA) composed of 10% (v/v) heat inactivated FBS, 2% penicillin/streptomycin, 1% amphotericin B (all from PAA, Austria). The cells were cultured and conditioned at 5% CO_2 moist atmosphere at 37°C. In addition, the media for NG108-15 cells were supplemented with HAT media supplement (50x) Hybri-Max and the cells were primed prior to its use. (R)-(+)-α-lipoic acid was purchased from Sigma Aldrich whereas mesuagenin c was isolated via a neuroprotective-assay guided and fractionation method with the the Phytochemistry Lab, Department of Chemistry, University of Malaya. The LPS is phenol extracted from *E. coli* serotype O55:B5 and the source strain is CDC 1644-70 (Sigma Aldrich). Morphological analysis was performed with a fluorescence microscope (Leica Inverted Fluorescence Microscope, DM16000B) and flow cytometric analysis was acquired with BD Accuri C6 Flow Cytometry and BD CFlow® Software. Both mesuagenin c and (R)-(+)-α-lipoic acid were dissolved in dimethyl sulphoxide (DMSO) (stock solution of 20 mM). The cells were pretreated with various concentrations of mesuagenin c or (R)-(+)-α-lipoic acid for 2 h prior to the LPS exposure for 24 h. Cells exposed to vehicle alone (10% FBS DMEM, DMSO ≤ 0.5% v/v) were used as the control group.

3.3.2 Cell viability assay

The protective effects of mesuagenin c and (R)-(+)-α-lipoic acid against LPS-induced toxicity in BV-2 cells was evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide] assay. Cells were plated at a total density of 1 x 10^4 cells/well in a 96-well plates. The cells were left to adhere for 24 h and pretreated with serial concentrations of mesuagenin c or (R)-(+)-α-lipoic acid with and without LPS (0.5 µg/mL) for another 24 h. 20 µL MTT solution (Sigma Aldrich) was added into each well and incubated at 37°C for 4 h. The absorbance was measured by using a microplate reader (ASYS UVM340) at 570 nm (and a reference wavelength of 650 nm).

3.3.3 Nitric oxide (NO) assay

The BV-2 cells were plated and subjected to the above described treatments. The supernatant of each sample was collected to measure the total NO level. NO levels were determined using the Pierce® NO Quantification Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). The absorbance was read using a microplate reader (ASYS Hitech UVM340) at 540 nm and the NO level was determined accordingly to the nitrate standard curve according to the manufacturer guidelines.

3.3.4 Measurement of intracellular ROS level

LPS exposure induces cellular oxidative stress by escalating the intracellular ROS level. Hence, the modulation of intracellular ROS level by mesuagenin c and (R)-(+)-α-lipoic acid was measured using the fluorescent probe 2',7'-dichlorfluorescein-diacetate (DCFH-DA). Cells were plated at a density of 1x10^6 cells/ml in 60mm culture dishes and subjected to the above described pretreatment prior to LPS exposure. Cells were harvested, rinsed and incubated with 10 µM of DCFH-DA for 30 min at 37°C in cell loading medium. The fluorescence signal was measured using flow cytometer.

3.3.5 Determination of Prostaglandin E_2 (PGE_2) level

Microglial cells were cultured in 96-well plates, pretreated with different concentrations of mesuagenin c and (R)-(+)-α-lipoic acid for 1 h and then further stimulated with LPS for 24 h. The culture supernatant of each sample was collected to determine PGE_2 level

by using the Pierce® Prostaglandin E2 (PGE2) Competitive ELISA kit (Thermo Fisher Scientific, Rockford, IL, USA).

3.3.6 Western blot analysis

NG108-15 cells were plated on 60 mm culture dishes and subjected to the designated treatment. Cells were then collected, rinsed with PBS and centrifuged. Protein extracts were prepared by lysing the cells using RIPA buffer (Thermo Fisher Scientific, Rockford, IL, USA). The protein concentration was determined using the Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA). 20 μg of each protein sample was electrophoresed on 7 - 15% SDS–PAGE. Proteins on the gel were transferred onto a nitrocellulose membrane and blocked with 5% BSA. Membrane was then probed with the following primary antibodies: anti-pAkt, anti-Akt, anti-ERK1/2, anti-pERK1/2, anti-IκBα, anti-pIκBα, anti-lamin B, anti-NF-κB p65, anti-pNF-κB p6, and anti-murine exodus-2(CCL21) at 4°C overnight. All of the antibodies with exception for anti-murine exodus-2 CCL21 (Peprotech) were purchased from Cell Signaling and diluted at 1:1000. Protein bands were visualized by enhanced chemiluminescence (ECL) substrate solution kit (Bio-Rad) for 1 minute. Proteins were quantified with Bio-1D software as a proportion of the signal of the housekeeping protein band (β-actin and lamin B).

3.3.7 CCL21 knockdown in NG108-15 cells

The knockdown of CCL21 or Exodus was generated by using siRNA that were commercially obtained from Life Technology. The siRNAs are available at three different sequence reference, which are at 170, 261 and 373 sequence on the mRNA and labeled as E_1, E_2 and E_3, respectively. On the day prior to transfection, the cells were seeded at a density of 0.25 x 10^6/mL in a 60mm^2 culture dishes in DMEM supplemented with 10% FBS without antibiotics. The cells were left to adhere overnight and were transfected with the three different siRNAs at concentration 20 – 30 nM using

Lipofectamine® RNAiMAX in DMEM-serum and antibiotic free medium. For the control experiments, cells were transfected with 30 nM of scrambled siRNA. The cells were left to incubate for 4 h at 37°C in a 5% CO_2. Following this, the transfection complexes were removed, cell were washed with PBS twice and incubated with complete DMEM medium (24 - 48 h) for the knockdown analysis.

3.3.8 Real-time quantitative polymerase chain reaction (qPCR) analysis

Cells were plated and exposed to the designated treatment. Total RNA was isolated using RNAqueous-4PCR kit (Applied Biosystem) based on the manufacturer's protocol. CCL21 genes expression were evaluated by one-step SYBR Green relative Q-PCR (RotorGene-6000 System, Qiagen) and normalized to HMBS (hydroxymethylbilane synthase) as reference gene. The reactions were carried out in a total volume of 25 µL using the SensiMix One-Step Kit (Quantace). The PCR amplification conditions for CCL21 was 40 cycles of 10 seconds at 95 ^{o}C, 45 seconds at 45 ^{o}C and 10 seconds at 72 ^{o}C. The fluorescence threshold Ct values were calculated and the ΔCt values were determined using the formula $\Delta Ct = Ct_{CCL21} - Ct_{HMBS}$. The ΔΔCt values were then calculated based on formula ΔΔCt = ΔCt treated - ΔCt untreated. The expression level of CCL21 in the treated cells was measured relative to the level observed in the wild-type cells and was quantitated using formula $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). The primer sequences are shown in Table 3.1.

Table 3.1 The primer sequence for CCL21 and HMBS

Genes	Function	Sequence
CCL21	Forward	5'-TGC CTT AAG TAC AGC CAG AAG-3'
	Reverse	5'-TTC CTC AGG GTT TGC ACA TAG-3'
HMBS	Forward	5'-CCG AGC CAA GGA CCA GGA TA-3'
	Reverse	5'-CTC CTT CCA GGT GCC TCA GA-3'

3.3.9 Cytokines and chemokines antibody array

BV-2 cells were plated in a 6-well plates and left to adhere overnight. The cells were subjected to the designated treatment and the culture media were collected for cytokines and chemokines analysis by using the RayBio® Custom Mouse Antibody Array (RayBiotech Inc., Norcross, GA, USA). The total protein concentration for each sample was determined and standardized using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). The array experiments were conducted according to he manufacturer's protocol (RayBiotech Inc., Norcross, GA, USA). The sub-array slide was sent to the manufacturer for scanning and data extraction. The extracted data was then analyzed using the RayBio® Analysis Tool.

3.3.10 Co-culture of BV-2 and NG108-15 cells

To investigate the protective effects of R-(+)-α-lipoic acid and mesuagenin c in neuron-glia system, the BV-2 cells were co-cultured with a neuronal model, NG108-15 cells. The NG108-15 cells were seeded onto the bottom surface of the 6-well plates at a density of (1.5×10^6 cells). The BV-2 microglial cells were plated on 96-(CellCrown™) or 6-plate (BD Biosciences) inserts (0.4-μm pore size) at a density of 2×10^5 cells, respectively. These inserts were placed on top of the wells containing the NG108-15 culture. The BV-2 cells were pretreated with either R-(+)-α-lipoic acid or mesuagenin c, further stimulated with LPS (24 h) and the neuronal cell viability was evaluated using MTT assay. To further examine the neuro-glia communication, the BV-2 cells were co-cultured against NG108-15 cells. The BV-2 cells (2×10^5 cells) were plated on the cell culture insert while the NG108-15 cells (1.5×10^6 cells) were seeded on the bottom surface of 6-well plate. The neuron-glia model was subjected to the designated treatment for 24 h and following this, the samples media (conditioned media) from the NG108-15 cells culture were collected. Next, the BV-2 cells were

cultured by using the conditioned media. Following this, the BV-2 cells were then subjected to flow cytometric Cytokine Bead Array (BD Biosciences, USA).

3.3.11 Flow cytometric cytokine bead array (CBA)

The production of cytokines was measured using the Cytokine Bead Array (BD Biosciences, San Jose, CA, USA). In brief, following the designated treatment, 50 µL of culture medium was collected and mixed with the cytokine capture beads. The mixture was then mixed with PE-conjugated detection antibodies to form sandwich complexes. The desired cytokines) were then measured by flow cytometer and data were analyzed by using FCAP Array™ software with comparison to the mouse cytokines standards' curves. To further validate the NF-κB and cytokines regulation, the cells were pretreated with ethyl 3,4-dihydroxycinnamate (10 µM) prior to LPS exposure and the production of cytokines was then measured and compared with the LPS-treated group.

3.3.12 Data analysis

All the experimental data are expressed in mean ± standard error (S.E.). Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) and followed by Dunnett's test. *P-values* below 0.05 were considered to be statistically significant.

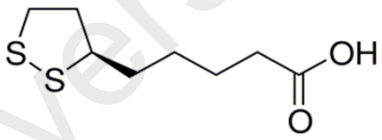
3.4 Results

3.4.1 (R)-(+)-α-lipoic acid and mesuagenin c protected BV-2 cells against LPS-induced toxicity

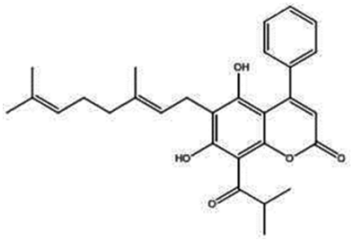
The protective effects of (R)-(+)-α-lipoic acid and mesuagenin c (Figure 3.1(a) against LPS-induced toxicity were evaluated by MTT cell viability assay. Exposure to LPS alone (0.5 µg/mL) significantly reduced the cell viability to ~70 %. Treatment with (R)-(+)-α-lipoic acid alone showed no significant toxicity towards the viability of BV-2 cells (Figure 3.1(b). Treatment with mesuagenin c alone showed that mesuagenin c at

0.5 - 20 µM did not induce significant cytotoxicity towards the BV-2 cells (Figure 3.1(c). However, treatment with mesuagenin c at 40 µM reduced the BV-2 cell viability to 90.25 ± 1.17 %, nevertheless, the reduction of cell viability was not significantly different than untreated cells, which indicated a non-toxic level at this concentration. Toxicity study by using normal cells on in *in vivo* model would provide better understanding that can clarify the toxicity of mesuagenin c. Conversely, pretreatment with R-(+)-α-lipoic acid or mesuagenin c significantly increased the BV-2 cells viability as compared to LPS-treated cells only. Moreover, pretreatment with (R)-(+)-α-lipoic acid (100 µM) and mesuagenin c (20 µM) fully protected the cells against LPS (cell viability up to 103.45 ± 1.95 % and 101.67 ± 1.05 %, respectively). More importantly, pretreatment with (R)-(+)-α-lipoic acid and mesuagenin c at all concentration increased the BV-2 cell viability as compared to LPS- treated cells which exemplified their protective effects in mitigating LPS-induced toxicity in BV-2 cells.

(A)



R-(+)-α-Lipoic acid



Mesuagenin C

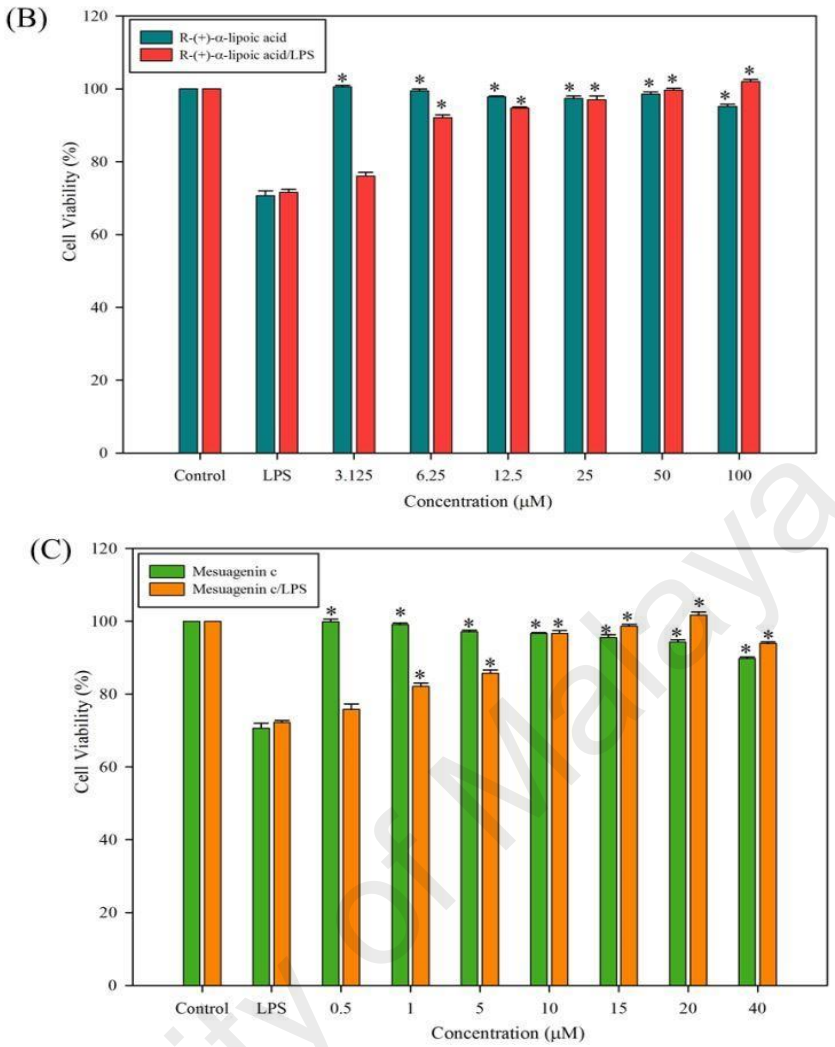


Figure 3.1 The protective effects of (R)-(+)-α-lipoic acid and mesuagenin c against LPS-induced toxicity in BV-2 cells. (A) Structure of (R)-(+)-α-lipoic acid (left) and mesuagenin c (right). (B) Pretreatment with (R)-(+)-α-lipoic acid and (C) Mesuagenin c increased in BV-2 cell viability prior to LPS exposure (500 ng/mL) for 24 h. Values are means ± S.E. from (n=6). *P < 0.05 indicates significantly different values from LPS-treated group.

3.4.2 (R)-(+)-α-lipoic acid and mesuagenin c reduced intracellular ROS, NO and PGE_2 level by attenuating COX-2 and iNOS in BV-2 cells

The ability of (R)-(+)-α-lipoic acid and mesuagenin c to suppress LPS-induced oxidative stress in BV-2 cells was first investigated by measuring the production of intracellular ROS, NO and PGE_2 level. Following LPS exposure, the NO production was significantly increased up to 6-folds as compared to the untreated control group (Figure 3.2(a)). Likewise, the level of PGE_2 production was detected at low level in untreated cells (116 ± 14.7 pg/mL), but remarkably increased following exposure to

LPS (1264.3 ± 30.6 pg/mL) (Figure 3.2(b)). However, pretreatment with (R)-(+)-α-lipoic acid (1 - 100 µM) and mesuagenin c (1 - 20 µM) significantly attenuated the production of NO and PGE_2 in a dose-dependent manner (Figure 3.2(a) and (b)). Upon LPS stimulation, the intracellular ROS level in the BV-2 cells increased significantly as depicted by the right shift of the red histogram (Figure 3.2(c) and (d)). However, following pretreatment with (R)-(+)-α-lipoic acid (1 - 100 µM) and mesuagenin c (1 - 20 µM), the production of intracellular ROS was suppressed (left shift of the blue histogram) in a dose-dependent manner as shown in the bar graph (Figure 3.2(c) and (d)). These observations first indicated that the anti-inflammatory activity of (R)-(+)-α-lipoic acid and mesuagenin c could be attributed to their antioxidative activity in BV-2 cells. To further determine the mechanisms by which (R)-(+)-α-lipoic acid and mesuagenin c reduced NO and PGE_2 production, its inhibitory effects on LPS-induced iNOS and COX-2 expression was investigated. LPS-stimulated BV-2 cells showed significant increase in COX-2 and iNOS expression as compared to control untreated cells (Figure 3.2 (e) and (f)). However, following pretreatment with (R)-(+)-α-lipoic acid and mesuagenin c, the expression of COX-2 and iNOS protein were significantly decreased in a dose-dependent manner (Figure 3.2 (e) and (f)). These results suggested that (R)-(+)-α-lipoic acid and mesuagenin c attenuated the NO and PGE_2 production by suppressing the COX-2 and iNOS expression in BV-2 cells.

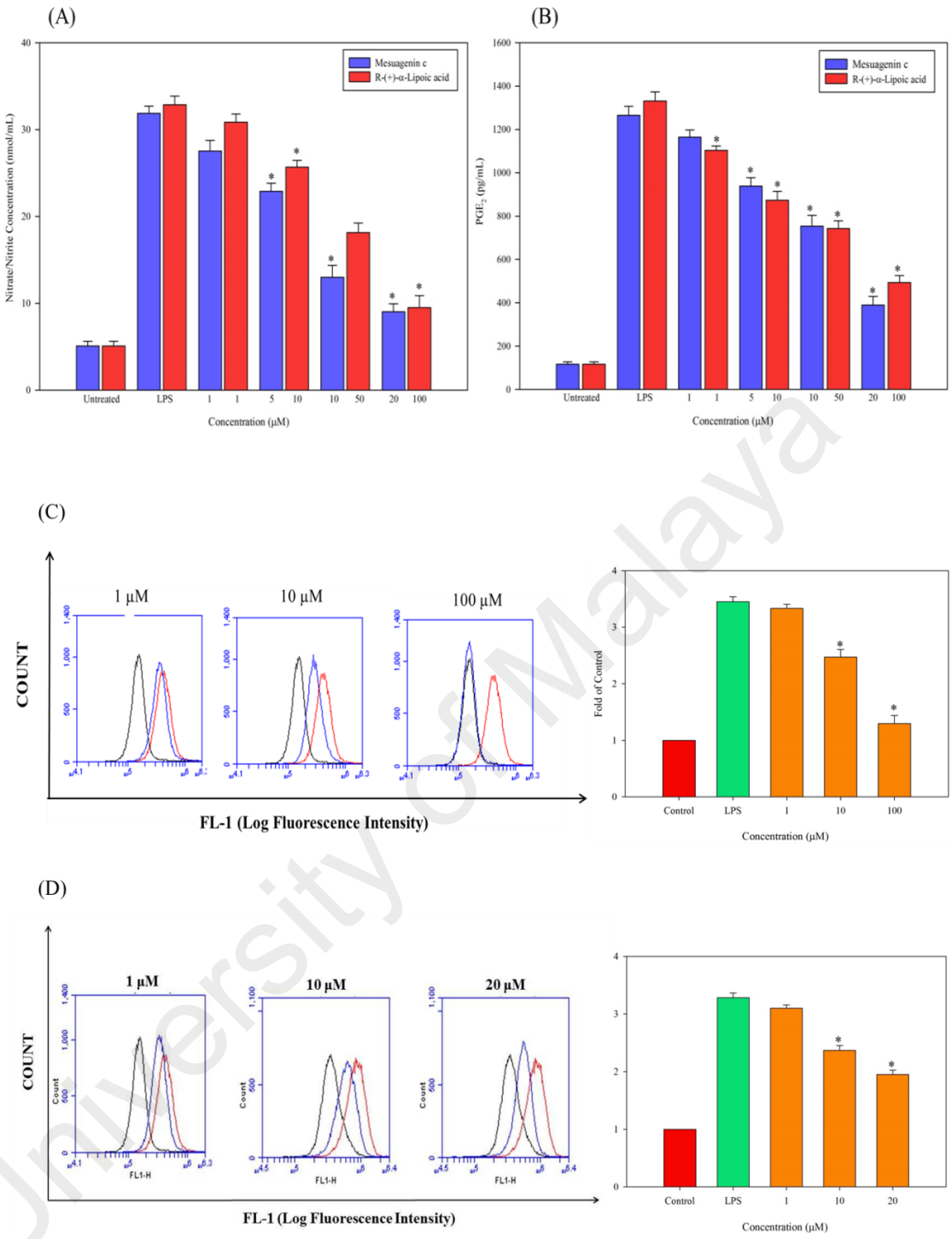


Figure 3.2 The effects of (R)-(+)-α-lipoic acid and mesuagenin c on LPS-induced the production of NO, PGE_2 and intracellular ROS. (A) Pretreatment with (R)-(+)-α-lipoic acid and mesuagenin c dose-dependently suppressed LPS-induced NO production in BV-2 cells. (B) (R)-(+)-α-lipoic acid and mesuagenin c reduced the production of PGE_2 in BV-2 cells (C) (R)-(+)-α-lipoic acid and (D) mesuagenin c reduced the intracellular ROS level in BV-2 cells following exposure to LPS (20 000 events were collected for each of the sample). Values are means ± S.E. (n=4). *P < 0.05 indicates significantly different values from LPS-treated group.

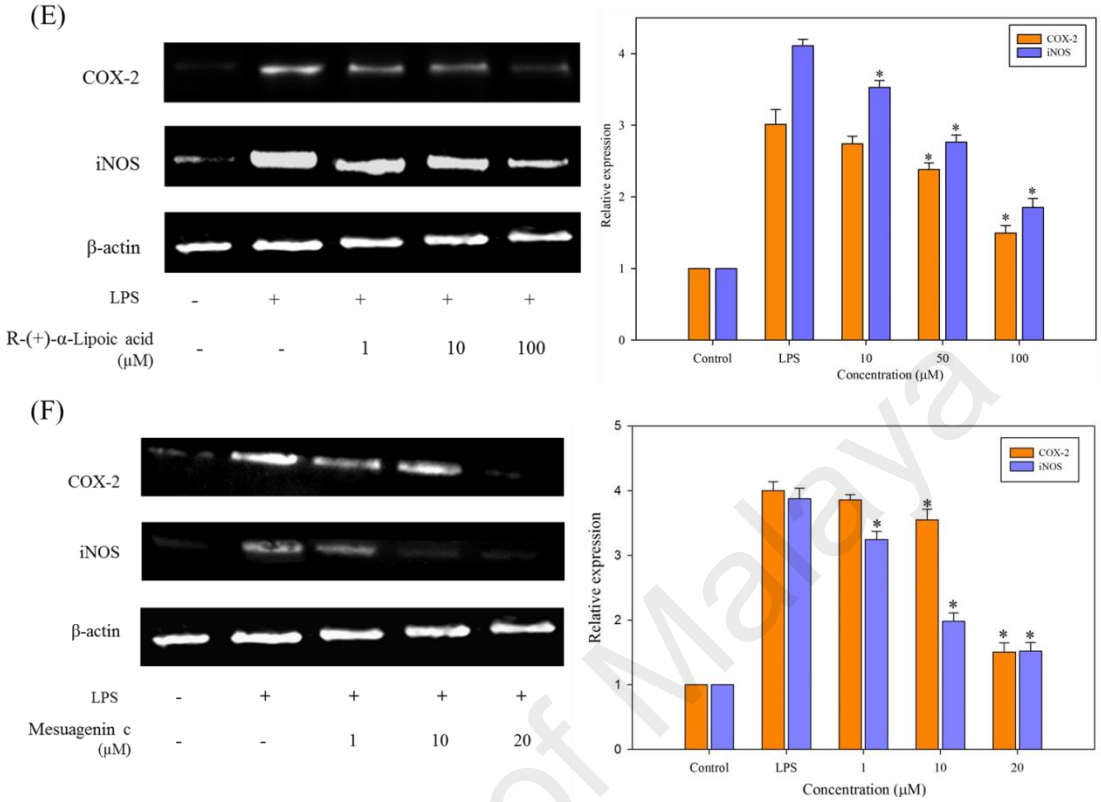


Figure 3.2, continued. (E) (R)-(+)-α-lipoic acid suppressed of LPS-induced iNOS and COX-2 expression. Western blotting: lane 1, DMEM control; lane 2, LPS (500 ng/mL); lane 3, LPS + MC (1 μM); lane 4, LPS + MC (10 μM); lane 5, LPS + MC (20 μM). (F) Mesuagenin c suppressed of LPS-induced iNOS and COX-2 expression. Western blotting: lane 1, DMEM control; lane 2, LPS (500 ng/mL); lane 3, LPS + R-LA (1 μM); lane 4, LPS + R-LA (10 μM); lane 5, LPS + R-LA (100 μM).β-actin was used as housekeeping protein. Values are means ± S.E. (n=4). *P < 0.05 indicates significantly different values from LPS-treated group.

3.4.3 (R)-(+)-α-lipoic acid- and mesuagenin c-induced rapid PI3K-Akt activation and GSK-3β inactivation is essential in suppressing LPS-induced NO and PGE_2 in BV-2 cells

The activation of Akt is crucial in inhibiting GSK-3β via phosphorylation. GSK-3β is a highly conserved protein kinase that is constitutively active and promotes the production of inflammatory cytokines and chemokines. To investigate the involvement of PI3K-Akt and GSK-3β, the ability of (R)-(+)-α-lipoic acid and mesuagenin c to influence the PI3K-Akt/GSK-3β signaling pathway in LPS-stimulated BV-2 cells was investigated. Exposure to LPS (2 h) was shown to induce the initial activation of Akt as compared to untreated cells. Pretreatment with (R)-(+)-α-lipoic acid induced the rapid activation (phosphorylation) of Akt (Ser473) at 1 h and peaked at 2 h following exposure to LPS (Figure 3.3(a) and (c)). However, pretreatment with mesuagenin c induced the rapid activation of Akt (Ser473) as early as 30 min and peaked at 1 h as compared to (R)-(+)-α-lipoic acid-treated cells (Figure 3.3(b) and (c)). In addition, the rapid activation of Akt declined at 2 - 4 h, indicated a transient activation by (R)-(+)-α-lipoic acid and mesuagenin c. LPS exposure also resulted in the increase of GSK-3β with a low level of GSK-3β phosphorylation (Ser9) (Figure 3.3(d - f)). Pretreatment with (R)-(+)-α-lipoic acid and mesuagenin c induced the inactivation (phosphorylation) of GSK-3β (Ser9) at 1 h and peaked at 2 h when compared to LPS-stimulated cells (Figure 3.3(d) and (f)). More importantly, the levels of p-Akt and p–GSK-3β (Ser9) were shown to be higher in the both (R)-(+)-α-lipoic acid- and mesuagenin c-treated cells as compared to the LPS-treated only. To study the regulation between PI3K-Akt and GSK-3β, the BV-2 cells were pretreated with PI3K inhibitor (Wortmannin) and Akt inhibitor (API-2). The pretreatment with Wortmannin was shown to suppress (R)-(+)-α-lipoic acid- and mesuagenin c-induced rapid phosphorylation of Akt (Ser473) which suggested that PI3K mediated (R)-(+)-α-lipoic acid- and mesuagenin c-induced Akt phosphorylation (Figure 3.3 (a - c)).

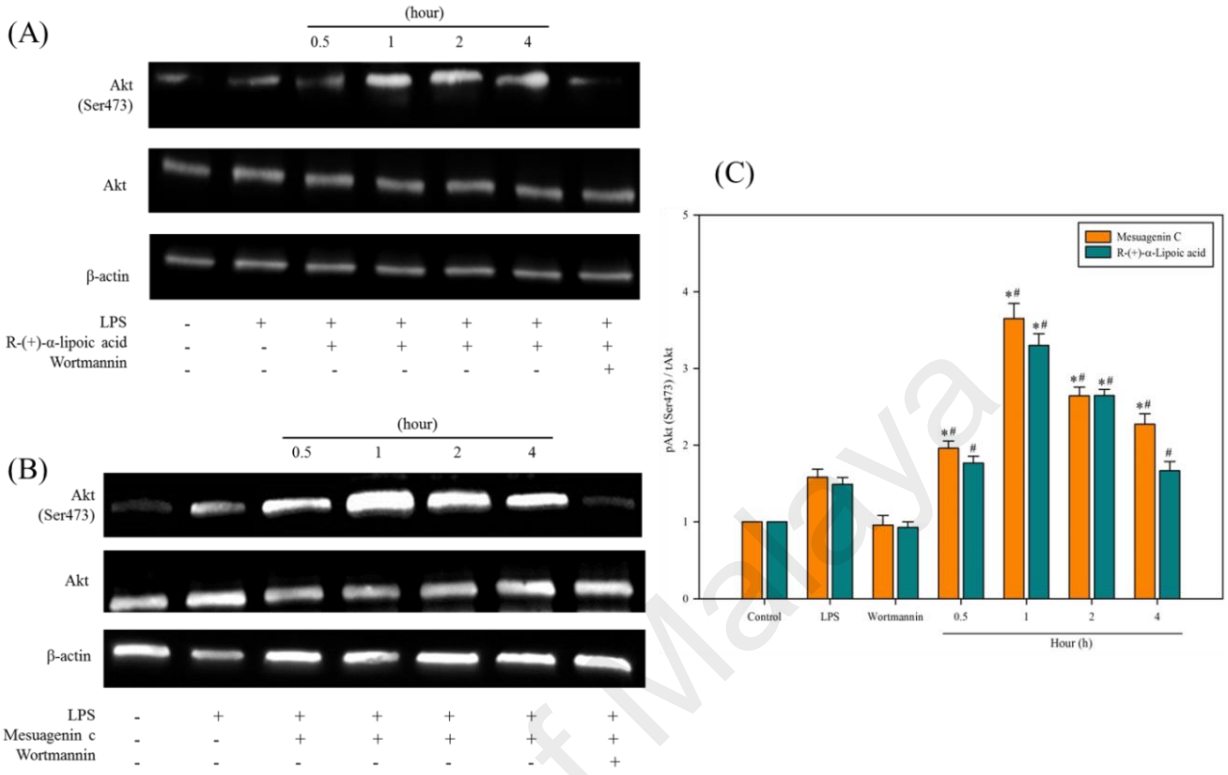


Figure 3.3 Western blot analysis of (A) (R)-(+)-α-lipoic acid (100 μM) and (B) Mesuagenin c (20 μM) on the activation of Akt(Ser473) and total Akt. Western blotting: lane 1, DMEM control; lane 2, LPS (500 ng/mL); lane 3, LPS + MC or R-LA (0.5 h) ; lane 4, LPS + MC or R-LA (1 h); lane 5, LPS + MC or R-LA (2 h); lane 6, LPS + MC or R-LA (4 h); lane 7, LPS + MC or R-LA (2 h) + Wortmannin. (C) Bar charts represent the ratio of pAkt/Akt. The Akt activation mediated by PI3K was validated with Wortmannin..

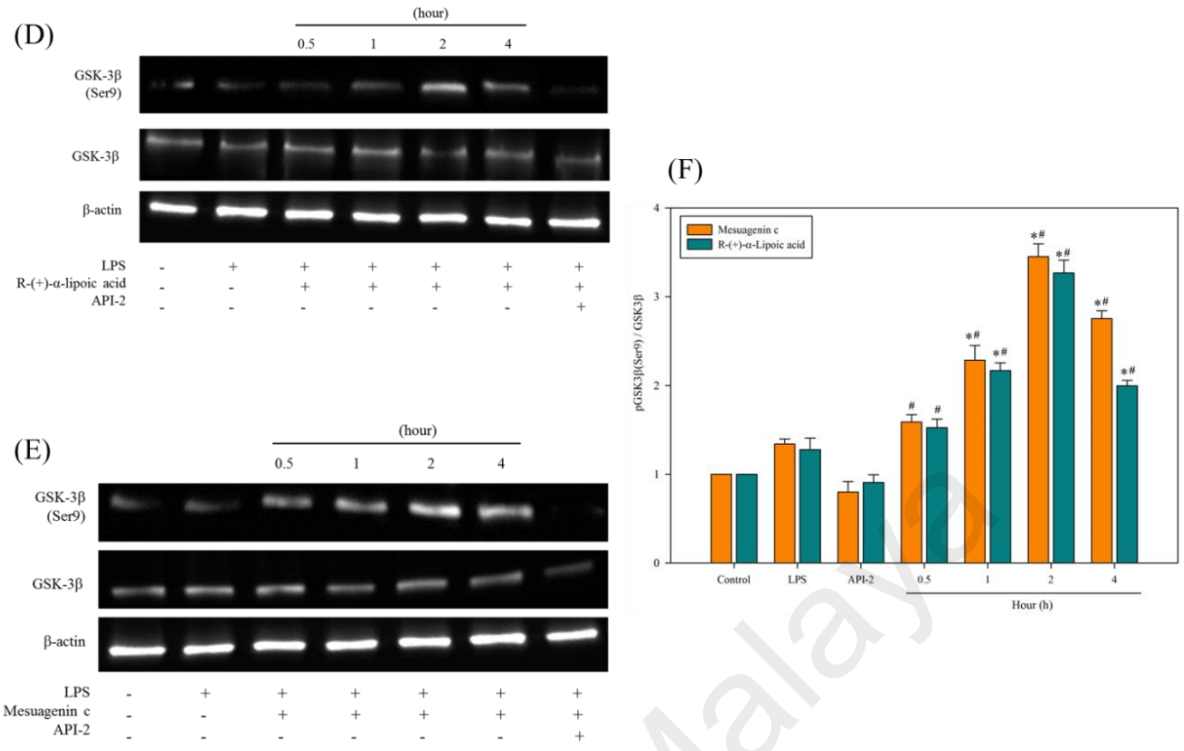


Figure 3.4 continued. (D) Western blot analysis of (R)-(+)-α-lipoic acid (100 µM) and (E) Western blot analysis of mesuagenin c (20 µM) on GSK-3β(Ser9) inactivation. Western blotting: lane 1, DMEM control; lane 2, LPS (500 ng/mL); lane 3, LPS + MC or R-LA (0.5 h) ; lane 4, LPS + MC or R-LA (1 h); lane 5, LPS + MC or R-LA (2 h); lane 6, LPS + MC or R-LA (4 h); lane 7, LPS + MC or R-LA (2 h) + API-2. (F) Bar charts represent the ratio of pGSK-3β/GSK-3β. The inactivation of GSK-3β through Akt activation was validated with pretreatment of API-2 (10 µM) prior to (R)-(+)-α-lipoic acid and mesuagenin c. Values are means ± S.E. from (n=4). *P<0.05 versus LPS, #P<0.05 versus API-2 or Wortmannin, significantly different as shown.

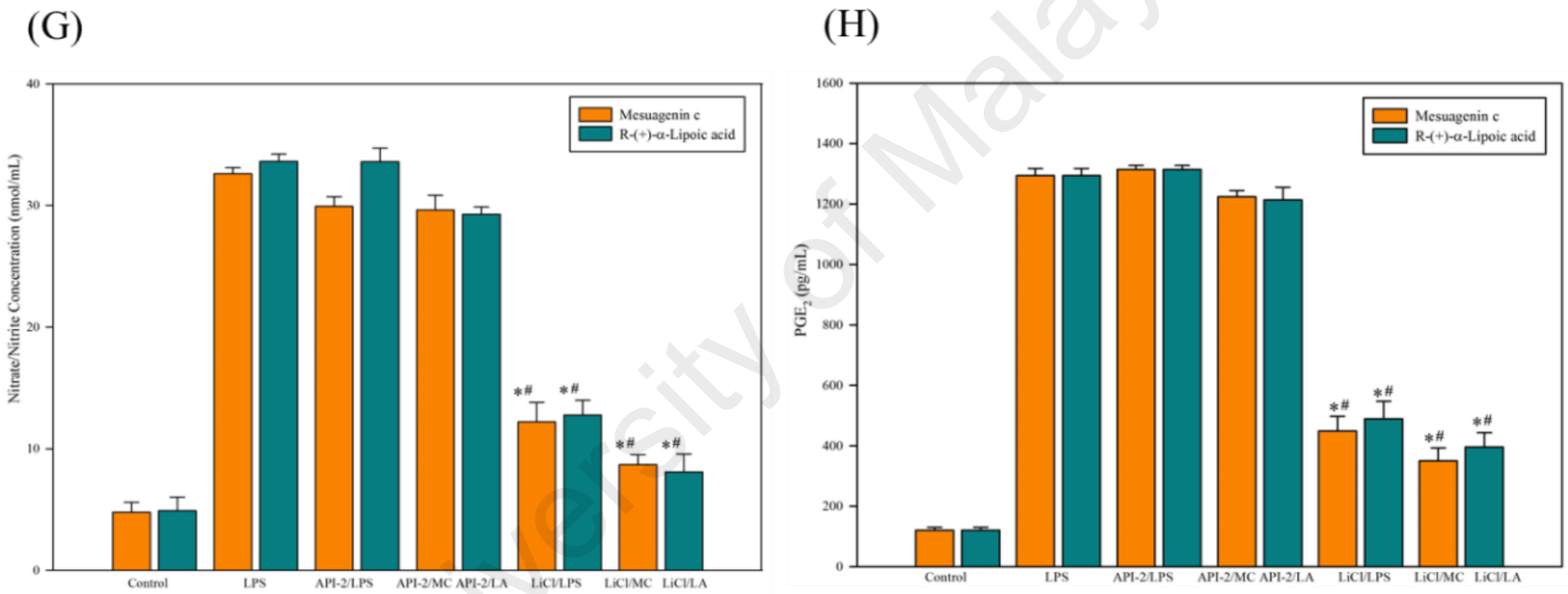


Figure 3.3, continued. (G) The addition of API-2 the inhibition of NO by (R)-(+)-α-lipoic acid (LA) and mesuagenin c (MC) whereas LiCl treatment reduced the NO level. (H) The addition of API-2 the inhibition of PGE2 by (R)-(+)-α-lipoic acid and mesuagenin c whereas LiCl treatment reduced the PGE2 level in BV-2 cells. Values are means ± S.E. from (n=4). *P<0.05 versus LPS, #P<0.05 versus API-2 or Wortmannin, significantly different as shown.

Interestingly, pretreatment with API-2 also suppressed (R)-(+)-α-lipoic acid- and mesuagenin c-induced the inactivation of GSK-3β (Ser9) and reduced the pGSK-3β/GSK-3β ratio (Figure 3.3 (d-f)). These observations suggested that (R)-(+)-α-lipoic acid- and mesuagenin c-induced activation of Akt is essential in inactivating GSK-3β in LPS-stimulated BV-2 cells which is consistent with other reports (De Oliveira et al., 2012; Koriyama et al., 2013b). To validate the anti-inflammatory effects of (R)-(+)-α-lipoic acid and mesuagenin c via Akt activation and GSK-3β inactivation, the BV-2 cells were pretreated with API-2 and lithium chloride (LiCl) and the level of NO and PGE_2 was measured. The addition of API-2 mitigated (R)-(+)-α-lipoic acid- and mesuagenin c-induced suppression of NO and PGE_2 in LPS-stimulated BV-2 cells (Figure 3.3 (g) and (h)). In contrast, the addition of LiCl and LiCl alone demonstrated significant reduction of NO and PGE_2 level (Figure 3.3 (g) and (h)) as compared to API-2 and LPS-treated groups. These observations further indicated that Akt activation and GSK-3β inactivation were responsible for the suppression of LPS-induced NO and PGE_2 by (R)-(+)-α-lipoic acid and mesuagenin c in BV-2 cells.

3.4.4 (R)-(+)-α-lipoic acid and mesuagenin c suppressed NF-κB p65 nuclear translocation through Akt activation and GSK-3β inactivation

The expression of inflammatory factors such as chemokines, cytokines, iNOS and COX-2 are mainly regulated by NF-κB. The nuclear translocation of p65 subunit or RelA is crucial in the transcription of various pro-inflammatory genes following exposure to LPS. Hence, the anti-inflammatory mechanism of (R)-(+)-α-lipoic acid and mesuagenin c through the inhibition of p65 nuclear translocation was investigated. Exposure to LPS was shown to mediate the NF-κB p65 nuclear translocation via the phosphorylation of IκBα in the cytosolic fraction which led to significant increase of pIκBα/IκBα and p65(nuclear)/p65(cytosolic) ratios (Figure 3.4). Conversely,

pretreatment with (R)-(+)-α-lipoic acid and mesuagenin c significantly suppressed the LPS-induced IκBα degradation and NF-κB p65 nuclear translocation in a dose dependent manner (Figure 3.4 (a-d)). These observations suggested that (R)-(+)-α-lipoic acid and mesuagenin c mediated their anti-inflammatory effect against LPS-stimulated BV-2 by suppressing the NF-κB p65 nuclear translocation. Interestingly, the addition of LiCl prior to LPS exposure also significantly suppressed IκBα degradation and NF-κB p65 nuclear translocation (Figure 3.4 (a-d)). Additionally, pretreatment with API-2 inhibited the ability of (R)-(+)-α-lipoic acid and mesuagenin c to suppress IκBα degradation and NF-κB p65 nuclear translocation (Figure 3.4 (a-d)). Nevertheless, treatment with API-2 and LiCl alone showed low alteration in p65(nuclear)/p65(cytosolic) ratios. The data suggest that the anti-inflammatory effects of (R)-(+)-α-lipoic acid and mesuagenin c are mediated by their ability to mimic the action of the known GSK-3β inhibitor against LPS-induced NF-κB p65 translocation. These further substantiated that (R)-(+)-α-lipoic acid and mesuagenin c induced anti-inflammatory effect against LPS in BV-2 cells mainly by suppressing the NF-κB p65 translocation through the activation of Akt and inactivation of GSK-3β (Ser9).

3.4.5 (R)-(+)-α-lipoic acid and mesuagenin c suppressed galectin-3, pro-inflammatory cytokines and chemokines production by augmenting IL-4 and IL-10 through PI3K-Akt/GSK-3β pathway

Aberrant microglial activation is normally succeeded by excessive production of pro-inflammatory mediators such as pro-inflammatory cytokines, chemokines and galectin-3 (Gal3). Following exposure to LPS, the production of anti-inflammatory cytokines, IL-4 and IL-10 were reduced significantly while pro-inflammatory cytokines (IFN-γ, IL-1β, IL-2, IL-6, IL-12 and TNF-α) production was significantly augmented (Figure 3.5 (a) and b)). Moreover, LPS exposure also elevated the production of pro-inflammatory chemotactic chemokines (CCL2, CCL3, CCL4, CCL5/RANTES and CCL12) and Gal3 as compared to control untreated cells (Figure 3.5 (a) and (c).

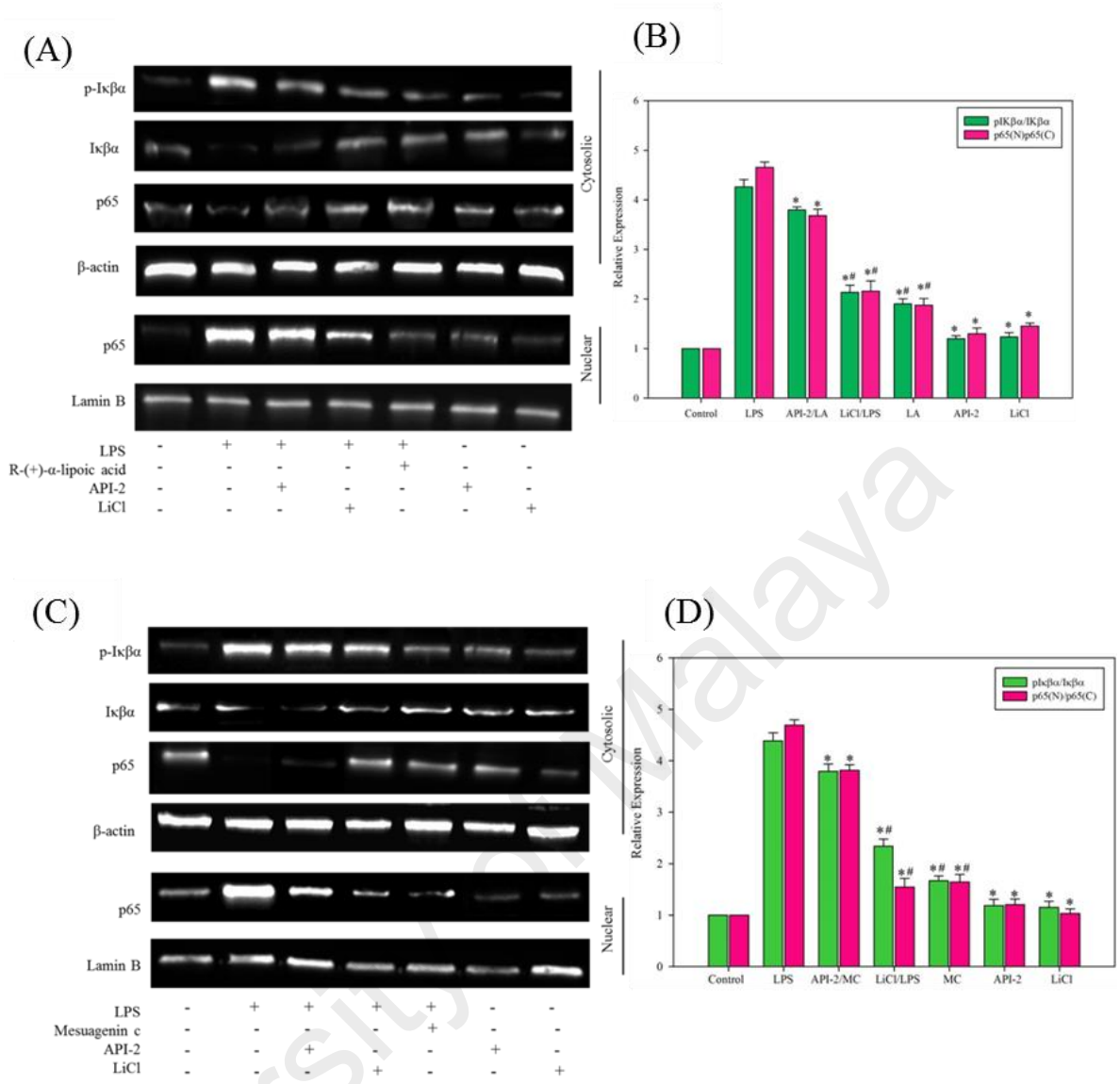


Figure 3.5 The suppression of NF-κB p65 nuclear translocation through activation of Akt and inactivation of GSK-3β (Ser9) by (A) Western blot analysis of; (B) Bar chart of (R)-(+)-α-lipoic acid on NF-κB p65 nuclear translocation. Western blotting, lane 1, DMEM control; lane 2, LPS (500 ng/mL); lane 3, LPS + API-2; lane 4, LPS + LiCl; lane 5, LPS + R-LA (100 μM); lane 6, API-2; lane 7, LiCl. (C) Western blot analysis of; Western blotting, lane 1, DMEM control; lane 2, LPS (500 ng/mL); lane 3, LPS + API-2; lane 4, LPS + LiCl; lane 5, LPS + MC (20 μM); lane 6, API-2; lane 7, LiCl. (D) Bar chart of mesuagenin c on NF-κB p65 nuclear translocation. The addition of API-2 inhibited the suppression of NF-κB p65 nuclear translocation while treatment with LiCl inhibited LPS-induced NF-κB p65 nuclear translocation similar to pretreatment with (R)-(+)-α-lipoic acid and mesuagenin c. Values are means ± S.E. (n=4). *P<0.05 versus LPS, $^{\#}$P<0.05 versus API-2, significantly different as shown.

(A)

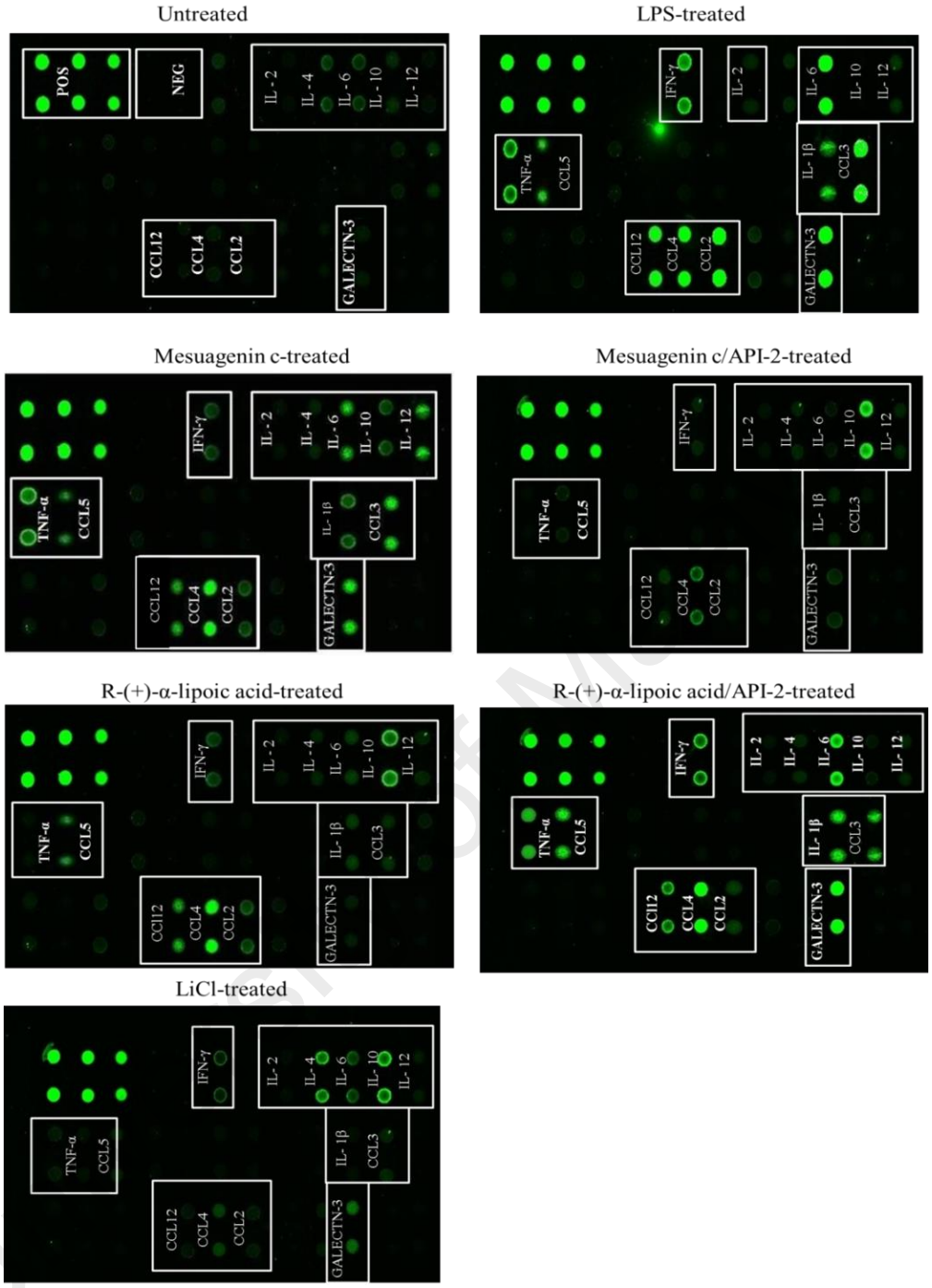
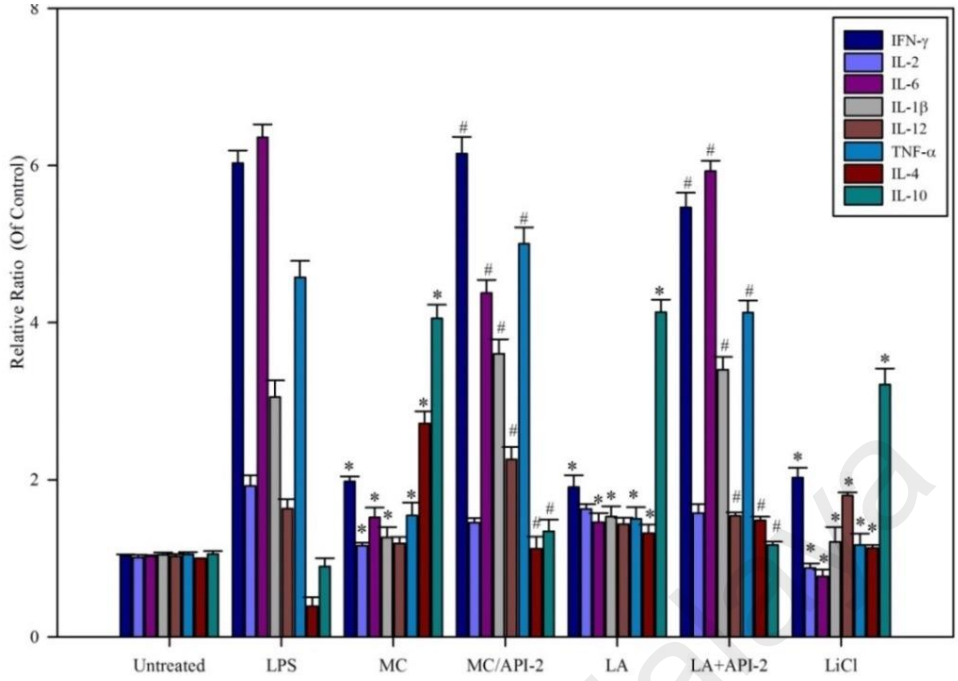


Figure 3.6 The modulatory effects of (R)-(+)-α-lipoic acid and mesuagenin c on Galectin-3, inflammatory cytokines and chemokines production through PI3K-Akt/GSK-3β pathway. (A) Protein array slide image analysis of inflammatory cytokines, chemokines and Gal3 by (R)-(+)-α-lipoic acid and mesuagenin c.

Pretreatment with (R)-(+)-α-lipoic acid significantly increased IL-10 production which decreased the production of the aforementioned pro-inflammatory cytokines (Figure 3.5 (a) and (b)). In contrast, pretreatment with mesuagenin c significantly increased the production of both IL-4 and IL-10 which significantly suppressed the production of the pro-inflammatory cytokines. Moreover, both (R)-(+)-α-lipoic acid and mesuagenin c suppressed LPS-induced production of chemokines and Gal3(Figure 3.5 (a) and (b)).

To elucidate the modulation of cytokines, chemokines and Gal3 production, API-2 was added prior to (R)-(+)-α-lipoic acid and mesuagenin c treatment and compared with LiCl treatment alone. Addition of API-2 significantly increased the production of pro-inflammatory cytokines, chemotactic chemokines and Gal3 as compared to (R)-(+)-α-lipoic acid-treated and mesuagenin c-treated groups (Figure 3.5 (a)-(c)). Contrary to this, LiCl treatment significantly reversed this observation where the production of pro-inflammatory cytokines, chemotactic chemokines and Gal3 were significantly reduced when compared to API-2/(R)-(+)-α-lipoic acid- and API-2/mesuagenin c-treated cells (Figure 3.5 (a)-(c)). Moreover, the addition of LiCl only increased the IL-10 production as compared to pretreatment with mesuagenin c. This observation further corroborated that the suppression of Gal3 and the modulation inflammatory cytokines and chemokines by (R)-(+)-α-lipoic acid and mesuagenin c is mediated via Akt activation and GSK-3β inactivation pathway.

(B)



(C)

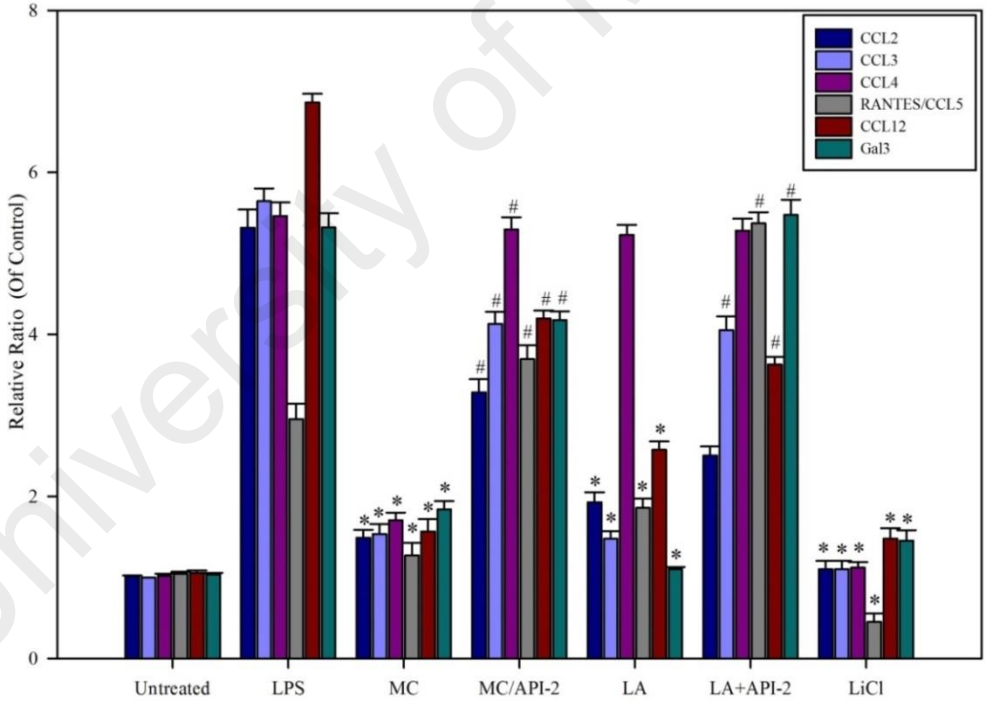


Figure 3.5, continued. (B) Bar chart demonstrated that (R)-(+)-α-lipoic acid (LA) and mesuagenin c (MC) increased IL-4 and IL-10 which reduced pro-inflammatory cytokines. The addition of API-2 suppressed the reduction of pro-inflammatory cytokines while LiCl increased IL-10 and reduced pro-inflammatory cytokines. (C) Bar chart demonstrated that (R)-(+)-α-lipoic acid and mesuagenin c reduced chemokines and Gal3. The addition of API-2 suppressed the reduction of chemokines and Gal3. LiCl treatment alone reduced the production of chemokines and Gal3. Values are means ± S.E. (n=3). * $P<0.05$ versus LPS, $^{\#}P<0.05$ versus (R)-(+)-α-lipoic acid or mesuagenin c, significantly different as shown.

3.4.6 CCL21 knockdown enhanced (R)-(+)-α-lipoic acid and mesuagenin c protective effects against LPS and inflammatory responses in neuron-glia co-culture system through Akt activation

To determine the role of CCL21 in neuron-glia communication, the CCL21 gene that is expressed in endangered neurons was knocked down in NG108-15 cells. The NG108-15 cells were subjected to CCL21 siRNAs at three different locations to increase the efficiency of CLL21 knockdown. This triple knockdown resulted in 85% inhibition of CCL21 expression (Figure 3.6 (a) and (b)) that was sustained for 4 days. The protective effects of (R)-(+)-α-lipoic acid and mesuagenin c on activated microglia-mediated neurotoxicity was investigated under BV-2 and NG108-15 co-culture conditions. The BV-2 cells were pretreated with either (R)-(+)-α-lipoic acid or mesuagenin c, stimulated with LPS and incubated for 24 h. The BV-2 cells that were exposed to LPS resulted in a significant decrease in NG108-15 cell viability (Figure 3.6(c)). The NG108-15 cells viability was increased following pretreatment of BV-2 cells with (R)-(+)-α-lipoic acid and mesuagenin c which suggested that the inhibition of LPS-induced inflammation in microglia mitigated neuronal cell death. In addition, when the BV-2 cells were pretreated with API-2, the protective effects of (R)-(+)-α-lipoic acid and mesuagenin c were suppressed and the NG108-15 cell viability was decreased (Figure 3.6 (d)). These results confirmed that the activation of Akt by (R)-(+)-α-lipoic acid and mesuagenin c in the co-culture system is important in protecting the NG108-15 cells against LPS-induced inflammation in BV-2 cells.

To further investigate the neuron-glia communication, the BV-2 cells were co-cultured with the transfected NG108-15 cells. The wild-type and transfected cells were plated for co-culture experiments and subjected to the designated treatment as described. Next, the conditioned media from each of the sample group were transferred and used to culture a new batch of BV-2 cells. The conditioned media from all groups were recollected and further analyzed with cytometric cytokine bead array. In Figure 3.6 (e) and (f), the BV-

2 cells that were cultured with LPS-conditioned and recombinant CCL21-conditioned media displayed significant reduction of IL-4 and IL-10 that increased the level of IL-6 and TNF-α. Contrary to this, BV-2 cells that were cultured with either (R)-(+)-α-lipoic acid- or mesuagenin c-conditioned media returned the IL-4 and IL-10 level to normal level, higher than the normal cells. Furthermore, this significant increase of IL-4 and IL-10 led to the reduction of IL-6 and TNF-α level (Figure 3.6 (e) and (f)). Moreover, the level of IL-6 and TNF-α was significantly reduced in the $CCL21^-$ groups that were treated with either (R)-(+)-α-lipoic acid or mesuagenin c when compared to all groups. Furthermore, following the knockdown of CCL21, the IL-10 level was shown to be significantly higher in the both $CCL21^-$/(R)-(+)-α-lipoic acid- and $CCL21^-$/mesuagenin c-treated cells when compared to (R)-(+)-α-lipoic acid- and mesuagenin c-treated group (Figure 3.6 (e) and (f)). In addition, the $CCL21^-$/(R)-(+)-α-lipoic acid-treated group also demonstrated a significant increase in IL-4 level when compared to (R)-(+)-α-lipoic acid-treated group. The results indicated that the suppression of inflammation coupled with CCL21 inhibition in neurons enhances the anti-inflammatory activity of (R)-(+)-α-lipoic acid and mesuagenin c in neuron-glia system.

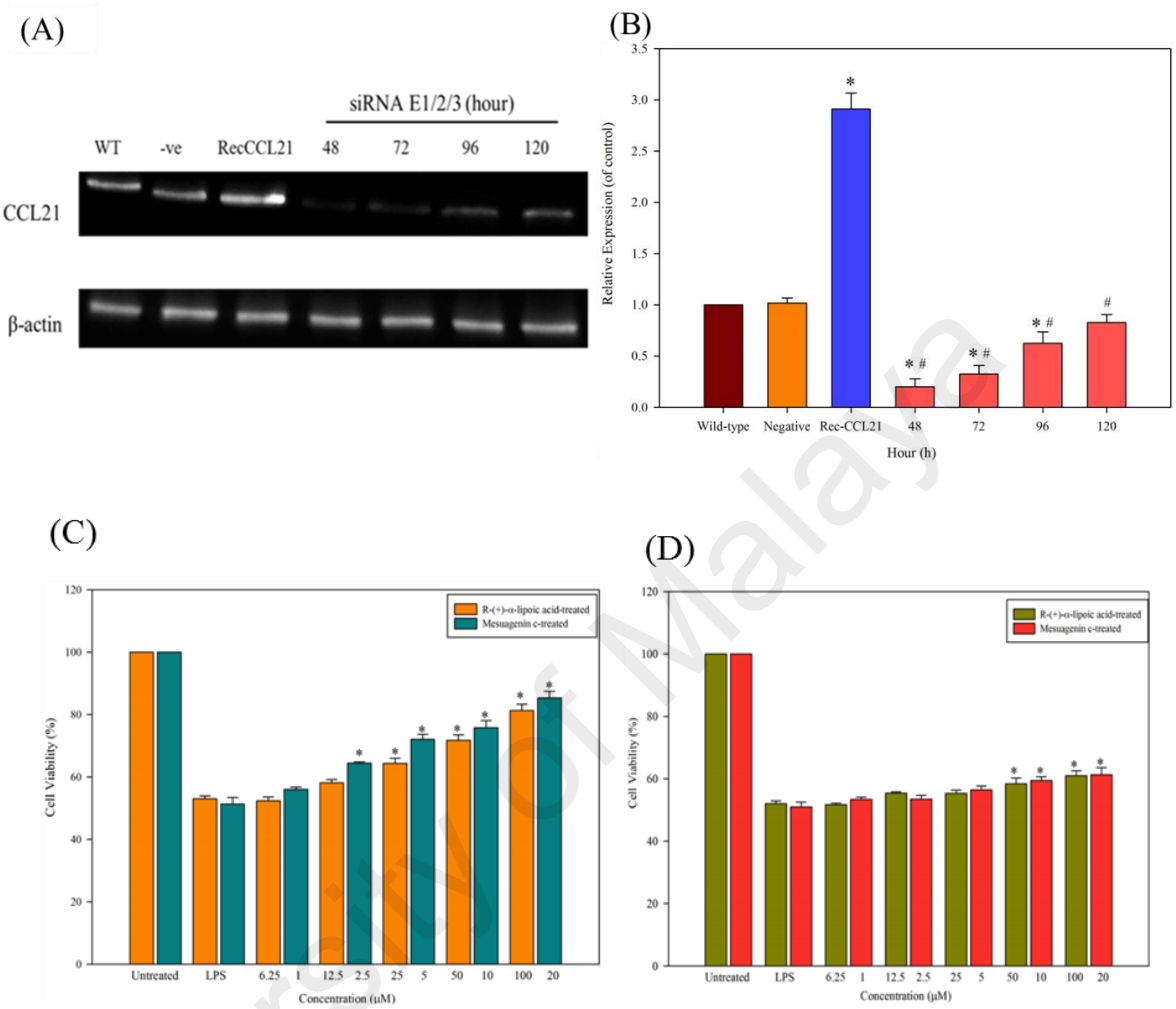
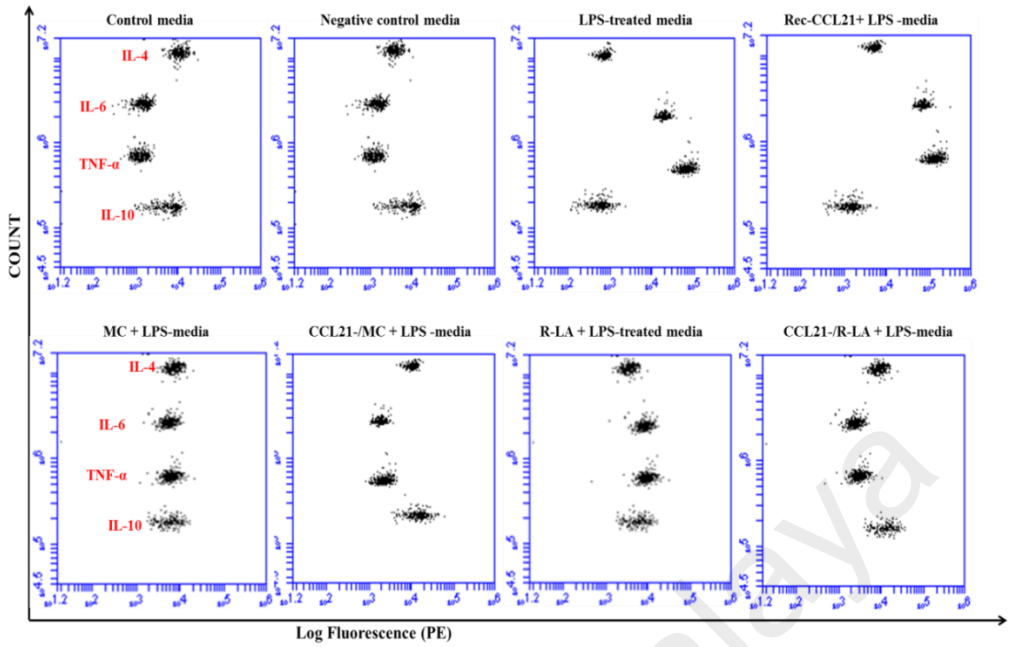


Figure 3.6 The suppression of LPS-induced toxicity and neuron-glia inflammation by R-(+)-α-lipoic acid and mesuagenin c. (A) Western blot analysis; Western blotting, lane 1, wild-type; lane 2, negative control, lane 3, recombinant CCL21; lane 4, $CCL21^{-}$ (48 h); lane 5, $CCL21^{-}$ (72 h); lane 6, $CCL21^{-}$ (96 h); lane 7, $CCL21^{-}$ (120 h). (B) bar charts represent the CCL21 expression after triple knockdown (48 – 120 h). (C) Dose-dependent increase in co-cultured NG108-15 cell viability following pretreatment with (R)-(+)-α-Lipoic acid and mesuagenin c in BV-2 cells. (D) Addition of API-2 diminished the protective effects of (R)-(+)-α-lipoic acid and mesuagenin c and reduced the co-cultured NG108-15 cells viability.

*P<0.05 versus untreated or LPS-treated, #P<0.05 recombinant CCL21, significantly different as shown.

(E)



(F)

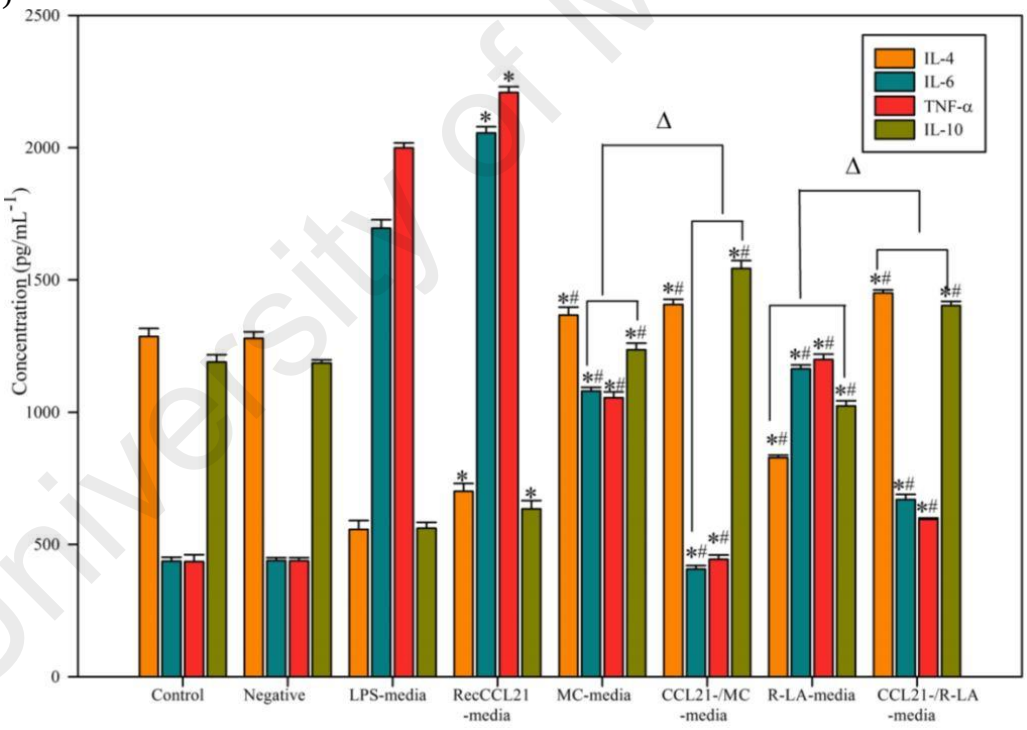


Figure 3.6, continued. (E) Representative flow cytometric dot plots (2100 events were collected according to BD Biosciences TH1/TH2/TH17 CBA protocol) of TNF-α, IL-4, IL-6 and IL-10 following direct conditioned media co-culture of NG108-15 cells with BV-2 cells as compared to LPS-treated and knocked down groups. (F) Bar charts represrent the different level of IL-4 and IL-10 that modulated the level of IL-6 and TNF-α as compared to LPS-treated and knocked down groups. Values are means ± S.E. from (n=3). Values are means ± S.E. (n=4). *P < 0.05 versus LPS-treated, $^{\#}$P < 0.05 versus Rec-CCL21 and $^{\Delta}$P< 0.05 versus (R)-(+)-α-lipoic acid (R-LA) or mesuagenin c (MC)-treated group, significantly different as shown.

3.5 Discussion

Aberrant microglial activation often constitutes the sign of chronic neuroinflammation that is often followed with neuronal death in neurodegenerative diseases (Glass et al., 2010). On this account, the suppression of aberrant microglia activation may provide clinical therapeutic strategy for inflammation-mediated neurodegenerative disorders. Nonetheless, a deeper understanding of the molecular machinery of microglial activation is still clearly needed in deciphering the target molecules for the intervention of neurodegenerative diseases. This present study demonstrated that (R)-(+)-α-lipoic acid and mesuagenin c protected both BV-2 and NG108-15 cells against LPS by modulating NF-κB, cytokines, chemokines and galectin-3 through PI3K-Akt activation and GSK-3β inactivation. Moreover, the inhibition of CCL21 enhanced the anti-inflammatory effects of (R)-(+)-α-lipoic acid and mesuagenin c against LPS in the neuron-glia model.

LPS can lead to prolonged microglial activation with increased production of various inflammatory mediators, such as ROS and RNS. Microglia in the healthy brain do not express iNOS, but following inflammatory stimuli, they become activated and express iNOS that augments the production of NO (Huang et al., 2009b). Overproduction of NO is extremely harmful as it induces an irreversible inhibition of cytochrome *c* oxidase leading to elevation of ROS generation and hence, disrupts the mitochondrial respiratory chain (Brown, 2010). Excessive ROS can lead to oxidative stress, loss of cellular functions and ultimately cell death by inducing kinase phosphorylation, increase activation of transcription factors that enhance the expression of inflammatory mediators (Schieber & Chandel, 2014). COX-2 is a key enzyme in the synthesis of PGE_2 during inflammation and serves as a factor that mediates superoxide-induced cell death in primary cortical neurons (Im, Kim, Paik, & Han, 2006). More importantly, COX-2 and iNOS expression are modulated by LPS and their sustained up-

regulation in microglia contributes to progressive neuronal damage. The data showed that (R)-(+)-α-lipoic acid and mesuagenin c attenuated the production of NO, ROS and PGE_2 in a dose-dependent manner following LPS exposure (Figure 3.2) relevant to its previous reported anti-oxidative property in neuronal model (Chan et al., 2012; Kamarudin et al., 2014). In this regard, both (R)-(+)-α-lipoic acid and mesuagenin c significantly inhibited the expression of iNOS and COX-2 which suggests suppression of NO and PGE_2 are mainly attributed to the inhibition of iNOS and COX-2 expression. Interestingly, pretreatment with API-2 but not LiCl reversed inhibition of NO and PGE_2 by (R)-(+)-α-lipoic acid and mesuagenin c. Pretreatment with LiCl did not alter or inhibit the ability of (R)-(+)-α-lipoic acid and mesuagenin c to suppress NO and PGE_2 production. These findings first indicated that the anti-inflammatory activity of (R)-(+)-α-lipoic acid and mesuagenin c is regulated by Akt activation and GSK-3β inactivation.

PI3K-Akt is one of the principal signaling pathways responsible in the regulation of LPS-induced inflammation through TLR4 activation (De Oliveira et al., 2012; Fukao & Koyasu, 2003). The activation of PI3K-Akt was shown to induce a negative regulation on LPS-induced IL-6 and TNF-α production from bone marrow macrophages and limit LPS-induced inflammation in human monocytic cells (Fang et al., 2004; Guha & Mackman, 2002). Moreover, recently the pharmacological inhibition of PI3K-Akt and the downstream target protein (GSK-3β and mTOR) are responsible in reducing LPS-induced inflammation in primary microglia that inhibits COX-2, mPGES-1 and prostanoid formation (De Oliveira et al., 2012). The current data revealed that pretreatment with (R)-(+)-α-lipoic acid and mesuagenin c induced a rapid phosphorylation of Akt (Ser473) and GSK-3β (Ser9) higher than LPS-treated group (Figure 3.3 (a) and (b)). The activated Akt (Ser473) has diverse downstream targets which includes mTOR, FoxO1 and GSK-3β (O'Neill, 2013). The phosphorylated Akt mediates its inhibitory action by phosphorylating GSK-3β at serine 9 and renders it

inactive. Even though GSK-3β is known to be involved in inflammation mediated TLR signaling, nevertheless, the molecular mechanisms of TLR-mediated GSK-3β activation are still unclear. GSK-3β can positively and negatively affect an array of transcription factors that are important in the production of inflammatory factors and thus, its inhibition is thought to be protective against a plethora of inflammatory stimuli (Beurel et al., 2010; Grimes & Jope, 2001; Hoeflich et al., 2000). The addition of Wortmannin and API-2 was shown to suppress the ability of (R)-(+)-α-lipoic acid and mesuagenin c to inactivate GSK-3β and demonstrated that the inactivation of GSK-3β is dependent on Akt activation.

The present study is similar with another reported study that showed lipoic acid induced anti-inflammatory effect against LPS in BV-2 cells and blocked the expression of iNOS and COX-2 by inactivating GSK-3β (Ser9) that is dependent on Akt (Koriyama et al., 2013b). Therefore, the current data suggest that the anti-inflammatory actions of (R)-(+)-α-lipoic acid and mesuagenin c could share some similarity with the anti-inflammatory activity of lithium, a GSK-3β inhibitor used to treat bipolar disorder. Lithium was reported to induce serine-phosphorylation of GSK-3β through a feedback loop that involves the regulation of protein phosphatase-1 (Zhang et al., 2003) or by the activation of Akt (Chalecka-Franaszek & Chuang, 1999). Recently, lithium was demonstrated to attenuate LPS-induced microglial inflammation by suppressing TLR-4 via activation of PI3K/Akt and FoxO1 (Dong et al., 2014). Based on the present data, the addition of LiCl alone or in combination with (R)-(+)-α-lipoic acid and mesuagenin c resulted in the suppression of LPS-induced inflammation in BV-2 cells. Moreover, the mode of inhibition was found to be similar with the action of LiCl that rapidly activated Akt and inactivated GSK-3β, suggesting that the compounds might share the similar mechanisms. Nonetheless, LiCl has also been shown to exert anti-inflammatory action against LPS-stimulated microglia thorugh the rapid activation of PI3K/Akt and FoxO1.

Hence, the current data might suggest that the anti-inflammatory actions of (R)-(+)-α-lipoic acid and mesuagenin c in some parts mimic the anti-inflammatory activity of LiCl. Additionally, the both compounds could also activate the rapid activation of Akt via FoxO1 regulation, and thus, further studies should investigate this possibility. Moreover, the ability of both compounds to partially mimic the action of LiCl in some parts, might suggest that they both can pass through the cell membrane and act directly on the target proteins. However, this might need further clarification and further studies using computational structural homology analysis that can simulate this hypothesis can provide the necessary information of their mechanisms. Since both compounds induced serine-phosphorylation of GSK-3β through Akt phosphorylation, additionally, their ability to regulate the activity of protein phosphatase-1 should also be investigated to justify their ability to mimic some of LiCl mode of actions. Nevertheless, the current data are in line with these reports and confirmed that (R)-(+)-α-lipoic acid and mesuagenin c suppressed iNOS and COX-2 expression and subsequent NO and PGE_2 production via the rapid activation of PI3K-Akt and GSK-3β inactivation.

GSK-3β is extremely important in the activation and suppression of various transcription factors such as c-Jun, CREB and NF-κB that are critical in regulating the production of pro- and anti-inflammatory cytokines, chemokines, COX-2 and iNOS. NF-κB is an important transcription factor that regulates the expression of the genes involved inflammatory responses, cell survival and cell proliferation (Hayden & Ghosh, 2012; Mincheva-Tasheva & Soler, 2013). NF-κB transcriptional activity is tightly regulated via its interaction with IκB that keep it sequestered in an inactive form in the cytoplasm. The degradation of IκB induced by IKK releases multiple NF-κB dimers particularly the primary target, the p65:p50 heterodimer and hence, allowing its translocation into the nucleus for subsequent gene transcription (Hayden & Ghosh, 2012). The inactivation of GSK-3β also inhibits the translocation of NF-κB p65 into

cell nuclei and suppressed TLR-mediated inflammatory responses (Beurel et al., 2010). Previous study reported that GSK-3β-deficient mice induced NF-κB inactivation and lithium treatment suppressed the transactivation of NF-κB in the wild-type group (Hoeflich et al., 2000). Following the activation of PI3K-Akt and inactivation of GSK-3β, (R)-(+)-α-lipoic acid and mesuagenin c suppressed LPS-induced phosphorylation of IκBα and NF-κB p65 nuclear translocation (Figure 3.4). Additionally, pretreatment with API-2 prior to (R)-(+)-α-lipoic acid and mesuagenin c treatment demonstrated reduced inhibition of NF-κB p65. These observations suggested (R)-(+)-α-lipoic acid and mesuagenin c might induce the activation of Akt protein that can indude the self-limiting feedback mechanism that suppressed the NF-κB signaling. Nevertheless, the exact mechanisms by which the compounds induced the activation of Akt through directinteraction of activation of receptors (GPCRs and TRKs) should be investigated to clarify these observations. Contrary to this, the addition of LiCl suppressed LPS-induced IκBα degradation and NF-κB p65 nuclear translocation. Moreover, the genetic deletion of GSK-3β was shown to suppress IKK-induced phosphorylation and ubiquitination of IκB and hence, inhibited NF-κB and COX-2 expression induced by TNF-α (Takada, Fang, Jamaluddin, Boyd, & Aggarwal, 2004). The inhibition of GSK-3β affects NF-κB p65 transcriptional activity by reducing its binding ability which increases the binding of CREB protein to the limited nuclear co-activator CREB binding protein (CBP) in the nucleus and promotes NF-κB sequestration in the cytosol (Grimes & Jope, 2001; Lei et al., 2011). In addition, the inhibition of GSK-3β suppressed the production of heat-inactivated *Staphylococcus aureus*-induced TNF-α and reduced the biosynthesis of iNOS/NO in BV-2 cells (Cheng et al., 2009). More importantly, our present data support these findings and suggest that pretreatment with (R)-(+)-α-lipoic acid and mesuagenin c inhibited the LPS-induced NF-κB p65 translocation through the activation of PI3K-Akt and inactivation of GSK-3β (Ser9).

NF-κB, cytokines and chemokines can be reciprocally regulated during inflammation (Hayden & Ghosh, 2012; Mincheva-Tasheva & Soler, 2013). However, the aberrant regulation among NF-κB, cytokines and chemokines can result in chronic neuroinflammation that leads to neuronal damage (Hayden & Ghosh, 2011; Kamarudin et al., 2014; Minami et al., 2006). Following the inhibition of GSK-3β and NF-κB, pretreatment with mesuagenin c significantly decreased LPS-induced production of pro-inflammatory cytokines and chemokines in BV-2 cells by augmenting the IL-4 and IL-10 production (Figure 3.5 (a) and (c)). Pretreatment with (R)-(+)-α-lipoic acid also demonstrated the reduction of pro-inflammatory cytokines and chemokines but only through the augmentation of IL-10. Similarly, LiCl treatment suppressed the production of pro-inflammatory cytokines and chemokines by increasing IL-10 production. During inflammation, various anti- and pro-inflammatory cytokines that are produced simultaneously can be reciprocally regulated. For instance, IL-4 and IL-10 modulate the inflammatory response in microglial cells towards Aβ by regulating the production of pro-inflammatory cytokines and chemokines (IL-1α, IL-1β, IL-6, TNF-α and MCP-1) (Szczepanik, Funes, Petko, & Ringheim, 2001). Moreover, GSK-3β negatively regulate IL-10 production with subsequent inhibition of pro-inflammatory cytokines and chemokines (Huang et al., 2009a). In contrast, the addition of API-2 significantly reversed these observations, suggesting that Akt activation by (R)-(+)-α-lipoic acid and mesuagenin c is essential in suppressing LPS-induced production of pro-inflammatory cytokines and chemokines in BV-2 cells. The current findings further support the notion that the activation of PI3K-Akt/GSK-3(α/β) increased the production of IL-10 in suppressing the expression of pro-inflammatory cytokines (Antoniv & Ivashkiv, 2011).

Galectin-3 (Gal3) has been reported to be expressed in microglia following exposure to inflammatory stimuli (Burguillos et al., 2015; Lalancette-Hébert et al., 2012). Recently, secretion of Gal3 was shown to trigger the BV-2 cells activation

though TLR4-dependent inflammatory response and required for the complete activation of TLR4 upon LPS exposure (Burguillos et al., 2015). Furthermore, aldosterone was reported to induce Gal3 secretion in THP-1 and RAW 264.7 cells through mineralocorticoid receptors via PI3K/Akt and NF-κB transcription signaling pathways (Lin et al., 2014). Pretreatment with (R)-(+)-α-lipoic acid and mesuagenin c significantly suppressed LPS-induced the production of Gal3 in BV-2 cells (Figure 3.5 (a) and (c)). Moreover, the data demonstrated that both (R)-(+)-α-lipoic acid and mesuagenin c-induced the activation of PI3K-Akt and inhibition of GSK-3β was responsible for the suppression of LPS-induced the production of Gal3 in BV-2 cells.

One of the main aspects in the normal brain function is the bidirectional interaction between neurons and its neighboring glial cells. During neuroinflammation, microglia secrete various inflammatory mediators that are toxic or protective towards neurons (Suzumura, 2013; Tambuyzer et al., 2009). Likewise, the injured neurons can also transmit diverse signals such as chemokines (CCL5/RANTES and CCL21) that can influence the activation of microglial cells (Rostène et al., 2011). Although the release of inflammatory mediators is aimed to exterminate threats through microglial activation, however, their aberrant regulation and production can lead to persistent neuronal damage. Hence, a well-regulated bidirectional communication between neurons and glial cells are extremely important in maintaining a healthy CNS system. LPS exposure can indunce microglia to exacerbate inflammation by releasing pro-inflammatory cytokines that can modulate the production of other cytokines and chemokines (Matsumoto et al., 2014; Smith et al., 2012). Numerous studies have reported that various chemokines synthesized in neurons and activated microglial cells play crucial role in physiological and pathological conditions (Réaux-Le Goazigo et al., 2013; Rostène et al., 2011).

In the last decade, a special attention has been given to CCL21 involvement in brain inflammation and inflammation-mediated neuropathic pain. For instance, CCL21 was reported to be rapidly induced in cortical neurons and NG108-15 cells following glutamate-induced excitotoxicity that induced the distant and specific activation of microglia cells (Biber et al., 2001; de Jong et al., 2005). Moreover, the over expression of CCL21 was shown to trigger a massive brain inflammation in transgenic mice within three days after the expression onset (Chen et al., 2002a). In this study, the knockdown of CCL21 in NG108-15 cells was hypothesized to reduce the neuron-glia inflammatory responses and therefore, enhance the anti-inflammatory effects of R-(+)-α-lipoic acid and mesuagenin c. The direct inhibition of BV-2 activation by (R)-(+)-α-lipoic acid and mesuagenin c was first shown to reduce the LPS-induced neuronal cell death in the co-culture NG108-15 cells (Figure 3.6 (c)). Moreover, the level of IL-6 and TNF-α in BV-2 cells that were cultured with (R)-(+)-α-lipoic acid and mesuagenin c conditioned media was lower as compared to LPS- and recombinant CCL21-treated group. Additionally, the IL-4 and IL-10 levels were increased higher than the untreated cells by (R)-(+)-α-lipoic acid and mesuagenin c conditioned media whereas BV-2 cells that were cultured with LPS-conditioned media and recombinant CCL21-conditioned media displayed lower level of IL-4 and IL-10 as compared to (R)-(+)-α-lipoic acid and mesuagenin c. This indicated that both (R)-(+)-α-lipoic acid and mesuagenin c inhibited inflammatory responses in neurons following LPS-induced microglia inflammation by augmenting the anti-inflamamtory cytokines that suppressed the pro-inflamamtory cytokines levels. As postulated, the knockdown of CCL21 enhanced the reduction of pro-inflammatory factors in BV-2 cells cultured with CCL21^{-}/(R)-(+)-α-lipoic acid- and CCL21^{-}/mesuagenin c-conditioned media by significantly increasing the production of IL-4 and IL-10. The expression of CCL21 in the brain is solely found in endangered neurons and therefore, provides the basis for CCL21 to be a candidate for neuron-

microglia signaling. Moreover, CCL21 can be found in vesicle-like structures that are distributed throughout neurons and suggested to be a neuronal signal to induce microglia activation at distant sites from a primary lesion (de Jong et al., 2005).The present findings suggest that the knockdown of CCL21 enhances the anti-inflammatory acitivity of (R)-(+)-α-lipoic acid and mesuagenin c and therefore, hihglights it as potential target molecule that can attenuate aberrant inflammatory communication between neuron and glia.

The overproduction of cytokines and chemokines following LPS exposure in microglia can lead to the disintegration of the BBB which can induce peripheral leukocytes and macrophages trafficking (Dimitrijevic et al., 2006; Minami et al., 2006). Hence, the anti-inflammatory effect of (R)-(+)-α-lipoic acid and mesuagenin c is thought to mitigate the disruption of BBB following chronic inflammation. However, the compactness of endothelial BBB layers with a transelectrical resistance >1500 Ω cm2 simply limits entry various molecules, particularly potential therapeutics into the brain, which may exacerbate the chronic inflammation itself (Masserini, 2013; Petty & Lo, 2002). Nevertheless, the BBB is a dynamic biological entity, although it limits the entry of most pharmaceuticals, small hydrophilic compounds with a mass lower than 150 Da and highly hydrophobic compounds with a mass lower than 400– 600 Da that can cross the membrane by passive diffusion (Santaguida et al., 2006) such as opiates (morphine and methadone), anxiolytics (diazepam), SSRIs (paroxetine) and antipsychotics (chlorpromazine and promethazine) (Masserini, 2013). Moreover, larger molecules such as neurotrophin and cytokines can enter the brain via receptor-mediated endocytosis (M Rabanel, Aoun, Elkin, Mokhtar, & Hildgen, 2012; Minn et al., 2002; Zhang & Pardridge, 2001). (R)-(+)-α-lipoic acid with low molecular weight and partition coefficient, log P_{OW} of 3.40 has great potential to cross the BBB since it is mainly distributed to hydrophobic areas, in particular the lipid bilayers of the

endothelial cells and thus, aide its uptake via cellular diffusion or endocytosis. Moreover, it is known that (R)-(+)-α-lipoic acid can functions in both hydrophilic and lipophilic compartments of cells. In comparison, although the partition coefficient of mesuagenin c is unknown, however based on its hydrophobic propery since it is isolated from the hexane fraction, the compound could possess the ability to enter the BBB. However, this does not guarantee the ability of mesuagenin c to cross the BBB and hence, would compromise its anti-inflammatory, neuroprotective and neuritogenic activities in *in vivo* model.

3.6 Conclusion

While the initial activation of Akt by LPS induced inflammation in BV-2 cells, the rapid activation of PI3K-Akt that led to GSK-3β inactivation by (R)-(+)-α-lipoic acid and mesuagenin c inhibited NF-κB and suppressed the subsequent inflammatory responses. This self-limiting feedback mechanism by (R)-(+)-α-lipoic acid and mesuagenin c mimics the anti-inflammatory mechanisms of lithium by activating the PI3K-Akt and inhibiting GSK-3β. Morever, the inhibition of CCL21 augments the anti-inflammatory effects of (R)-(+)-α-lipoic acid and mesuagenin c against LPS-induced chronic inflammation in neuron-glia system. Collectively, (R)-(+)-α-lipoic acid and mesuagenin c protect both BV-2 and NG108-15 cells against LPS by modulating NF-κB, cytokines, chemokines and galectin-3 through PI3K-Akt activation and GSK-3β inactivation that is enhanced by CCL21 inhibition (Figure 3.7).

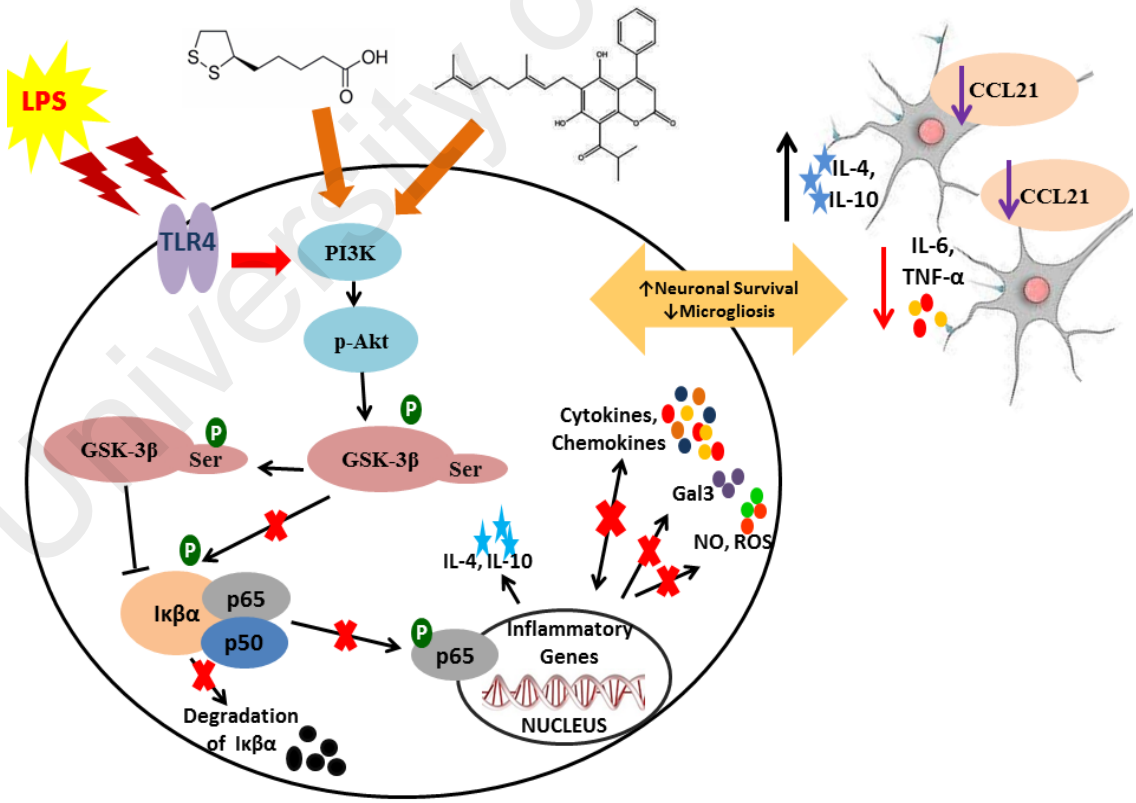


Figure 3.7 (R)-(+)-α-lipoic acid and mesuagenin c suppress the inflammation by modulating NF-κB, cytokines, chemokines and Gal-3 by activating PI3K-Akt and inactivating GSK-3β in LPS-stimulated BV-2 co-cultured with NG108-15 cells.

CHAPTER 4:

Mesuagenin c from *Mesua kunstleri* mitigated H_2O_2-induced cell death in NG108-15 cell through PI3K-AKT/GSK-3B pathway and suppression of NF-KB-cytokines

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A majort part of this chapter has been published in Evidence-Based and Complementary Alternatives Medicine (2012) - Special Issue: Therapeutic Approaches to Neuroprotective Activity by Complementary and Alternative Medicines

4.1 Introduction

A decade after stepping into the new millennium, neurodegenerative diseases still denote as one of world most arduous and appalling health issues as a result of increasing demographic fluctuation towards the aged population as well as growing apprehension for a better quality of life. Such alertness, together with the huge social and economic costs of the disease, has ignited in depth research efforts to combat or at least delaying the onset of neurodegenerative diseases (Fukushima *et al*., 2011). In a normal cycle of tissue proliferation, the aged and older cells are set to decease in order to give way for the generation of new cells. Such setting of programmed cell death or better known as apoptosis shown to be the prime cause in neurodegenerative diseases as a number of these diseases are characterized by a progressive fading away of neurons.

Nowadays, the emergence of natural products as therapeutics agents has raised a global interest among scientists in developing newer but more importantly safer drugs particularly for neurodegenerative diseases. The bark of *Mesua kunstleri* was subjected to various extraction and fractionation as an attempt to study its potential neuroprotective activity. The hexane extract of *M. kunstleri* was further studied and subjected to neuroprotective activity-guided fractionation and isolation. This neuroprotective activity-guided fractionation and isolation study has driven to the isolation of five 4-phenylcoumarins of which mesuagenin c displayed the most potent neuroprotective activity against H_2O_2 and was further subjected to various assays to elucidate its neuroprotective mechanisms. To date, this is the first report of neuroprotective activity in NG108-15 cells by mesuagenin c. Thus, the novel neuroprotective mesuagenin c could lead to the discovery of novel molecules that potentially can act as new target sites in combating neurodegenerative diseases.

4.2 Literature review

Generally, apoptosis is regulated by the Bcl-2 family of proteins which are further grouped into anti-apoptotic proteins (Bcl-2, Mcl-1) and pro-apoptotic proteins (Bax, Noxa), the adaptor protein Apaf-1 (apoptotic protease-activating factor 1) and the cysteine-aspartyl-specific proteases family (caspases) (Hengartner, 2000; Ola et al., 2011). Apoptosis holds a cardinal role in the maturation of the nervous system through the interplay between both anti- and pro-apoptotic proteins (Yuan & Yankner, 2000). Basically, neurons will either render an adaptive response or initiate apoptosis when they are exposed to stress signals or apoptotic stimuli such as withdrawal of neurotrophic factor, ischemic stroke, misfolded proteins, mitochondrial-complex inhibition, excessive calcium entry and excitotoxicity (Bredesen et al., 2006; Mattson & Magnus, 2006; Yuan & Yankner, 2000). Therefore, the prevention of neuronal apoptosis confers a mode of neuroprotection in combating and delaying the onset of neurodegenerative diseases.

Over the year, accumulated research findings have demonstrated that oxidative stress through accumulation of ROS and RNS as one of the major culprits which can either directly or indirectly activating the neuronal cell death pathway (Bonda et al., 2010; Ermak & Davies, 2002; Ott, Gogvadze, Orrenius, & Zhivotovsky, 2007). For instance, ROS can cause cellular damage and subsequent cell death by oxidizing vital cellular components such as lipids, proteins, and DNA. Moreover, the brain is exposed throughout life to excitatory amino acids such as glutamate where its metabolism produces ROS, thereby promoting excitotoxicity (Ho, Ortiz, Rogers, & Shea, 2002; Ward, Rego, Frenguelli, & Nicholls, 2000). Moreover, high level of ROS can induce various signaling pathways and redox-sensitive signal transduction such as activation of NF-κB. Such events can lead to various cellular processes such as inflammatoion, cell proliferation , cell survival and cell death through the production of pro-inflammatory

factors such as cytokines and chemokines by NF-κB (Nakajima & Kitamura, 2013; Nakano et al., 2006; Siomek, 2012).

PI3K-AKT signalling pathway is one of the key regulators of cell cycle proliferation, growth, survival, protein synthesis, and glucose metabolism. Moreover, activation of PI3K-Akt pathway has been shown to confer protection against drug-induced damage in neurons (Wang et al., 2010b). The mammalian target of rapamycin or best known as mTOR is one of the common target of Akt protein which can positively regulates cell growth and protects cells from neuronal cell death (Wrighton, 2013). Glycogen synthase kinase 3 (GSK3) is a protein kinase that is generally active in resting cells and is kept suppressed following activation of several distinct pathways such as the PI3K-Akt, insulin, and the growth factor pathway (Beurel & Jope, 2006; Cohen & Frame, 2001). More importantly, GSK3 inhibitors have been shown to serve as powerful tools that can modulate the activation of transcription factors such as NF-κB and hence, the balance of both anti- and pro-inflammatory cytokines from pro-inflammatory to anti-inflammatory (Cohen & Goedert, 2004; Jope et al., 2007).

4.3 Materials and method

4.3.1 Cell culture and materials

The NG108-15 was obtained from the American Type Culture Collection (ATCC). The cells were cultured in DMEM medium and conditioned at 5% CO_2 moist atmosphere at 37°C. The media were supplemented with 10% (v/v) heat inactivated FBS, 2% penicillin/streptomycin, 1% amphotericin B. In addition, the media for NG108-15 cells were supplemented with HAT media supplement (50x) Hybri-Max and the cells were primed for 3-4 weeks prior to its use. The media were filter sterilized by using µm filter membrane. Morphological analysis was performed with a fluorescence microscope (Leica Inverted Fluorescence Microscope, DM16000B) and flow cytometric analysis

was acquired with BD Accuri C6 Flow Cytometry and BD CFlow® Software. All fractions and isolated compounds were dissolved in dimethyl sulphoxide (DMSO) to produce stock solution with concentration of 20mM. The cells were then pretreated with various concentrations of samples for 2 h prior to the exposure of H_2O_2. Cells exposed to vehicle alone (10% FBS DMEM, DMSO ≤ 0.5% v/v) were used as the control group. Mesuagenin c was isolated and supplied by the Phytochemistry Lab, Department of Chemistry, University of Malaya.

4.3.2 Cell viability assay

The neuroprotective effects of mesuagenin c on the cell viability of H_2O_2-stressed NG108-15 cells was analyzed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. NG108-15 cells were raised to confluency, harvested by accutase, rinsed with PBS and plated at a total density of 5 x 10^3 cells/well in a 96-well plate. The cells were left to adhere for 48 h and then preincubated for 2 h with mesuagenin c prior to H_2O_2 (2 mM) exposure for subsequent 10 h. To each well 20 µL MTT solution (5 mg/mL) (Sigma Aldrich) was added and incubated at 37°C for another 4 h. The cells were analyzed by using a microplate reader (ASYS UVM340) at 570 nm (with a reference wavelength of 650 nm).

Cell viability was calculated based on the formula below:

% of cell viability = [absorbance of treated cells/ absorbance of control cells] x 100%

4.3.3 Nuclear morphological analysis

A total of 0.5 x 10^6 cells were plated ito 60mm culture dishes and pretreated with mesuagenin c (12.5 – 50 µM) for 2 h before exposure to H_2O_2 (400 µM) for 10 h. After treatment, cells were harvested and washed with PBS. Hoechst 33342 (Sigma Aldrich), a DNA fluorochrome (10 µg/mL) was added, followed by PI (2.5 µg/mL) and the cells were further incubated for 15 min at 37°C. The cells were then observed by

fluorescent microscope (Leica Inverted Fluorescence Microscope, DM16000B) and approximately 100 cells from five random microscopic fields were counted. Cells that exhibited intense blue fluorescence were considered to be at an early apoptosis stage. In contrast, cells that were dual stained with H33342 and PI were considered to be at the late apoptosis stage.

4.3.4 Total intracellular glutathione (GSH) content

Similarly, NG108-15 cells were seeded into 60 mm culture dishes and left for 48 h to adhere. The cells were then subjected to the designated treatment. Cells were rinsed, harvested and pellet was collected by centrifugation. The pellet was washed with ice-cold PBS and resuspended in 500 μL of 5% 5-sulfosalicylic acid (SSA, Sigma Aldrich). The cell suspension was then centrifuged at 10 000 rpm for 15 min and supernatant was collected to be used in the intracellular glutathione assay using 96-well plate format. The supernatant was added into each well containing GSH standards (Sigma Aldrich), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB, Sigma Aldrich), and NADPH (Calbiochem) in phosphate buffer. The reaction was immediately initiated by the addition of glutathione reductase (Sigma Aldrich). The final concentrations of the reaction mixture were 95 mM potassium phosphate buffer (pH 7.0), 0.95 mM EDTA, 0.038 mg/mL (48 μM) NADPH, 0.031 mg/mL DTNB, 0.115 units/mL glutathione reductase, and 0.24% 5-SSA. Absorbance was read at 1 min interval for 15 min at 405 nm with Oasys UVM340 microplate reader. The GSH concentration in each sample was calculated and compared with GSH standard curve.

4.3.5 Mitochondrial membrane potential ($\Delta\psi m$)

To quantify the change in mitochondrial membrane potential ($\Delta\psi m$), the lipophylic cationic fluorescent compound JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolylcarbocyanine iodide) dye was used accordingly to the manufacturer's

protocol (Stratagene). JC-1 possesses the ability to penetrate the plasma membrane and enters the cytosol of viable cell where it binds to intact mitochondrial membranes with large $\Delta\psi m$ to form J-aggregates which emit red fluorescence at 585 nm when excited. During membrane depolarization these J-aggregates dissociate into monomeric form which emits green color at 530 nm. Cells were harvested, washed and stained with JC-1 for 15 min at 37°C. Cells were then washed with PBS and $\Delta\psi m$ was measured by flow cytometry (BD FACScalibur) for the detection of red and green fluorescence signals. JC-1 aggregates (red fluorescence) within the mitochondria of healthy cells were detected in the FL-2 channel whereas JC-1 monomers (green fluorescence) in the cytoplasm of apoptotic cells were detected in the FL-1 channel.

4.3.6 Cytomteric detection of phosphatidylserine externalization

Annexin V-FITC/PI (BD) staining was performed to aid the detection of the apoptotic cells with translocated phosphatidylserine. Cells were stained with fluorescein isothiocyanate (FITC)-labelled annexin V (green fluorescence), simultaneously with dye exclusion of PI (negative for red fluorescence). Similarly, cells were plated into each 60 mm culture dishes and subjected to the designated. Cells were then harvested, gently washed, and resuspended in annexin V binding buffer (BD). Next, cells were stained with annexin V-FITC and PI in the dark at room temperature for 15 min. Cells were evaluated by using flow cytometry (BD FACScalibur). The differentiation of apoptotic and necrotic cells was based on the staining with PI.

4.3.7 Measurement of intracellular ROS level

Cellular oxidative stress induced upon exposure to H2O2 and its modulation by R-LA was measured using fluorescent probe 2',7'-dichlorfluorescein-diacetate (DCFH-DA). Cells were plated and subjected to similar pretreatment of LA prior to the addition of H_2O_2. Cells were harvested, rinsed and incubated with 10 µM of DCFH-DA for 30 min

at 37◦C in cell loading medium. The fluorescence signal was measured using flow cytometer.

4.3.8 Cytometric immunofluorescence staining of Bax and Bcl-2 protein

Neuronal apoptosis is regulated by the interplay between the pro- (Bax, Bik) and anti-apoptotic (Bcl-2, Bcl-xL) proteins. The expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 was determined by using flow cytometric immunofluorescence staining according to the manufacturer protocol (Santa Cruz Biotechnology, Inc). Cells were plated and subjected to the designated treatment. The cells were then harvested, washed with ice-cold PBS, fixed and permeabilized using Intracellular flow cytometry (FCM) System (Santa Cruz Biotechnology, Inc). Cells were resuspended and aliquoted ($1.0x10^6$ cells/mL) into 100 µl using FCM wash buffer. Next, cells were either incubated with 20 µL of fluorescein isothiocyanate (FITC)-conjugated Bax mouse monoclonal antibody (Santa Cruz Biotechnology, Inc) or rabbit IgG isotype control (Abcam) for 1 h. Similarly, for the expression of Bcl-2, cells were incubated with either PE-conjugated Bcl-2 mouse monoclonal antibody (Santa Cruz Biotechnology, Inc) or rabbit IgG isotype control (Abcam) for 1 h. Cells were then washed and resuspended in 500 µL FCM wash buffer. Cells were analyzed using BD Accuri C6 Flow Cytometry and BD CFlow® Software.

4.3.9 Assessment of Caspase-3/7 & -9 activities

The caspase-3/7 & -9 activities were evaluated by using Carboxyfluorescein FLICA Apoptosis Detection Kit based on the manufacturer's protocol (Immnunochemistry Technologies, LLC). The enzyme activity was measured based on inhibitor probes, FAM-DEVD-FMK (FLICA) and FAM-LEHD-FMK, for caspase-3/7 and -9, respectively. These probes bind covalently and irreversibly to the active site of the active caspase heterodimer emitting the green fluorescent signal detected by the FL-1

which is a direct measure of the number of the active caspase enzymes. For this assay, the cells were pretreated with mesuagenin C (12.5 – 50 µM) for 2 h and subsequently exposed to H_2O_2 (2 mM) for 10 h. Likewise, cells were harvested, washed with PBS, resuspended in media and stained with 30X FLICA solutions. The cells were then incubated at 37ºC under 5% CO_2 for 1 h and washed twice with 1X FLICA washing solution. Cells were then resuspended in a 400 µL washing buffer and fixed by the addition of 40 µL fixative solution for flow cytometry analysis. Cells were analyzed using BD Accuri C6 Flow Cytometry and BD CFlow® Software.

4.3.10 Western blot analysis

NG108-15 cells were plated on 60 mm culture and subjected to the designated treatment. Following this, the cells were then collected, rinsed with PBS and centrifuged. Protein extracts were prepared by lysing the cells RIPA buffer (Thermo Fisher Scientific, Rockford, IL, USA). The protein concentration was determined using the Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA). 20 µg of each protein sample was electrophoresed on 7 - 12% SDS–PAGE. Proteins on the gel were transferred onto a nitrocellulose membrane and blocked with 5% BSA. The membrane was then probed with the following primary antibodies: anti-β-actin, anti-pAkt (Thr308, Ser473), anti-Akt, anti-pGSK-3β(Ser9), anti-GSK-3β, anti-pmTOR,anti-mTOR (Cell Signaling Technology, Danvers, MA, USA) (Cell Signaling, USA) at 4°C overnight followed by appropriate HRP-conjugated secondary antibody. Protein bands were visualized by enhanced chemiluminescence (ECL) substrate solution kit (Bio-Rad) for 1 minute. Proteins were quantified with Bio-1D software as a proportion of the signal of the housekeeping protein band (β-actin).

4.3.11 NF-κB p65 translocation assay

Cells were plated onto coverslips and subject to designated treatments. After treatment, cells were gently rinsed and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Cells were rinsed with PBS and blocked with blocking buffer (5% BSA, 0.5% Triton X-100 in PBS) for an hour. The cells were then incubated with rabbit anti-NF-κB p65 (Cell Signaling, USA) for overnight followed with incubation with FITC-conjugated goat anti-rabbit secondary antibody (Pierce Antibodies, Rockford, IL, USA) for 2 h, respectively. The cells were washed and then analyzed by using a fluorescence microscope.

4.3.12 Cytokines measurement

The production of cytokines was measured by using Cytokine Bead Array (BD Biosciences, San Jose, CA, USA). In brief, following the designated treatment, 50 µL of culture medium was collected and mixed with the cytokine capture beads. The mixture was then mixed with PE-conjugated detection antibodies to form sandwich complexes. The desired cytokines (IL-6, IL-10 and TNF-α) were then measured by flow cytometer and data were analyzed by using FCAP Array™ software with comparison to the mouse cytokines standards' curves. To further validate the NF-κβ – cytokines regulation, the cells were pretreated with ethyl 3,4-dihydroxycinnamate (10 µM) prior to H_2O_2 exposure and the production of cytokines was then measured and compared with the H_2O_2-treated group.

4.3.13 Data analysis

All the experimental data are expressed in mean ± standard error (S.E.). Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) and followed by Dunnett's test. P-values below 0.05 were considered to be statistically significant.

4.4 Results

4.4.1 Mesuagenin c protected NG108-15 against H_2O_2-induced cell death

Neuroprotective effects of mesuagenin c against H_2O_2-induced cell death were evaluated with the MTT assay. Exposure to H_2O_2 (10 h), significantly reduced the NG108-15 cell viability to 45.41 ± 3.42%. However, following pretreatment with mesuagenin c (3.12 – 200 µM), the cells viability was significantly increased in a dose-dependent manner. Pretreatment with mesuagenin c at 12.5 µM induced the the initial significant protection against H_2O_2 (58.41 ± 3.45%) (Figure 4.1). It was demonstrated hat pretreatment with mesuagenin c (25, 50 and 100 µM) significantly increased the NG108-15 cell viability to 61.11 ± 2.21%, 64.19 ± 1.92% and 71.95 ± 0.95%, respectively. Pretreatment with mesuagenin c at 200 µM was shown to induce the highest protection level (78.99 ± 3.49%). More importantly, pretreatment with mesuagenin c was shown to protect the NG108-15 cells effectively against H_2O_2 exposure and therefore, merit its protective mechanism investigation in NG108-15 cells.

4.4.2 Mesuagenin c suppressed H_2O_2-induced nuclear morphologic changes in NG108-15 cells

To determine whether the cell death was due to apoptosis, NG108-15 cells were stained with Hoechst 33342/PI. As depicted in Figure 4.2, control (untreated) cells without H_2O_2-treatment were uniformly stained and displayed equally disseminated chromatin, normal organelle and intact cell membrane. In contrast, cells that were treated with 400 µM H_2O_2 for 10 h (Figure 4.2) illustrated archetypal characteristics of apoptotic cells including the condensation of chromatin, shrinkage of nuclei and presence of apoptotic bodies with intense blue fluorescence. However, pretreatment (2 h) with varying concentrations of mesuagenin c (12.5-50 µM) markedly reduced the level of H_2O_2-induced nuclei morphological alterations and the number of cells with nuclear condensation and fragmentation was significantly decreased (Figure 4.2).

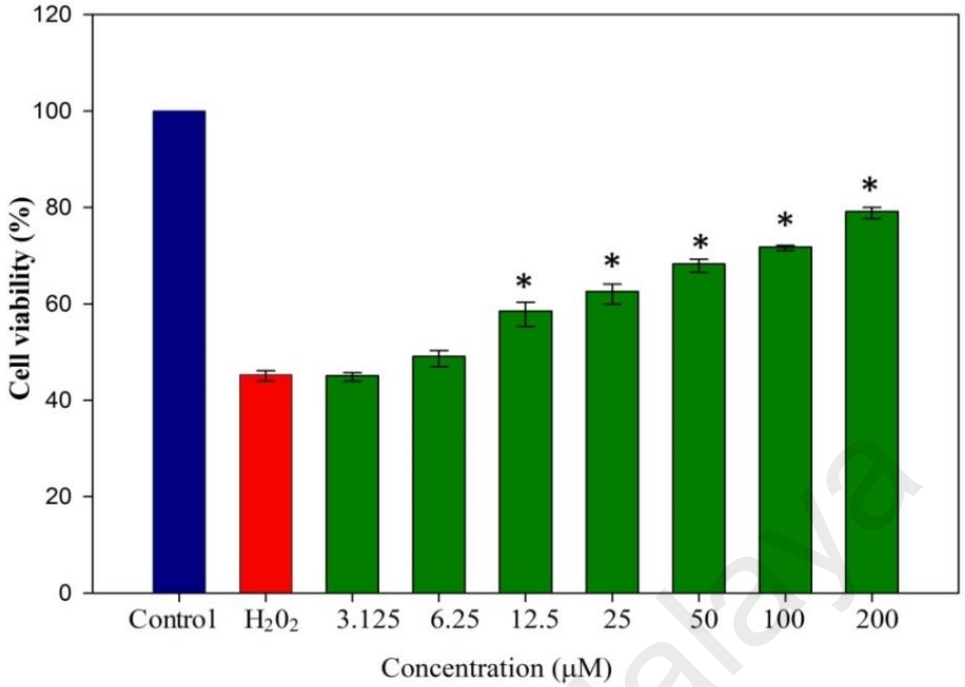


Figure 4.1 Dose-dependent increase in cell viability by pretreatment with mesuagenin c in H_2O_2-induced cell death prior to H_2O_2 exposure for 10 h. Values are mean ± S.E. from at least three independent experiments. The asterisk indicated significantly different values from H_2O_2-treated cells (*P<0.05).

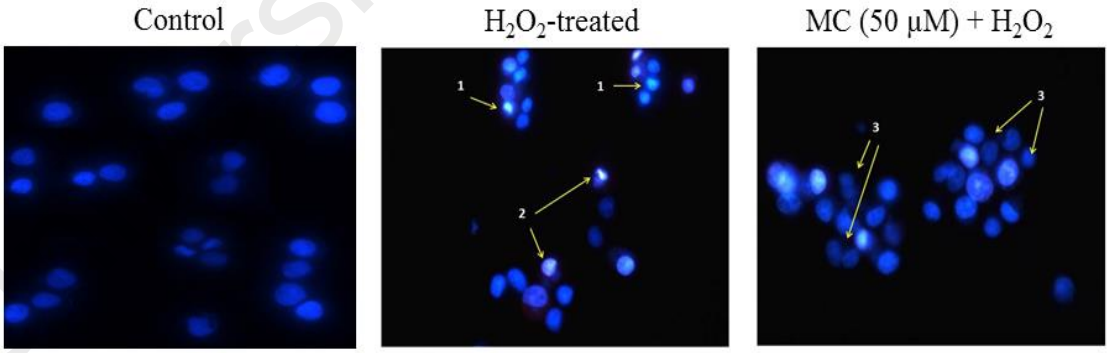


Figure 4.2 Mesuagenin c prevented H_2O_2-induced morphological changes in NG108-15 cells. Viable cell nuclei were evenly stained while H_2O_2-treated cells show reduced nuclear size, chromatin condensation and the nuclei were unevenly stained with intense blue fluorescence (arrow 1) which indicates early apoptotic cells. Cell nuclei that were dual stained with Hoechst 33342 and PI (arrow 2) were considered to be at their late apoptosis. Pretreatment with mesuagenin c reducted of apoptotic cell number (arrow 1) and increasing number of evenly stained nuclei indicating viable cells (arrow 3, Magnification 200X).

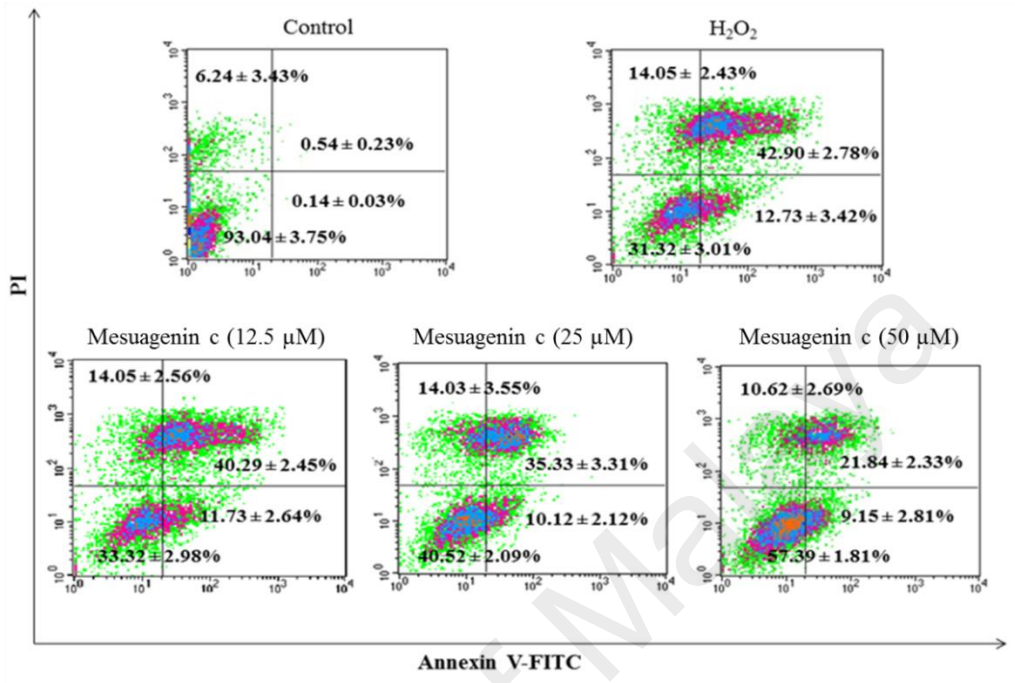
4.4.3 Mesuagenin c mitigated the externalization of phosphatidylserine

The addition of H_2O_2 (400 µM) significantly increased both annexin V^+/PI^- (early apoptosis, lower right quadrant) and annexin V^+/PI^+ (late apoptosis, upper right quadrant) cell populations to 11.73 ± 3.42% and 42.90 ± 2.78%, respectively, as indicated in Figure 4.3(a). In contrast, pretreatment with mesuagenin c (50 µM) decreased the early apoptotic cell population to 9.15 ± 1.65% and late apoptotic cell population to 21.84 ± 2.33% (Figure 4.3(a)). Pretreatment with 12.5, 25 and 50 µM of mesuagenin c for 2 h followed with H_2O_2 insult dose-dependently reduced the cumulative early and late apoptotic cell population from 54.63 ± 2.67% to 51.99 ± 2.55%, 45.45 ± 2.72% and 30.99 ± 3.09%, respectively, as shown in Figure 4.3(b). It was also noted that the pretreatment increased the viable cell population (lower left quadrant) from 38.30 ± 3.01% (H_2O_2-treated cells) to 57.39 ± 1.81% (Figure 4.3(a)). Based on these results, mesuagenin v prevented the effects of H_2O_2-induced apoptosis, signifying its potential neuroprotective ability in the present model.

4.4.4 Mesuagenin c increased the intracellular GSH level

Since the level of intracellular GSH plays a pivotal role in the neuroprotection, the effect of mesuagenin c on the intracellular GSH content in the NG108-15 cells challenged with H_2O_2 was determined. The intracellular GSH level for each concentration of mesuagenin c (12.25-50 µM) was determined from a standard curve constructed using known amounts of GSH (0.5-0.00195 nmoles). Exposure of NG108-15 cells with H_2O_2, significantly decreased the GSH level to 10 ρmoles as compared to 269 ρmoles in the untreated cells. In agreement with the protective effect of mesuagenin c, a significant increase in the intracellular GSH level in mesuagenin c-treated cells was observed. Pretreatment with mesuagenin c (12.25-50 µM), increased the level of intracellular GSH dose-dependently, yielding a 16.2-fold increase at the highest concentration of 50 µM, as compared to the H_2O_2-treated cells (Figure 4.4).

(A)



(B)

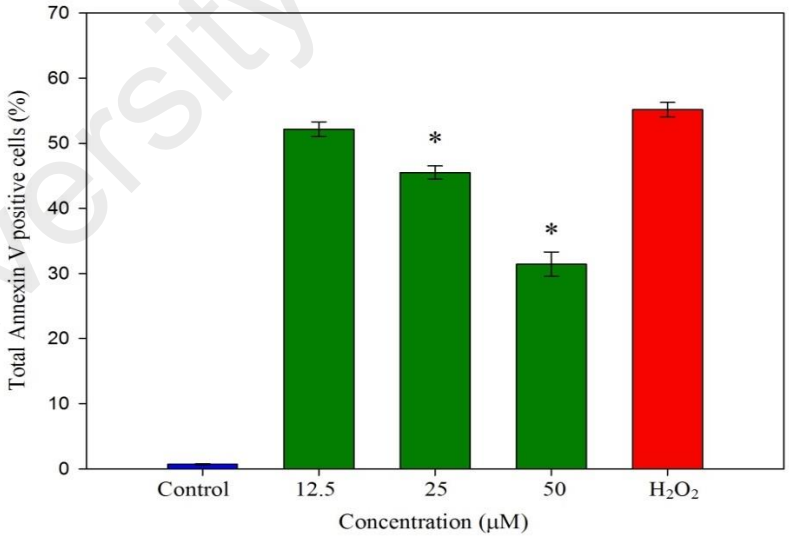


Figure 4.3 The effect of mesuagenin c on the externalization of PS in NG108-15. (A) The different distribution of NG108-15 cells stained with annexin V-FITC /PI in a dual parametric dot plots of PI fluorescence (Y-axis) versus Annexin V-FITC fluorescence (X-axis). (B) Bar chart indicate the proportion of annexin V positive cells (Annexin V^+/PI^- and Annexin V^+/PI^+) as compared to H_2O_2-treated cells. Values are mean ± S.E. from at least three independent experiments. The asterisk indicated significantly different values from H_2O_2-treated cells (*$P<0.05$).

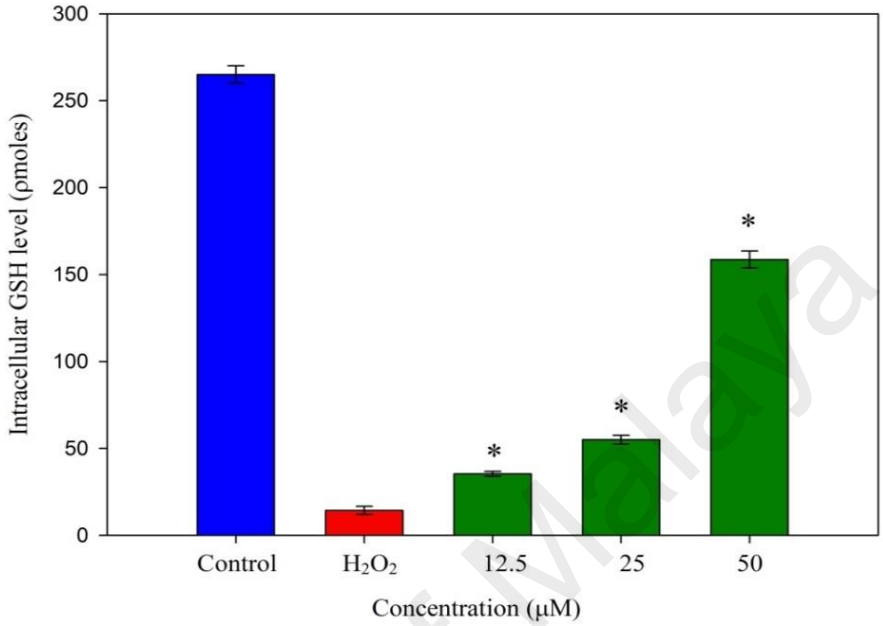


Figure 4.4 The effect of mesuagenin c on total intracellular GSH level. Pretreatment with mesuagenin c dose-dependently aggrandized intracellular GSH level after H_2O_2 challenge in the NG108-15 cells. Values are means ± S.E. from at least three independent experiments. The asterisk indicated significantly different values from H_2O_2-treated cells (*$P<0.05$).

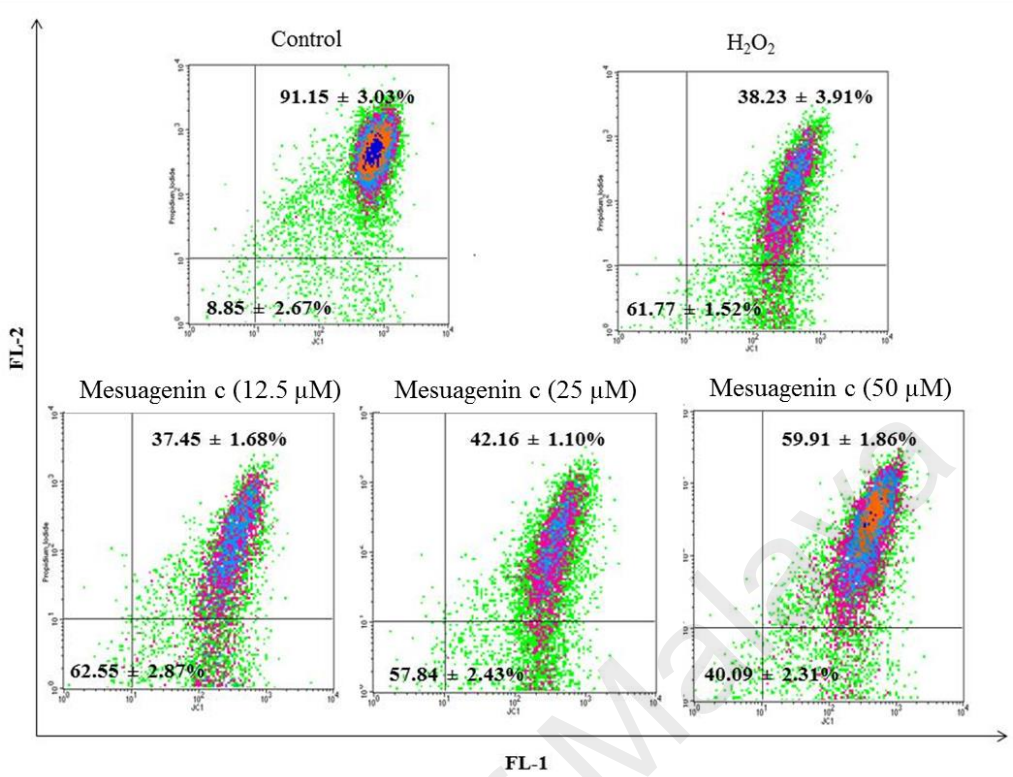
4.4.5 Mesuagenin c attenuated *Δψm* dissipation in NG108-15 cells

The dissipation of *Δψm* is an apoptotic hallmark in the initiation of apoptosis, exemplifies one of the early events occurring during apoptosis. A cationic lipophilic fluorescent probe known as JC-1 was used to indicate the loss of *Δψm*. This lipophilic dye enters mitochondria in proportion to the membrane potential. JC-1 will form J-aggregates at the high intramitochondrial concentrations induced by higher *Δψm* values. From the data attained, there was an alteration of fluorescence signal from the upper right quadrant to the lower right quadrant which leads to a lower red fluorescence signal (38.23 ± 3.91%) and higher green fluorescence signal (61.77 ± 1.52%) in H_2O_2-treated cells, signifying disruption of *Δψm* (Figure 4.5(a)). Nevertheless, this alteration was reversed by 2 h pretreatment with mesuagenin c in a dose-dependent manner as shown in Figure 4.5(b) where NG108-15 cells treated with 50 µM mesuagenin c (*P<0.01) overturned the effect of H_2O_2, shifting the fluorescence signal from lower right (40.09 ± 2.31%) to the upper right (59.91 ± 1.86%) as indicated by JC-1 flourescence ratio bar chart (Figure 4.5(a)).

4.4.6 Mesuagenin c modulated Bax and Bcl-2 protein expression

The ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2 is commonly associated with the activation of caspases, such as caspase-3 which executes apoptosis. To investigate the effects of mesuagenin c on Bax/Bcl-2 ratio, NG108-15 cells were first pretreated with different concentrations of mesuagenin c (12.5, 25 and 50 µM) for 2 h prior to exposure to H_2O_2 for 10 h. Our obtained data showed that H_2O_2-treated cell profiles shifted to the right in the Bax histograms resulting in 3.09-fold increase (Figures 4.6(a) and (b)) in the expression of Bax protein, while decreasing the level of Bcl-2 (Figures 4.6(c) and (d)). This led to a significant surge in the Bax/Bcl-2 ratio to 7.02-fold (Figure 4.6(e)). However, pretreatment with mesuagenin c (12.5, 25 and 50 µM) significantly inhibited

(A)



(B)

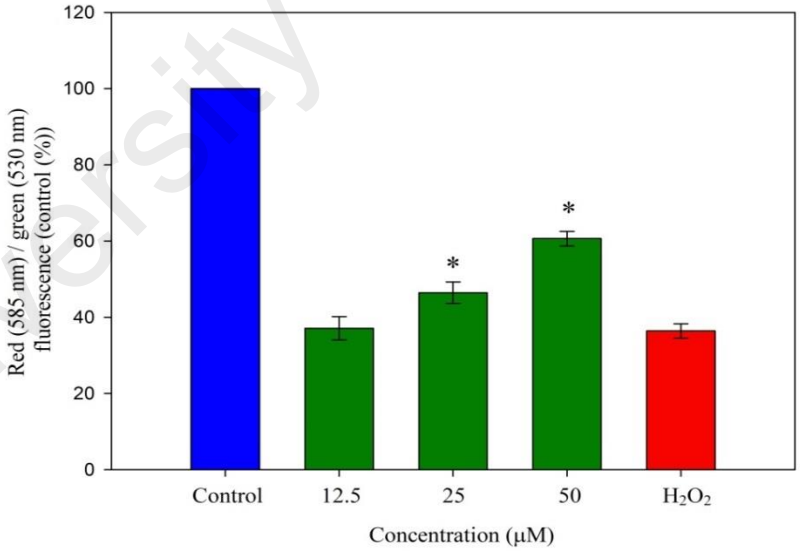


Figure 4.5 Flow cytometry analysis showing alterations in the *Δψm* of NG108-15 cells. Upon completion of treatment, the cells were stained with JC-1 and the alteration in *Δψm* was analyzed as mentioned in methods. (A) Representative dot plots of JC-1 aggregates (FL-2 Red fluorescence) vs. JC-1 monomers (FL-1 green fluorescence). (B) Bar chart showing the percentages of relative fluorescence intensity of *Δψm* in control, H_2O_2-only and varying pretreatment concentrations of mesuagenin c in NG108-15 cells. Values are mean ± S.E. from at least three independent experiments. The asterisk indicated significantly different values from H_2O_2-treated cells (*P<0.05).

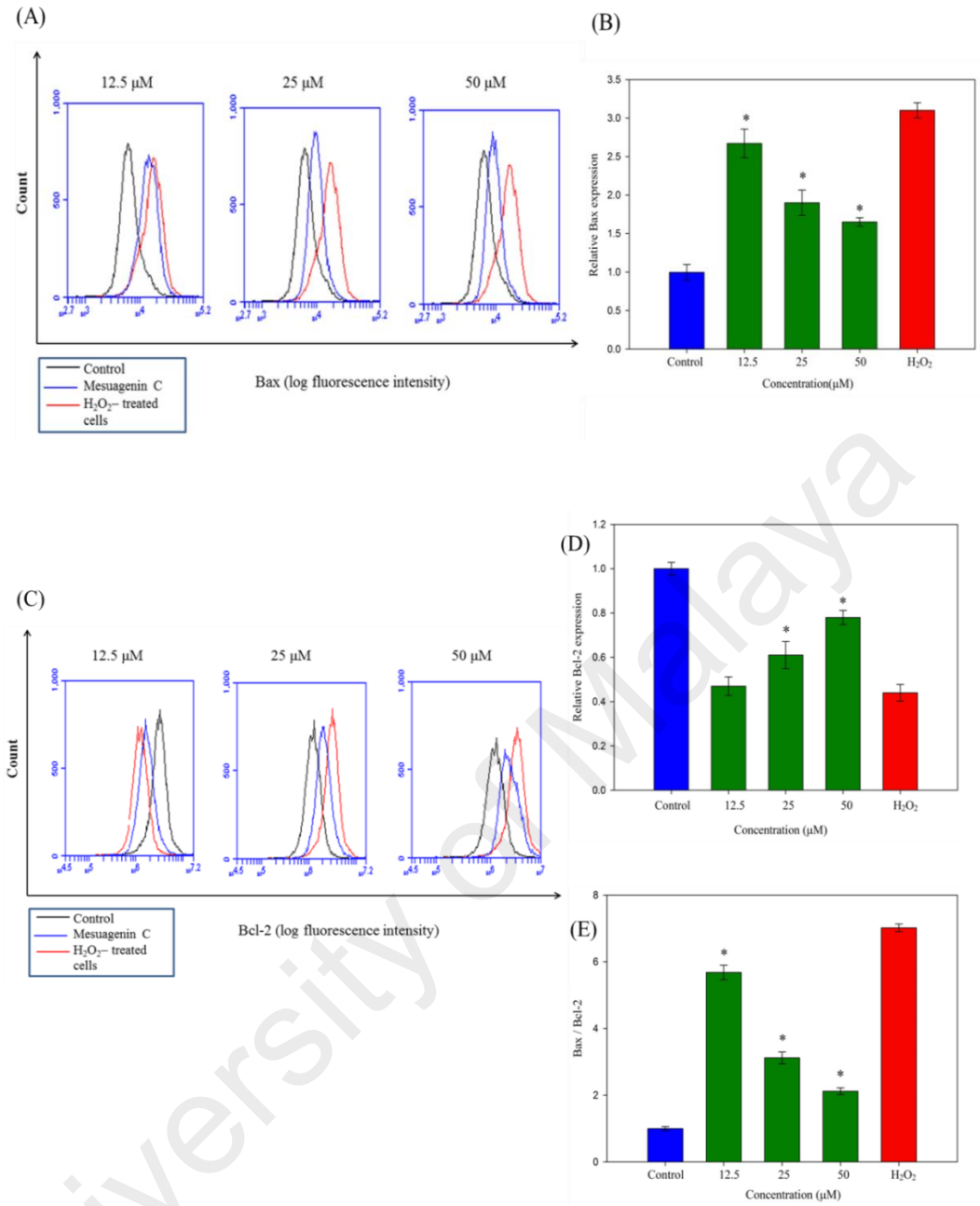


Figure 4.6 Mesuagenin c modulated Bax and Bcl-2 protein expression in H_2O_2-treated NG108-15 cells. (A) Representative overlay of histograms showing Bax-associated immunofluorescence. (B) Bar chart represents dose-dependent downregulation of Bax protein expression after pretreatment with mesuagenin c. (C) Representative overlay of histograms showing Bcl-2-associated immunofluorescence. (D) Bar chart represents dose-dependent elevation of Bcl-2 expression after pretreatment with mesuagenin c. (E) Bar chart represents dose-dependent attenuation of Bax/Bcl-2 ratio. Values are mean ± S.E. from at least three independent experiments. The asterisk indicated significantly different values from H_2O_2-treated cells (*P<0.05).

the H_2O_2-induced increase of Bax (Figures 4.6(a) and (b)) and decrease of Bcl-2 protein expression (Figures 4.6(c) and (d)) dramatically when compared to H_2O_2-treated cells. This is further demonstrated by the lowering in the ratio of Bax/Bcl-2 from 5.68 to 2.12 (Figure 4.6(e)). On that account, mesuagenin c may prevent the NG108-15 cells from entering neuronal apoptosis.

4.4.7 Mesuagenin c inhibited activation of caspase-3/7 and -9

To investigate the involvement of caspase-dependent and caspase-independent pathways in H_2O_2-induced apoptosis, the NG108-15 cells were subjected to H_2O_2 treatment (400 µM) for 10 h and the caspase-3/7 and -9 activities were measured using flow cytometry analysis. The results in Figures 4.7(a) and (c) showed that H_2O_2-treated cell profiles shifted to the right in caspase-3/7 and -9 histograms indicating significant aggrandization of caspase-3/7 (566.76 ± 13.59%) and caspase-9 (372.97 ± 11.62%) activities when compared to untreated control cells. The effects of mesuagenin c on induced by H_2O_2induced caspase-3/7 and -9 activities were then investigated. The findings demonstrated that pretreatment with mesuagenin c (50 µM) for 2 h partially inhibited the elevated activities of caspase-3/7 and -9 induced by H_2O_2. The caspase-3/7 and -9 activities were found to be significantly reduced to 221.59 ± 10.66% (Figure 4.7(b)) and 171.39 ± 10.56% (Figure 4.7(d)), respectively, (*$P<0.01$) as compared to the H_2O_2-treated cells. The reduction of caspase-9 activity further explains the attenuation of the caspase-3/7 activation which reversed the H_2O_2-induced apoptosis effects. The data suggested that mesuagenin c may suppress caspase-9 activation followed by inhibition of caspase-3/7 via protecting mitochondrial membrane integrity and suppressing cytochrome c release from the mitochondrial inter-membrane space. The results implied that H_2O_2-induced apoptosis was mediated by caspase-dependent intrinsic apoptotic pathways.

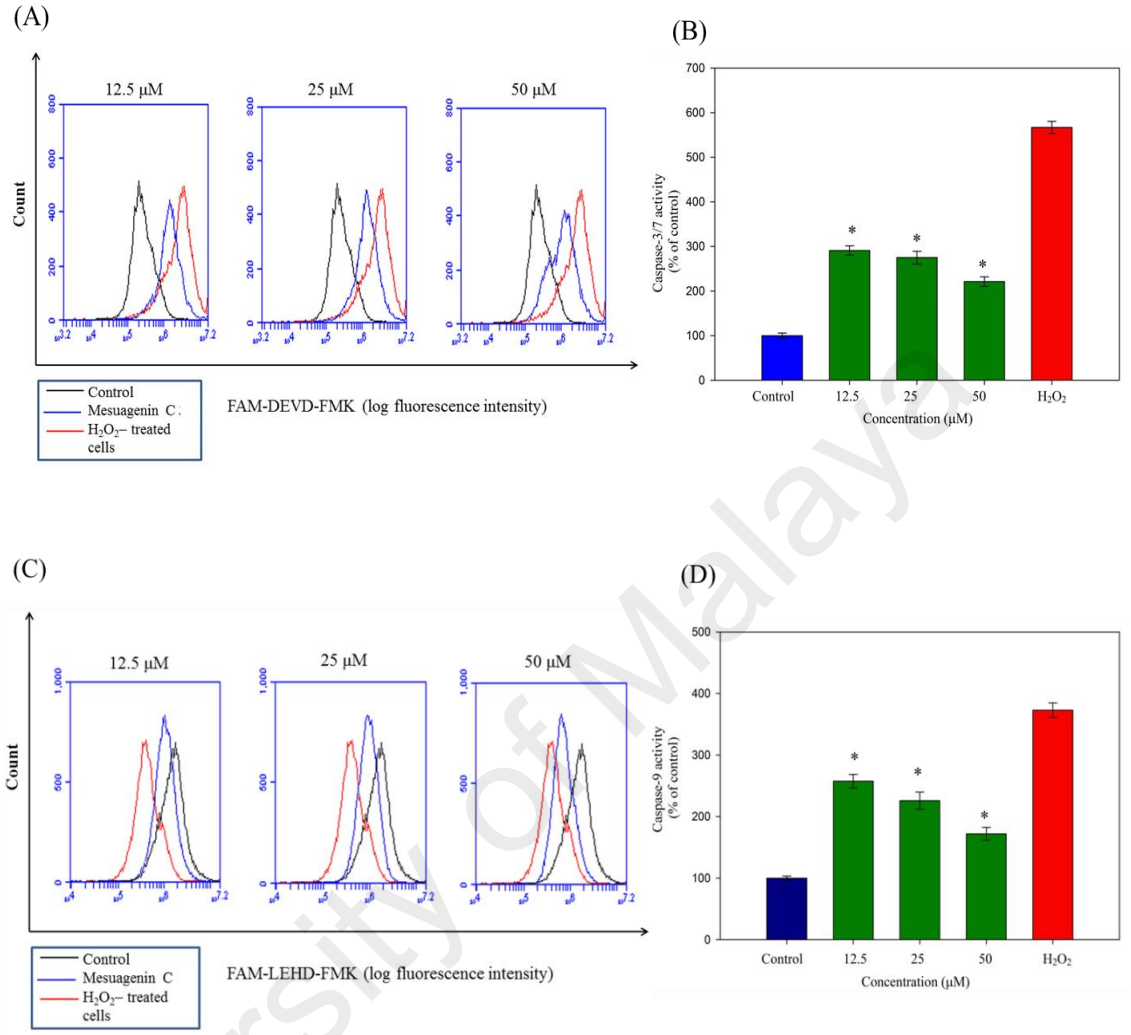


Figure 4.7 Mesuagenin c prevented H_2O_2-induced activation of caspase-3 and caspase-9 in H_2O_2-treated NG108-15 cells. (A) Representative overlay of histograms showing caspase-3/7-associated immunofluorescence. (B) Bar chart represents dose-dependent reduction in caspase 3/7 activity after pretreatment with mesuagenin c. (C) Representative overlay of histograms showing caspase-9 associated immunofluorescence. (D) Bar chart represents dose-dependent reduction in caspase-9 activity after pretreatment with mesuagenin c. Values are mean ± S.E. from at least three independent experiments. The asterisk indicated significantly different values from H_2O_2-treated cells (*P<0.05).

4.4.8 Mesuagenin c induced activation of PI3K-Akt via mTORC2

PI3K-Akt pathway plays imperative roles in the regulation of neuronal survival. Exposure to H_2O_2 resulted in significant reduction inactivation of Akt (Thr308 and Ser473) when compared to untreated cells. Nonetheless, following pretreatment with mesuagenin c, the activation of Akt at both Thr308 and Ser473 was signifantly increased (Figure 4.8(a)) which led to an increase of both pAkt(Thr308)/Akt and pAkt(Ser473)/Akt protein ratios (Figure 4.8(a) and (c)). Full activation of Akt can be achieved via phosphorylation by the mTORC2 which contains the regulatory proteins Rictor and mTOR. In accordance with this, mesuagenin c treatment increased the Rictor protein expression (Figure 4.8(f)) as compared to H_2O_2-treated cells. Furthermore, mesuagenin c demonstrated an increase in the phosphorylated mTOR expression (Figure 4.8(d)) leading to an increase in pmTOR/mTOR protein ratio (Figure 4.8(e). As an attempt to validate the involvemnet of PI3K-Akt, inhibitor of PI3K (Wortmannin) and Akt (API-2) were added prior to mesuagenin c treatment. The addition of Wortmannin and API-2 significantly decreased the NG108-15 cells viability and hence, attenuated the neuroprotective activity of mesuagenin c (Figure 4.8(h)).

4.4.9 Mesuagenin c modulated cytokines production by inactivating GSK-3β and NF-κB p65 translocation

To further corroborate the participation of Akt downstream targets other than mTORC2, the regulation of GSK-3β was then determined. GSK-3β is reported to be essential in regulating NF-κB activity since it can promote rapid NF-κB p65 translocation. Based on this, the activation of PI3K-Akt by mesuagenin c was postulated to mediate the inhibition of GSK-3β which will be followed by suppression of NF-κB p65 translocation. A significant increase in GSK-3β expression accompanied by a reduction of pGSK-3β (Ser9) was observed in H_2O_2-treated cells (Figure 4.9(a)), but these changes were prevented when the NG108-15 cells were pretreated with mesuagenin c

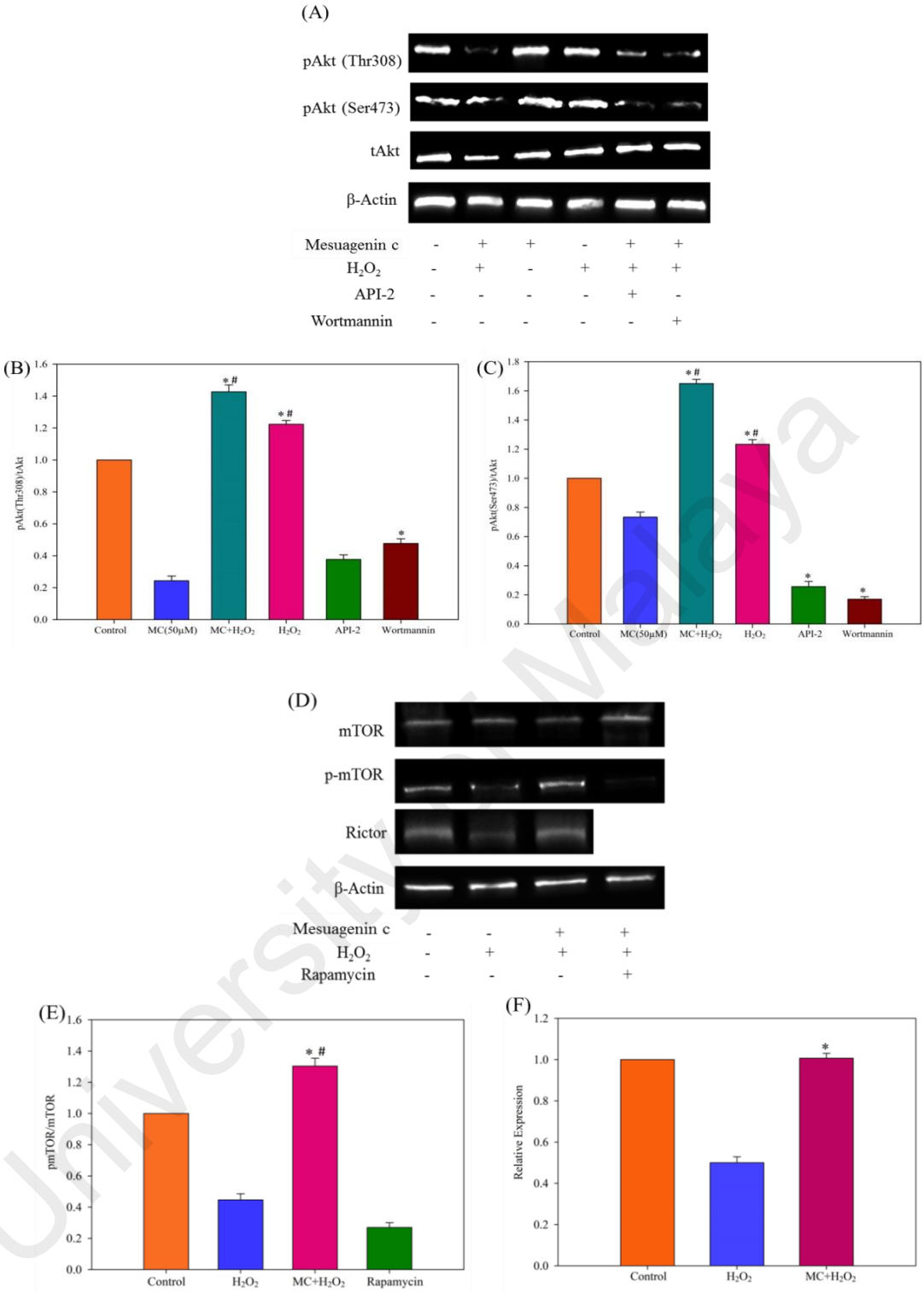


Figure 4.8 (A) Western blot analysis of mesuagenin c on Akt(Th308, Ser473) in the absence or presence of H_2O_2. The PI3K-Akt activation was validated by API-2 (10 µM) or Wortmannin (0.5 nM) for 1 h prior to mesuagenin c and H_2O_2 exposure. Lane 1, DMEM control; lane 2, H_2O_2 + MC; lane 3, MC; lane 4, H_2O_2; lane 5, H_2O_2 + MC + API-2; lane 6, H_2O_2 + MC + wortmannin. (B) pAkt(Ser473)/tAkt ratio (C) pAkt(Th308)/tAkt ratio (D) Western blot analysis of mesuagenin c on mTOR and rictor expression in NG108-15 cells. Lane 1, DMEM control; lane 2, H_2O_2 ; lane 3, H_2O_2 + MC (50 µM); lane 4, H_2O_2+ MC + rapamycin. (E) pmTOR/mTOR ratio (F) rictor.β-actin was used as housekeeping protein. Values are means ± S.E. (n=3). *P<0.05 versus H_2O_2, $^{\#}$P<0.05 versus API-2 or Wortmannin, significantly different as shown.

(G)

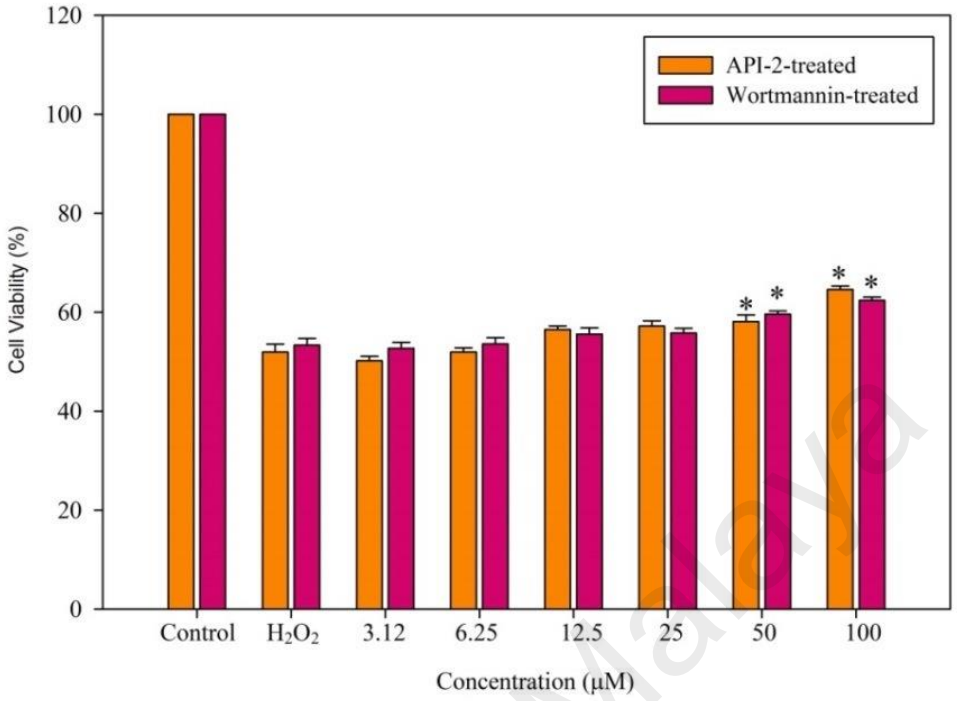


Figure 4.8, continued. (G)Viability of NG108-15 cells pretreated with mesuagenin c and API-2 or Wortmannin. Values are means ± S.E. (n=3). *P<0.05 versus H_2O_2, significantly different as shown.

(Figure 4.9(b)). Pretreatment with mesuagenin c (50 µM) was shown to suppress the translocation of NF-κB p65. Similarly, the immunofluorescence data substantiated that treatment with mesuagenin c inhibited the translocation and activation of NF-κB p65 in NG108-15 cells that were exposed to H_2O_2 (Figure 4.9(c)). Next, the effects of mesuagenin c on the production of major inflammatory cytokines (TNF-α, IL-6 and IL-10) were analyzed since GSK-3β and NF-κB activation is reciprocally regulated with the production of cytokines. Following H_2O_2 exposure, TNF-α and IL-6 production was augmented significantly as compared to control untreated cells (Figure 4.9 (d) and (e)). Moreover, these changes were accompanied by the significant reduction of antiinflammatory cytokine, IL-10. Upon inhibition of GSK-3β and suppression of NF-κB p65, mesuagenin c treatment aggrandized the production of antiinflammatory cytokine, IL-10 which then resulted in further diminution of TNF-α and IL-6 as compared to H_2O_2-treated cells. To further validate the regulation of NF-κB-cytokines modulated by mesuagenin c, the NG108-15 cells were also pretreated with ethyl 3,4-dihydroxycinnamate, an NF-κB inhibitor. It was observed that pretreatment with ethyl 3,4-dihydroxycinnamate suppressed the production of IL-6 and TNF-α as well as IL-10 as compared to H_2O_2-treated groups. The results suggest that pretreatment with mesuagenin c induced neuroprotection against H_2O_2 by modulating both anti- and proinflammatory cytokines through their reciprocal regulation with NF-κB.

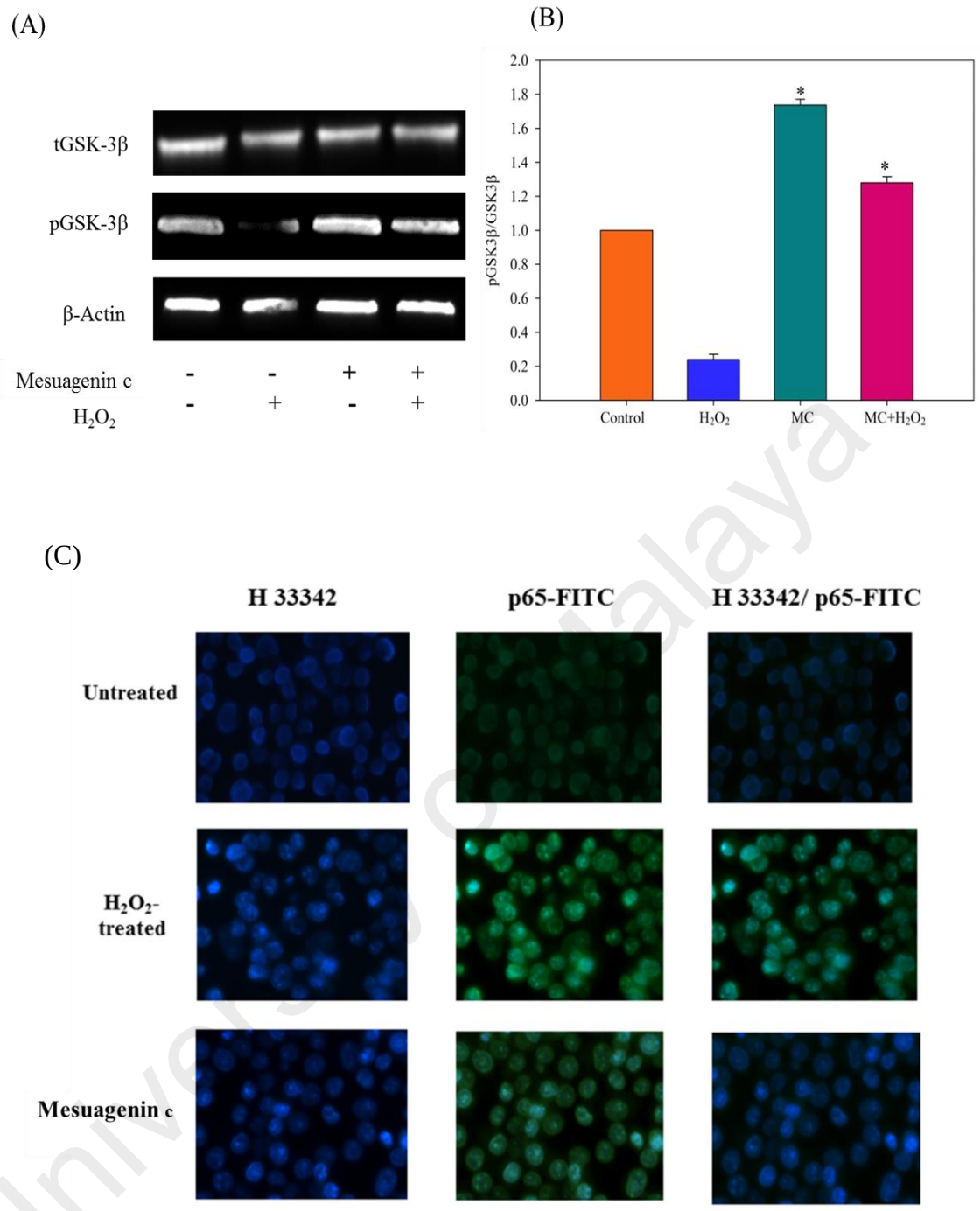


Figure 4.9 (A) Western blot analysis of mesuagenin c on pGSK-3β (Ser9) and total GSK-3β and NF-κB p65 translocation Western blotting, lane 1, DMEM control; lane 2, H_2O_2; lane 3, MC; lane 4, H_2O_2 + MC. (B) pGSK-3β(Ser9)/tGSK-3β ratio (C) Cells were pretreated with (mesuagenin c (50 µM) for 2 h before H_2O_2 addition for 4 h. Cells were stained with H33342 for nuclear visualization (blue) and NF-κB p65 translocation was visualised by fluorescence microscopy.(Magnification: 200x) Values are means ± S.E and the asterisk indicated significantly different values from H_2O_2-treated cells (*$P<0.05$, n=4). $^{\Delta}P<0.05$, significantly different as shown.

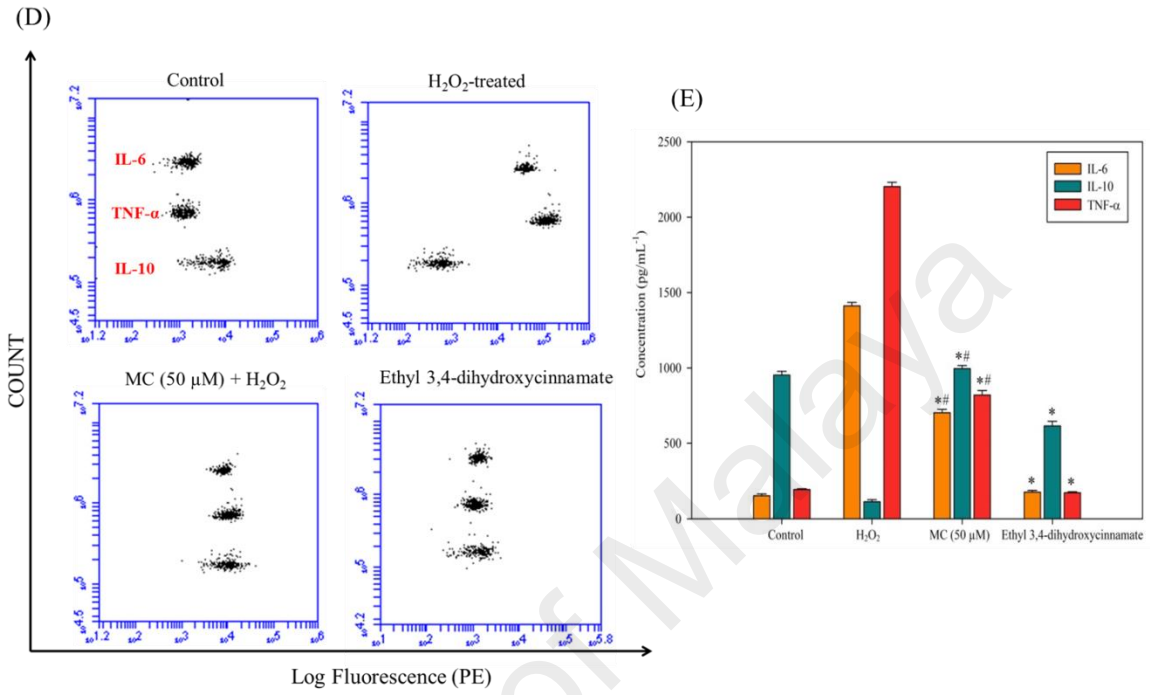


Figure 4.9, continued. (D) Pretreatment with mesuagenin c (MC; 50 µM) and ethyl 3,4-dihydroxycinnamate (10 µM) modulated the cytokines production following H_2O_2 exposure. Representative flow cytometric dot plots (2100 events were collected according to BD Biosciences TH1/TH2/TH17 CBA protocol) of TNF-α, IL-6 and IL-10. (E) Bar chart represents the concentration (pg/mL) of TNF-α, IL-6 and IL-10 modulated by (R)-(+)-α-lipoic acid and ethyl 3,4-dihydroxycinnamate following H_2O_2 exposure after 24 h. Values are means ± S.E and the asterisk indicated significantly different values from H_2O_2-treated cells (*P<0.01, n=4). $^{\Delta}$P<0.05, significantly different as shown.

4.5 Discussion

Cells can die by two major mechanisms: necrosis or apoptosis. Apoptosis is a gene-regulated phenomenon with the characteristic alterations of cellular structure including chromatin condensation, cell and nuclear shrinkage, oligonucleosomal DNA fragmentation and membrane blebbing (Gerschenson & Rotello, 1992). The neuroprotective effect of mesuagenin c was first verified using morphological analysis (Figure 4.2). Morphological changes associated with apoptotic cell death induced by H_2O_2 were characterized by the presence of shrunken cells, nuclear shrinkage, chromosome condensation and appearance of apoptotic bodies. On the contrary, these cellular events were evidently abrogated when the cells were pretreated with mesuagenin c. Moreover, flow cytometric annexin V-FITC/PI double staining revealed that mesuagenin c mitigated H_2O_2 induced cell death through apoptosis in NG108-15 cells. Having established this, the potential protective pathways were then studied.

Glutathione is the most luxuriant antioxidant in the brain which predominantly functions to detoxify H_2O_2 by decomposing it into water and oxygen and keeps the thiol groups of proteins in the reduced state (Mytilineou, Kramer, & Yabut, 2002). Oxidative stress that may result as a repercussion of elevated intracellular levels of reactive oxygen species (ROS) can lead to neuronal death (Ames, Shigenaga, & Hagen, 1993). The balance between generation of ROS and anti-oxidative processes can become perturbed as reported in aging (Beckman & Ames, 1998) and several neurological disorders such as Parkinson's disease and Alzheimer's disease (Dringen, 2000). A substantial growing line of evidence indicated that a high concentration of intracellular GSH protects cells against different ROS (Gutteridge & Halliwell, 2000). Glutathione depletion on the other hand, has been shown to directly modulate the dissipation of mitochondrial membrane potential and the activation of executioner caspases such as caspase-3/7 (Franco, Panayiotidis, & Cidlowski, 2007). Pretreatment with mesuagenin c

(50 µM) significantly increased the level of intracellular GSH by about 16-fold which may explain one of its antioxidative mechanisms in salvaging the NG108-15 cells from severe oxidative stress damage. This 60% restoration of GSH level by mesuagenin c is due to the fact that mesuagenin c at 50 µM protected 70% of the NG108-15 cells against H_2O_2 toxicity as shown in MTT assay, whereas concentration up to 200 µM induced protection up to 85% of the cells. Moreover, this partial restoration of GSH could be attributed to mesuagenin c which may serve an antioxidant with moderate potency but strong metal chelating activity against ROS. These antioxidative and metal chelating activities of mesuagenin c are presumably related and contributed to its antiapoptotic activity via mitochondrial-mediated apoptosis mechanisms. Decreased $\Delta\psi m$ and reduced ATP production are linked to mitochondrial-dependent apoptotic pathway and define the mitochondrial dysfunction (Hwang & Yen, 2008). Oxidative injury via H_2O_2 has been reported to dissipate $\Delta\psi m$, resulting in the rapid release of caspase activators such as cytochrome c into the cytoplasm, thus eliciting the apoptotic process (Jung et al., 2007; Xu, Li, & Liu, 2008). Concomitantly, results in this study have also established the ability of mesuagenin c to ameliorate the mitochondrial membrane potential after consequential loss of potential due to H_2O_2 challenge. These results suggest that mesuagenin c may play critical roles in preventing H_2O_2-induced apoptosis through its protective action on the mitochondria.

PI3K-Akt signaling pathway is one of the pathways that have been widely reported to confer neuronal survival and protecting neurons from damage (Abdul *et al.*, 2007, Wang *et al.,* 2010). Akt is a major downstream target of PI3K (You *et al*.,2009; Vauzour *et al*., 2007) where phosphorylation of Akt regulates various biological responses (Kim *et al*., 2001) such as cell proliferation and inhibition of apoptosis. Pretreatment with mesuagenin c suppressed H_2O_2-induced Akt inhibition by phosphorylating Akt at both Thr308 and Ser473 residues and increased the pAkt/Akt

ratio (Figure 3.8 (a-c)). Moreover, the addition of API-2 and Wortmannin significantly suppressed mesuagenin c-induced Akt phosphorylation which inhibited the protective effects of mesuagenin c and decreased the NG108-15 cell viability (Figure 4.8(g)). These observations then confirmed that mesuagenin c induced its protective effects against H_2O_2 by activating PI3K-Akt pathway. The full activation of Akt requires the phosphorylation of Ser473 residue by the mammalian target of rapamycin complex 2 (mTORC2). Pretreatment with mesuagenin c induced the phosphorylation of mTOR (Figure 4.8 (d) and (e)) and increased he expression of Rictor (Figure 4.8 (d) and (f)), an essential component of mTROC2 complex. Taken together, the data suggested that the protective mechanism of mesuagenin c is mediated by PI3K-Akt with the full activation of Akt protein through the mTORC2 complex. The activation of mTOR and PI3K-Akt is capable in suppressing neuronal apoptosis by preserving $\Delta\psi m$, inducing caspase inactivation and modulating inflammatory responses (Bhaskar et al., 2009; Guertin et al., 2006; Yap et al., 2008). Moreover, the activation of mTOR and PI3K-Akt modulates neuronal survival by regulating the Bcl-2 family of proteins (Shang et al., 2012; Shao et al., 2011).

The Bcl-2 family proteins are the critical regulators of the mitochondrial-mediated apoptosis by functioning either as promoters or inhibitors of the cell death process (Kuwana & Newmeyer, 2003). The two main members of Bcl-2 family; Bax and Bcl-2 play pivotal roles in the mitochondrial pathway of apoptosis. Bax has been associated in stimulating cell apoptosis, whereas Bcl-2 in inhibiting apoptosis (Haque et al., 2003; Zha & Reed, 1997). The interplay between Bax and Bcl-2 ratios determine the vulnerability of cells towards apoptosis (Adams & Cory, 2001). While Bcl-2 is responsible to maintain the mitochondrial integrity, Bax can destroy mitochondrial integrity and causes loss of mitochondrial membrane potential (Métrailler-Ruchonnet et al., 2007; Ola et al., 2011) which triggers the release of cytochrome *c* from the

mitochondrial transition pore (Chao & Korsmeyer, 1998). Pretreatment with mesuagenin c was shown to increase the Bcl-2 expression and decrease Bax expression which led to a reduction in Bax/Bcl-2 ratio (Figure 4.6(e)) as compared to H_2O_2-treated cells and therefore, suggests that mesuagenin c shifted the balance between pro- and anti-apoptotic proteins which then maintained the mitochondrial integrity toward cell survival. Furthermore, Bcl-2 overexpression can suppress cytochrome *c* release from the mitochondria which then inhibits activation of caspases and therefore, prevent apoptosis (Chao & Korsmeyer, 1998; Ola et al., 2011).

GSK-3β is a protein kinase that plays critical roles in cell development, metabolic homeostasis, neuronal growth and apoptosis since it targets and regulates important metabolic and signaling proteins, structural proteins and transcription factors (Beurel et al., 2010; Wang et al., 2011a). Inhibition of GSK-3β (Ser9) through Akt phosphorylation keeps it inactive and confers neuronal survival. In agreement with this, pretreatment with mesuagenin c increased the phosphorylation of GSK-3β (Ser9) as compared to H_2O_2-treated cells (Figure 4.9 (a) and (b)) and suggests that mesuagenin c may prevented neuronal cell death through inactivation of GSK-3β via PI3K-Akt activation. GSK-3β is an upstream mediator of executioner caspase, caspase-3, which can be activated in the apoptotic cells by both extrinsic and intrinsic pathways (Beurel & Jope, 2006). Moreover, an upsurge in the levels of pro-apoptotic proteins and/or a diminution in $\Delta\psi m$ and an opening of mitochondrial permeability transition pores, can lead to cytochrome *c* release from mitochondria (Crompton, 2000; van Loo et al., 2002). Once released, cytochrome *c* binds to the apoptosis protease activation factor (APAf-1) forming an apoptosome complex (Desagher & Martinou, 2000). This complex activates caspase-9 which will then cleave procaspase-3 and procaspase-7 that are responsible for the execution of apoptosis (Garrido et al., 2006). Following the activation of PI3K-Akt/GSK-3β pathway, mesuagenin c suppressed H_2O_2-induced the

activation of caspase-3/7 through the inhibition of caspase-9 and indicated that pretreatment with mesuagenin c protected the NG108-15 cells against H_2O_2 by inhibiting apoptosis through PI3K-Akt activation and GSK-3β inactivation. Since the activation of caspase-3/7 is for the execution of neuronal apoptosis (Cohen, 1997) therefore the suppressive effect of mesuagenin c on H_2O_2-induced caspase-3/7 activity further advocates that the mesuagenin c protective effect is a caspase-dependent mechanism.

The activation and inactivation of GSK-3β regulates an array of transcription factors that modulate the production of pro- and anti-inflammatory cytokines by promoting the binding of cAMP response element binding protein (CREB) while inhibiting the binding of NF-κB p65 subunit to the nuclear coactivator CREB binding protein (CBP) (Checker et al., 2012; Hayden & Ghosh, 2012; Heyen, Ye, Finck, & Johnson, 2000). NF-κB is an important regulator of DNA transcription that is activated in neurons and glial cells in a range of acute and chronic neurodegenerative conditions. NF-κB modulates the expression of genes that are involved in immune responses, inflammatory responses, cell survival and cell proliferation (Baker et al., 2011; Hayden & Ghosh, 2012). Furthermore, modulation of NF-κB activity is also influenced by neurotrophic factors (NGF, BDNF), cytokines, chemokines, and oxidative stress (Morgan & Liu, 2011; Tourniaire et al., 2013; Wang et al., 2011a). For instance, NF-κB and pro-inflammatory cytokines can reciprocally regulate each other activation. However, the aberrant activation of NF-κB can result in excessive production of inflammatory factors that may lead to neuronal cell death. Pretreatment with mesuagenin c inhibited the NF-κB p65 nuclear translocation (Figure 3.9 (c)) and this was followed with the suppression of pro-inflammatory cytokines production, TNF-α and IL-6 (Figure 4.9 (d) and (e)). Cytokines are among key modulators in inflammation via a complex and sometimes seemingly contradictory network of interactions. Major

pro-inflammatory cytokines such as IL-6 and TNFα are among the first pro-inflammatory cytokines to be expressed upon neuronal injury and their overproduction is highly detrimental towards neurons (Helmy et al., 2011; Rubio-Perez & Morillas-Ruiz, 2012). Furthermore, following GSK-3β activation or inactivation, a number of anti- and pro-inflammatory cytokines (TNF-α, IL-4, IL-6 and IL-10) may modulate each other production during inflammation (Hovsepian et al., 2013; Szczepanik et al., 2001). The present study further supported this view where inhibition of GSK-3β via PI3K-Akt by mesuagenin c demonstrated an increased of anti-inflammatory cytokine level, IL-10 that suppressed TNF-α and IL-6 as compared to H_2O_2-treated cells. Therefore, the data from this study further suggests that mesuagenin c induced the activation of PI3K-Akt/GSK-3β pathway which inhibited the NF-κB activation and attenuated the production of IL-6 and TNF-α by augmenting IL-10.

4.6 Conclusion

Mesuagenin c activated the PI3K-Akt/GSK-β pathway which subsequently mitigated mitochondrial-mediated caspase-dependent neuronal apoptosis by elevating the GSH level, *Δψm* and regulation of Bcl-2 and Bax proteins in conjunction with the attenuation of activated caspases. In addition, the inactivation of GSK-3β confered neuronal survival in NG108-15 cells through the augmentation of IL-10 that suppressed NF-κB and pro-inflammatory cytokines regulation. Based on these findings, mesuagenin c mitigated H_2O_2-induced neuronal cell death in NG108-15 cells by attenuating both mitochondrial-mediated caspase-dependent apoptosis and pro-inflammatorry cytokines via inhibition of GSK-3β through PI3K-Akt pathway (Figure 4.10)

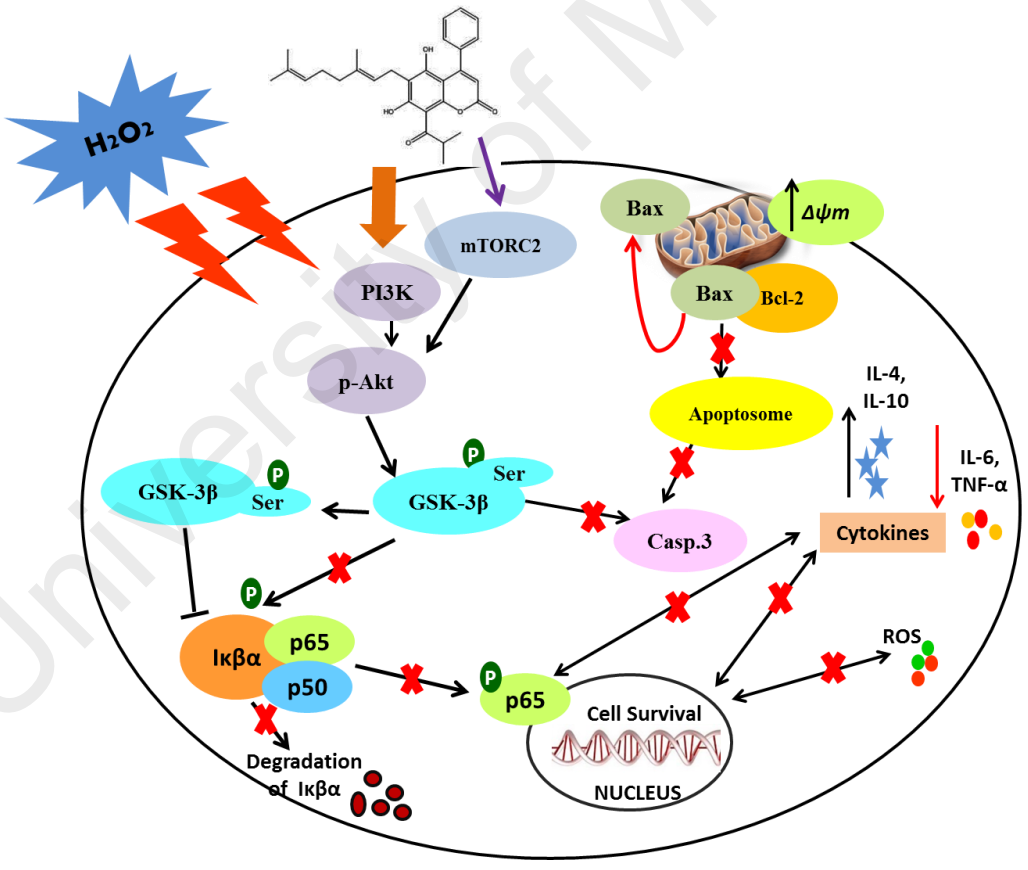


Figure 4.10 Schematic figure of the neuroprotective mechanisms of mesuagenin c in NG108-15 cells through actiavation of PI3K-Akt/GSK-3β and suppression of NF-κB-cytokines.

CHAPTER 5:

(R)-(+)-α-lipoic acid mitigated H_2O_2-induced cell death in NG108-15 cell through PI3K-AKT/GSK-3B pathway and suppression of NF-κB-cytokines.

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Published in Drug design, development and therapy. 2014 (8, 1765)

5.1 Introduction

The primary basis underlying neurodegenerative diseases is characterized mainly by typical aggregate-prone toxic proteins (Dasuri, Zhang, & Keller, 2013; Rubinsztein, 2006) and aberrant neuronal apoptosis, which involves oxidative stress, mitochondrial dysfunction, perturbation in calcium homeostasis and trophic-factor withdrawal (Brady & Morfini, 2010). Allied in a Pandora's box, the pathogenesis of these diseases is akin to one another largely at the subcellular level. Notwithstanding, the principal etiology of these neurodegenerative diseases remains unclear, ergo, voluminous research is centered on decoding its mystery which aims to provide therapeutic advancement which could offer a better quality of life.

Numerous empirical evidences have supported the intake of lipoic acid as nutraceutical since it is capable to evoke an array of cellular and molecular mechanisms in the prevention and treatment of chronic diseases. For instance, lipoic acid has been advocated as a potential anti-inflammatory agent for Alzheimer's disease (AD) (Maczurek et al., 2008), neuroprotective in various neuronal models (Fujita et al., 2008; Zaitone et al., 2012) and modulator of various genes involved in cell survival, inflammation and oxidative stress (Bitar et al., 2010; Salinthone et al., 2008). Recently, R-Lipoic acid was demonstrated to mitigate the accumulation of 4-hydroxy-2-nonenal (4HNE) and protect the retinal neurons against oxidative stress by inducing HO-1 activity-dependent mechanism (Koriyama et al., 2013a). The antioxidative property of (R)-(+)-α-lipoic acid has been suggested to contribute to its neuroprotective activities, albeit its conclusive molecular mechanisms are still not completely understood. In this study, (R)-(+)-α-lipoic acid was demonstrated to protect the NG108-15 cells against H_2O_2-induced cell death through PI3K-Akt/GSK-3β pathway which then suppressed the production of pro-inflammatory cytokines following GSK-3β mediated inhibition of NF-κB p65.

5.2 Literature review

Neurodegenerative diseases can be defined as the hereditary and sporadic conditions which can be characterized through selective loss of anatomically or physiologically related neuronal systems. Neuronal apoptosis can occurs in two common pathways either by an extrinsic or intrinsic pathway and each are characterized by the alterations in the mitochondria, the endoplasmic reticulum and cascade activation of caspases. In nervous system, the expression of individual Bcl-2 family members and their contribution to survival or death are developmentally regulated (Akhtar et al., 2004). Mitochondria play a critical role in cellular survival and apoptotic death which is highly regulated by the pro-apoptotic and anti-apoptotic members in Bcl-2 family. Study has reported that Bcl-2 maintained the mitochondrial integrity, while Bax destroyed the mitochondrial integrity and caused loss of mitochondrial membrane potential (Sharpe et al., 2004).

PI3K-Akt pathway has risen to prominence since it is involved in neuronal survival and confers protection against drug-induced damage in neurons (Wang et al., 2010b). PI3K protects neurons through its antiapoptotic effects mediated by its central downstream target, Akt, which regulates expression of apoptosis-related genes (Franke, 2008). The mammalian target of rapamycin (mTOR), a main downstream of PI3K-AKT is a protein kinase which functions as a sensor of extracellular signals that positively regulates cell growth and protects cells from apoptosis (Wrighton, 2013). GSK-3β is a protein that negatively regulated by PI3K-Akt pathway (Medina, Garrido, & Wandosell, 2011) where its inhibition confers neuroprotection by interfering with the intrinsic apoptosis signaling of the upstream apoptosome and production of inflammatory factors (Lei et al., 2011; Wang, Huang, Chen, Chang, & Kuo, 2010a).

ROS such as hydrogen peroxide (H_2O_2) is a highly reactive and diffusible free radical which can trigger various signaling pathways and redox-sensitive signal transduction that modulates cellular mechanisms for cell proliferation and survival, death and immune responses by inducing the production of pro-inflammatory factors such as cytokines through the activation of NF-κB (Nakajima & Kitamura, 2013; Nakano et al., 2006; Siomek, 2012). Furthermore, excessive secretion of pro-inflammatory cytokines has been established to be a crucial initiator of aberrant inflammatory responses in neurodegenerative diseases as they are transcriptionally and reciprocally regulated by NF-κB (Minami et al., 2006; Starossom et al., 2012). Cytokines are multitude of proteins which originate from the immune system and endogenously produced by the brain cells including microglia and neurons. In healthy adult brain, cytokines are expressed constitutively at low levels, however their production can be augmented in response to neuronal injury or infection (Helmy et al., 2011; Steinman, 2013; Verma, Nakaoke, Dohgu, & Banks, 2006) making them as key inflammatory factors in the brain (Cardona et al., 2008). Cytokines exert their biological functions through interactions with receptors on plasma membrane expressed by neuronal and glial cells which then activate diverse intracellular phosphorylative cascades responsible for apoptosis, cell survival, inflammatory responses and neural pathfinding (Deverman & Patterson, 2009; Rubio-Perez & Morillas-Ruiz, 2012).

5.3 Materials and method

5.3.1 Cell culture and material

The NG108-15 cells were obtained from ATCC, cultured in DMEM medium and conditioned at 5% CO_2 moist atmosphere at 37°C. The media were supplemented with 10% (v/v) heat inactivated FBS, 2% penicillin/streptomycin, 1% amphotericin B. The media were supplemented with HAT media supplement (50x) Hybri-Max and the cells were primed for 3-4 weeks prior to its use. (R)-(+)-α-lipoic acid (Sigma Aldirch) was dissolved in DMSO to produce stock solution with concentration of 20mM. The cells were pretreated with various concentrations of (R)-(+)-α-lipoic acid for 2 h prior to H_2O_2 exposure for 24 h. Cells exposed to vehicle alone (10% FBS DMEM, DMSO ≤ 0.5% v/v) were used as the control group. Morphological analysis was performed with a fluorescence microscope (Leica Inverted Fluorescence Microscope, DM16000B) and flow cytometric analysis was acquired with BD Accuri C6 Flow Cytometry and BD CFlow® Software.

5.3.2 Cell viability assay

The neuroprotective effects of (R)-(+)-α-lipoic acid against H_2O_2-induced apoptosis in NG108-15 cells was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cells were plated at a total density of $5x10^3$ cells/well in a 96-well plate. The cells were left to adhere for 48 h and treated with (R)-(+)-α-Lipoic acid (2 h) prior to H_2O_2 (400 µM) exposure for 24 h. 20 µL MTT solution (Sigma Aldrich) was added into each well and incubated at 37°C for another 4 h. The absorbance was measured by using a microplate reader (ASYS UVM340) at 570 nm (reference wavelength: 650 nm). The percentage of cell viability was calculated based on the formula below:

Viability = [absorbance of treated cells (As)/absorbance of control cells (AC)] x 100%

5.3.3 Nuclear morphological analysis

Cells (0.5 x 10^6 cells) were plated in a 60 mm culture dish and left to adhere. The cells were pretreated with (R)-(+)-α-Lipoic acid (12.5 - 50 μM) for 2 h prior to H_2O_2 exposure. The cells were then harvested and rinsed with PBS. Hoechst 33342 (5 μg/mL, Sigma Aldrich) was added, followed with PI dye (2.5 μg/mL) into each sample. The cells were incubated for 15 min and observed by fluorescence microscope. Cells that exhibited intense blue fluorescence were considered to be at an early apoptosis stage. In contrast, cells that were dual stained with H33342 and PI were considered to be at the late apoptosis stage.

5.3.4 Determination of glutathione/glutathione disulfide (GSH/GSSG) ratio

Cells (1 x 10^4 cells/well) were plated into a 96-well plate and left to adhere. Cells were subjected to the designated treatments and after 24 h, the solution in the well plate was discarded. 50 μL of Total Glutathione Lysis Reagent or Oxidized Glutathione Lysis Reagent were (Promega, Madison, WI, USA) added to each well and the plate was shaken for 5 min at room temperature. 50 μL of Luciferin Generation Reagent was added to all wells and incubated at room temperature for 30 min. 100 μL of Luciferin Detection Reagent was added and luminescence signal was then read. The GSH and GSSG concentration in each sample was calculated based on the GSH and GSSG standard curve.

5.3.5 Mitochondrial membrane potential (*Δψm*)

Alterations in *Δψm* were quantified by using a JC-1 kit according to the manufacturer's protocol (Stratagene, CA, USA). The analysis of the (R)-(+)-α-Lipoic acid effect on *Δψm* was evaluated as described previously (Chan et al., 2012). The green and red fluorescence signals were detected by flow cytometer. JC-1 aggregates which emitted red fluorescence signal within the intact mitochondria of healthy cells was detected in

the FL-2 channel, whereas JC-1 monomers with green fluorescence signal in the cytoplasm of apoptotic cells was detected in the FL-1 channel.

5.3.6 Measurement of intracellular ROS level

Cellular oxidative stress induced upon exposure to H_2O_2 and its modulation by (R)-(+)-α-Lipoic acid was measured using fluorescent probe 2',7'-dichlorfluorescein-diacetate (DCFH-DA). Cells were plated and subjected to similar pretreatment of LA prior to the addition of H_2O_2. Cells were harvested, rinsed and incubated with 10 µM of DCFH-DA for 30 min at 37°C in cell loading medium. The fluorescence signal was measured using flow cytometer.

5.3.7 Western blot analysis

NG108-15 cells were plated on 60 mm culture and subjected to the designated treatment. Following this, the cells were then collected, rinsed with PBS and centrifuged. Protein extracts were prepared by lysing the cells RIPA buffer (Thermo Fisher Scientific, Rockford, IL, USA). The protein concentration was determined using the Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA). 20 µg of each protein sample was electrophoresed on 7 - 15% SDS–PAGE. Proteins on the gel were transferred onto a nitrocellulose membrane and blocked with 5% BSA. The membrane was then probed with the following primary antibodies anti-β-actin, anti-Bax,anti-Bcl-2, anti-Bcl-xL, anti-pAkt (Thr308, Ser473), anti-Akt, anti-pGSK-3β(Ser9), anti-GSK-3β, anti-pmTOR,anti-mTOR, anti-rictor and anti-raptor (Cell Signaling Technology, Danvers, MA, USA) (Cell Signaling, USA) at 4°C overnight followed by appropriate HRP-conjugated secondary antibody. Protein bands were visualized by enhanced chemiluminescence (ECL) substrate solution kit (Bio-Rad) for 1 minute. Proteins were quantified with Bio-1D software as a proportion of the signal of the housekeeping protein band (β-actin).

5.3.8 NF-κB p65 translocation assay

Cells were plated onto coverslips and subject to designated treatments. After treatment, cells were gently rinsed and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Cells were rinsed with PBS and blocked with blocking buffer (5% BSA, 0.5% Triton X-100 in PBS) for an hour. The cells were then incubated with rabbit anti-NF-κβ p65 (Cell Signaling, USA) for overnight followed with incubation with FITC-conjugated goat anti-rabbit secondary antibody (Pierce Antibodies, Rockford, IL, USA) for 2 h, respectively. The cells were washed and then analyzed by using a fluorescence microscope.

5.3.9 Cytokines measurement

The production of cytokines was measured by using Cytokine Bead Array (BD Biosciences, San Jose, CA, USA). In brief, following the designated treatment, 50 µL of culture medium was collected and mixed with the cytokine capture beads. The mixture was then mixed with PE-conjugated detection antibodies to form sandwich complexes. The desired cytokines (IL-6, IL-10 and TNF-α) were then measured by flow cytometer and data were analyzed by using FCAP Array™ software with comparison to the mouse cytokines standards' curves. To further validate the NF-κβ – cytokines regulation, the cells were pretreated with ethyl 3,4-dihydroxycinnamate (10 µM) prior to H_2O_2 exposure and the production of cytokines was then measured and compared with the H_2O_2-treated group.

5.3.10 Data analysis

All the experimental data are expressed in mean ± standard error (S.E.). Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) and followed by Dunnett's test. P-values below 0.01 were considered to be statistically significant.

5.4 Results

5.4.1 (R)-(+)-α-lipoic acid protected against H_2O_2-induced cell death

Neuroprotective effects of (R)-(+)-α-lipoic acid against H_2O_2-induced cell death were evaluated with the MTT assay. Exposure to H_2O_2 (400 µM) significantly reduced the cell viability to 48.95 ± 2.82 %, therefore H_2O_2 at this concentration was selected for MTT assay. Following pretreatment with (R)-(+)-α-lipoic acid (3.12 - 100 µM), the cell viability was significantly increased in a dose-dependent manner. (R)-(+)-α-lipoic acid at 3.12 µM elicited the initial significant protection and increased the cell viability to 56.41 ± 1.87%. It was observed that pretreatment with varying concentrations (R)-(+)-α-lipoic acid (6.25, 12.5, 25 and 50 µM) significantly increased the cell viability to 67.06 ± 2.77%, 69.89 ± 1.72%, 74.95 ± 2.15% and 82.25 ± 2.97%, respectively. The highest protection level was achieved following pretreatment with (R)-(+)-α-lipoic acid at 100 µM which significantly increased the cell viability up to 89.37 ± 2.01% (Figure 5.1). Additionally, treatment with (R)-(+)-α-lipoic acid alone did not induce toxicity in NG108-15 cells (data not shown). More importantly, (R)-(+)-α-lipoic acid was shown to protect the NG108-15 cells effectively against H_2O_2 exposure in a dose-dependent manner.

5.4.2 (R)-(+)-α-lipoic acid suppressed nuclear morphological changes

Control untreated cells displayed evenly stained disseminated chromatin with intact nuclei with low blue fluorescence (Figure 5.2). H_2O_2-treated cells exhibited typical morphological characteristics of early apoptotic cells, which include condensed chromatin with fragmented DNA and the presence of apoptotic bodies with intense blue fluorescence (arrow 1, Figure 5.2). In addition, nuclei of NG108-15 cells were also dual-stained with both Hoechst 33342/PI indicating late apoptotic cell population (arrow 2, Figure 5.2). However, pretreatment with (R)-(+)-α-lipoic acid notably abated the H_2O_2-induced nuclear morphological changes where the number of cells with

nuclear condensation, fragmentation and the presence of apoptotic bodies were significantly decreased.

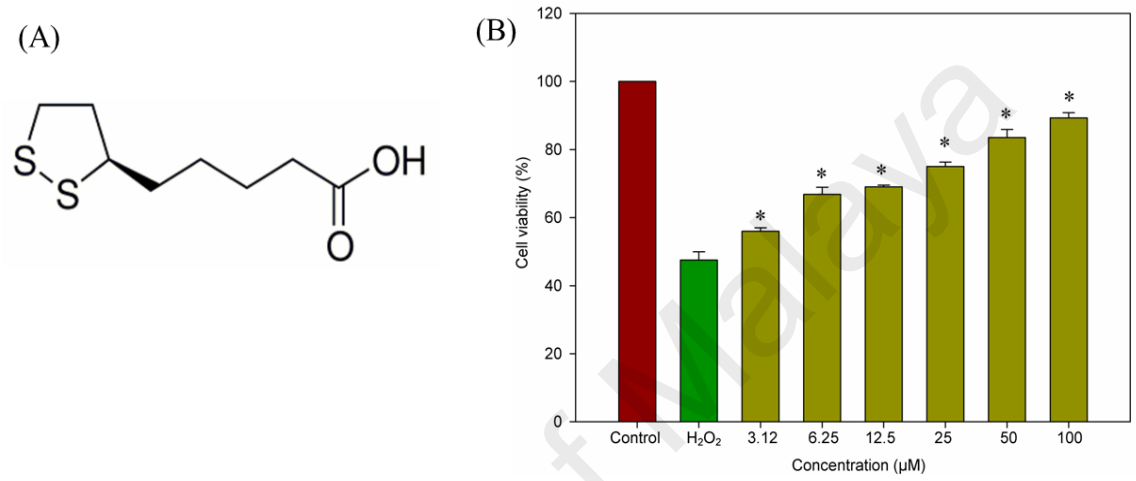

Figure 5.1 Neuroprotective effect of (R)-(+)-α-lipoic acid by MTT cell viability assay. (A) Structure of (R)-(+)-α-Lipoic acid. B) Dose-dependent increase in cell viability following pretreatment with (R)-(+)-α-Lipoic acid prior to H_2O_2 exposure (400 μM) for 24 h. Values are means ± S.E. and the asterisks indicated significantly different value from H_2O_2-treated cells (*P <0.01, n=9).

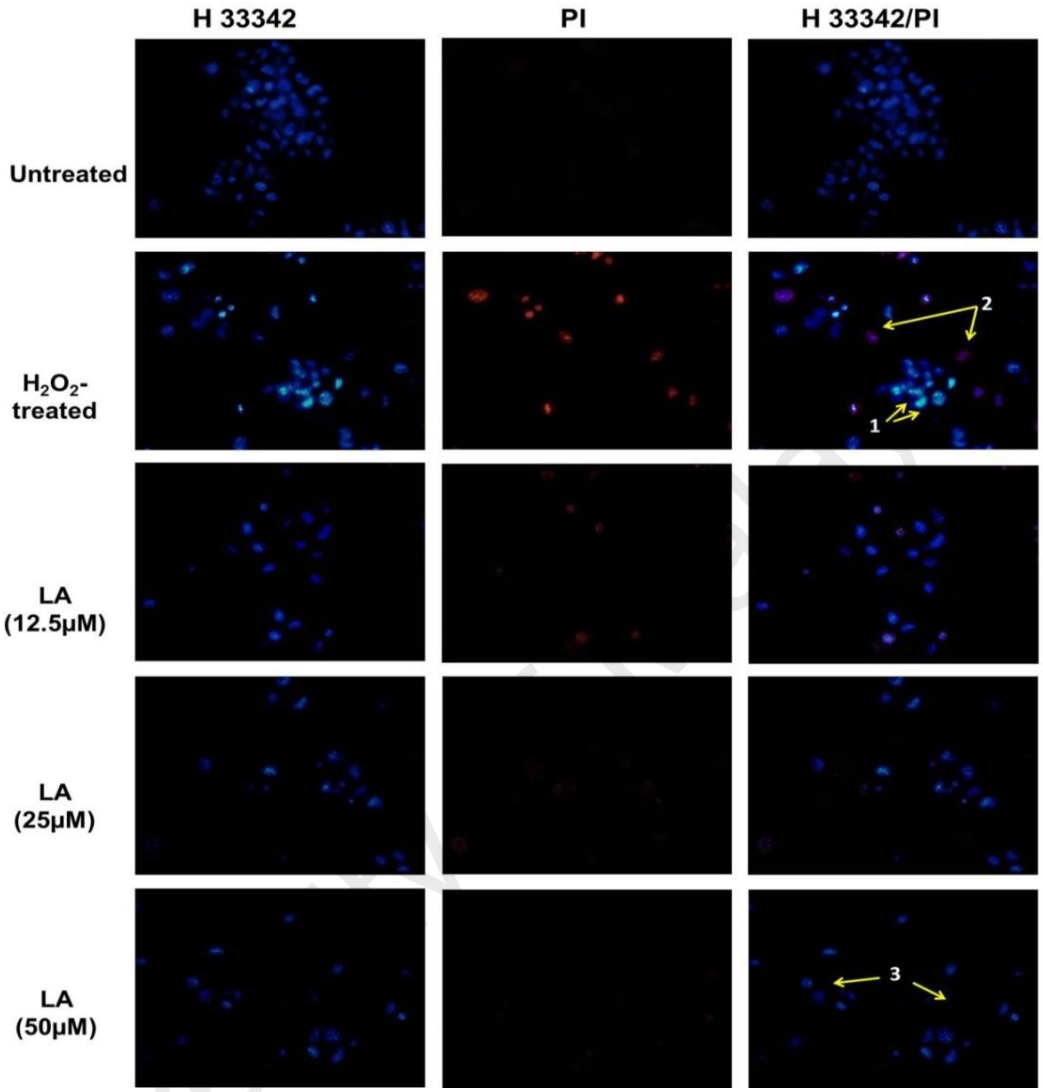

Figure 5.2 (R)-(+)-α-lipoic acid (LA) reduced the H_2O_2-induced morphological changes in NG108-15 cells. Control untreated cell nuclei were evenly stained. Images were taken with an average of 100 cells per field. Nuclei of H_2O_2-treated cells were stained with intense blue fluorescence indicating early apoptotic cells (arrow 1). The nuclei of late apoptotic cells were dual stained with Hoechst 33342/PI (arrow 2). Pretreatment with (R)-(+)-α-lipoic acid at 12.5 - 50 μM significantly abated the apoptotic cells but increased the number of evenly stained nuclei indicating viable cells (arrow 3). (Magnification 100X)

5.4.3 (R)-(+)-α-lipoic acid attenuated intracellular ROS by augmenting the GSH/GSSG ratio

GSH is an important intracellular antioxidant in the mammalian redox system which detoxifies H_2O_2 and keeps the thiol groups of proteins in the reduced state. Following oxidative stress, GSH will be oxidized into a dimer of two of the peptide elements linked by a disulfide bond between the cysteines known as oxidized glutathione (GSSG). The GSH/GSSG ratio has been used as an indicator of cell health and oxidative stress in neurodegenerative studies. Hence, the ratio of GSH/GSSG in the cells was investigated following pretreatment with (R)-(+)-α-lipoic acid and exposure to H_2O_2. The intracellular GSH level was determined from a standard curve using GSH standard (0.05-1000 nM) while the GSSG level was calculated based on GSSG standard curve derived from GSH standard curve according to the manufacturer's protocol. H_2O_2 exposure significantly decreased (Figure 5.3(a)) the reduced GSH level but elevated the GSSG level which led to the reduction of GSH/GSSG ratio to 1.15 fold as compared to 60.13 fold in untreated cells, (Figure 5.3(b)). However, pretreatment with (R)-(+)-α-lipoic acid (12.5 – 50 μM) significantly increased the reduced GSH level and decreased the GSSG level yielding 2.5, 5.09 and 27.14-fold increase of GSH/GSSG in a dose-dependent manner when compared to H_2O_2-treated cells (Figure 5.3(b). Following this, the intracellular ROS level in the NG108-15 cells was determined by flow cytometry analysis. It was demonstrated that exposure of NG108-15 cells to H_2O_2 increased the intracellular ROS production significantly as illustrated by the right shift in the histogram (Figure 5.3 (c) and (d)), 4.52–fold when compared to untreated cells. Notably, since pretreatment with (R)-(+)-α-lipoic acid was shown to elevate the GSH/GSSG ratio, it concomitantly suppressed the increase of intracellular ROS level by H_2O_2 in NG108-15 cells as evidenced by the shift to the left in the histogram (Figure 5.3 (c) and (d)).

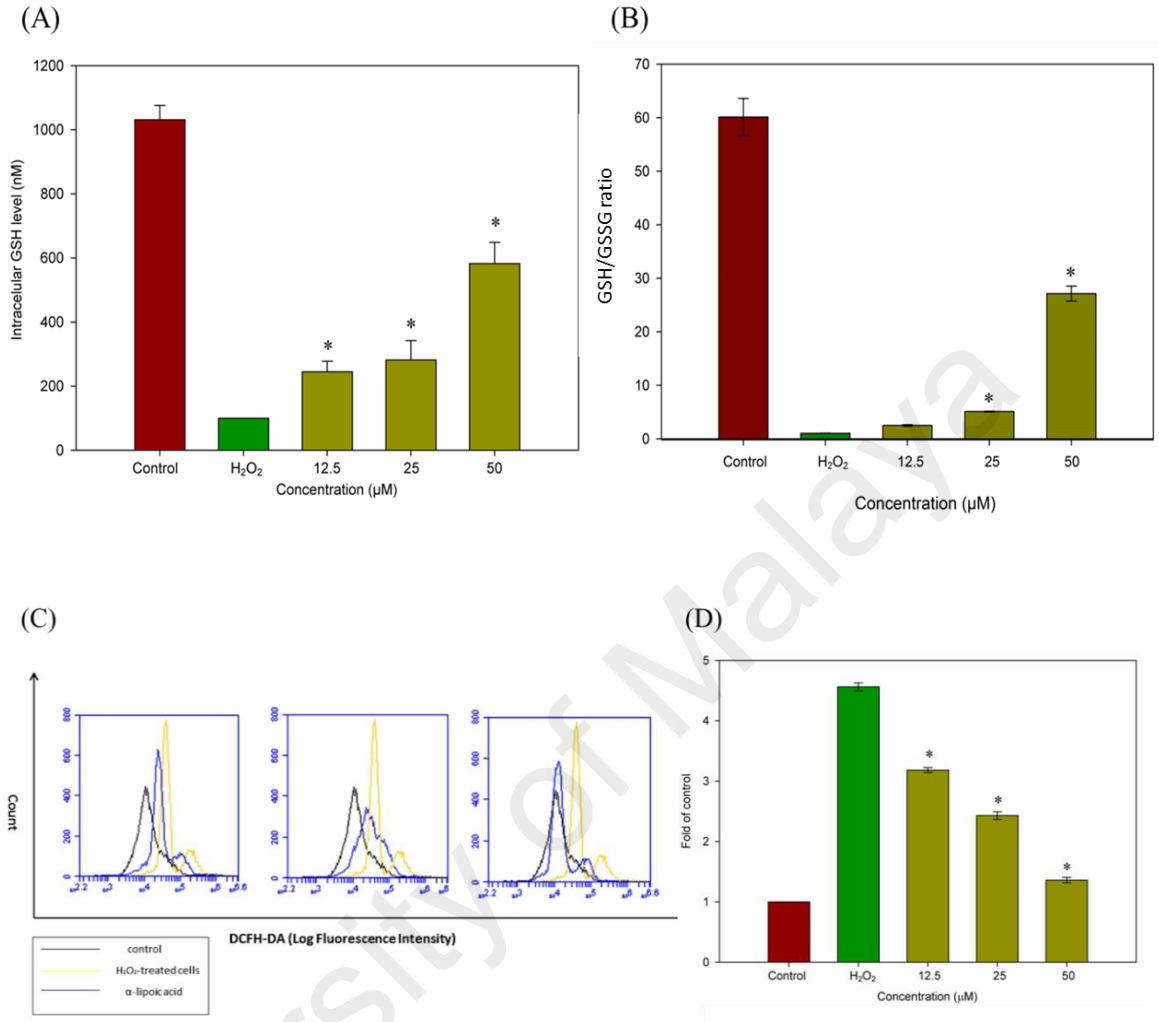

Figure 5.3 The effect of (R)-(+)-α-lipoic acid on GSH/GSSG and ROS levels. (A) Pretreatment with (R)-(+)-α-lipoic acid dose-dependently increased the reduced GSH and attenuated the production of GSSG level following H_2O_2 exposure. (B) Bar chart shows the GSH/GSSG ratio following H_2O_2 exposure and pretreatment with (R)-(+)-α-lipoic acid (C) Flow cytometric analysis demonstrated that (R)-(+)-α-lipoic acid reduced intracellular ROS level (D) Bar chart shows pretreatment with (R)-(+)-α-lipoic acid dose-dependently reduced intracellular ROS level in NG108-15 cells. Values are means ± S.E. and the asterisks indicated significantly different values from the H_2O_2-treated cells (*P < 0.01, n=4).

5.4.4 (R)-(+)-α-lipoic acid inhibited dissipation of *Δψm*

Changes in *Δψm* reflect the alteration of proton gradient across the inner mitochondrial membrane which is one of the key indicators of apoptosis. H_2O_2-treated cells exhibited changes in the fluorescence signal from the upper right quadrant to the lower quadrant which leads to a lower red fluorescence signal (23.09 ± 2.57 %) and higher green fluorescence signal (77.91 ± 1.47 %), indicating the dissipation in *Δψm* (Figure 4.4(a)). Treatment with (R)-(+)-α-lipoic acid significantly reversed dissipation in *Δψm* as shown in Figure 5.4(b). Pretreatment with (R)-(+)-α-lipoic acid at 50 μM suppressed the effect of H_2O_2-induced *Δψm* dissipation, resulting in the partial rescue of *Δψm* as shown by the increase of the upper quadrant to 62.86 ± 1.75 %. Although the inhibition of *Δψm* dissipation was partial, the protective effect against H_2O_2-induced dissipation of *Δψm* was significant. However, this protective effect of (R)-(+)-α-lipoic acid on *Δψm* could be further augmented with treatment at higher concentration (100 μM) since it shown higher protective effect in MTT assay.

5.4.5 (R)-(+)-α-lipoic acid modulated apoptotic proteins and caspase-3 expression

The increase in the ratio of proapoptotic protein to antiapoptotic protein is known to initiate apoptosis cascade via the activation of caspases. The data in Figure 5.5(a) showed that Bax expression was increased significantly in H_2O_2-treated NG108-15 cells. In addition, H_2O_2 reduced both Bcl-2 and Bcl-xL expression (Figure 5.5(a)) leading to a significant increase in both Bax/Bcl-2 and Bax/Bcl-xL ratios (Figure 5(b) and (c)), eventually resulting in apoptosis. Conversely, Bcl-2 and Bcl-xL expressions in (R)-(+)-α-lipoic acid-treated cells were elevated (Figure 5.5(a)) while Bax protein expression was suppressed. This led to a significant reduction in Bax/Bcl-2 and Bax/Bcl-xL ratios (Figure 5.5(b) and (c)). To examine the involvement of caspase, the

expression of procaspase-3 and cleaved caspase-3 were analyzed. The results showed that (R)-(+)-α-lipoic acid-treated cells expressed higher procaspase-3 as compared to H_2O_2-treated cells (Figure 5.5(a) and (d)). Moreover, the increase of cleaved caspase-3 in H_2O_2-treated cells was reduced following treatment with (R)-(+)-α-lipoic acid (Figure 5.5(a) and (d)).

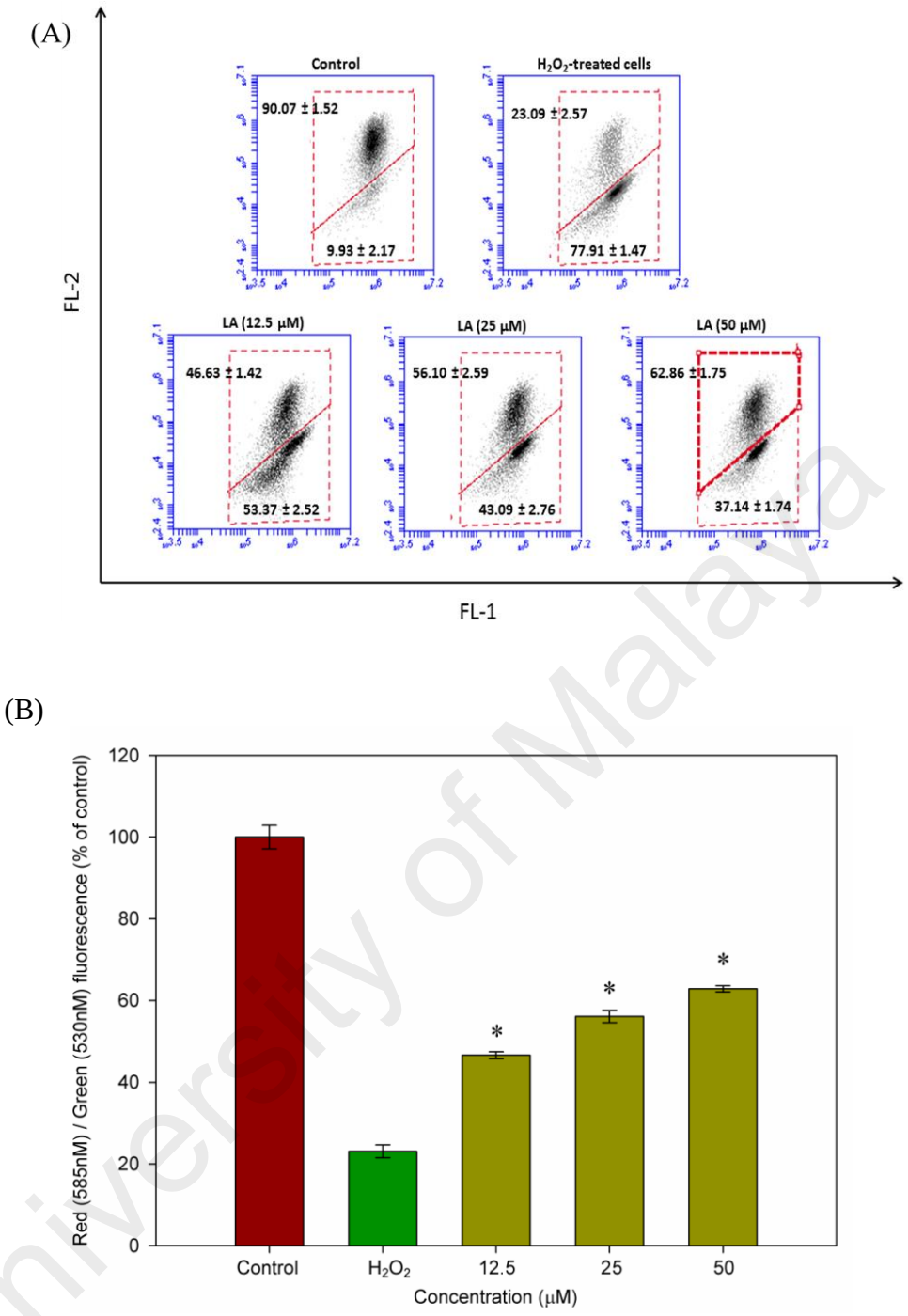

Figure 5.4 Flow cytometric analysis of *Δψm* in NG108-15 cells. (A) Representative dot plots of JC-1 aggregates (FL-2 Red fluorescence) versus JC-1 monomers (FL-1 green fluorescence). 20 000 events were collected for each of the sample. (B) Bar chart showing the percentages of relative fluorescence intensity of *Δψm* in control, H_2O_2-only and varying pretreatment concentrations of (R)-(+)-α-lipoic acid (LA) in NG108-15 cells. Values are means ± S.E. and the asterisk indicated significantly different values from H_2O_2-treated cells (*$P < 0.01$, n=4).

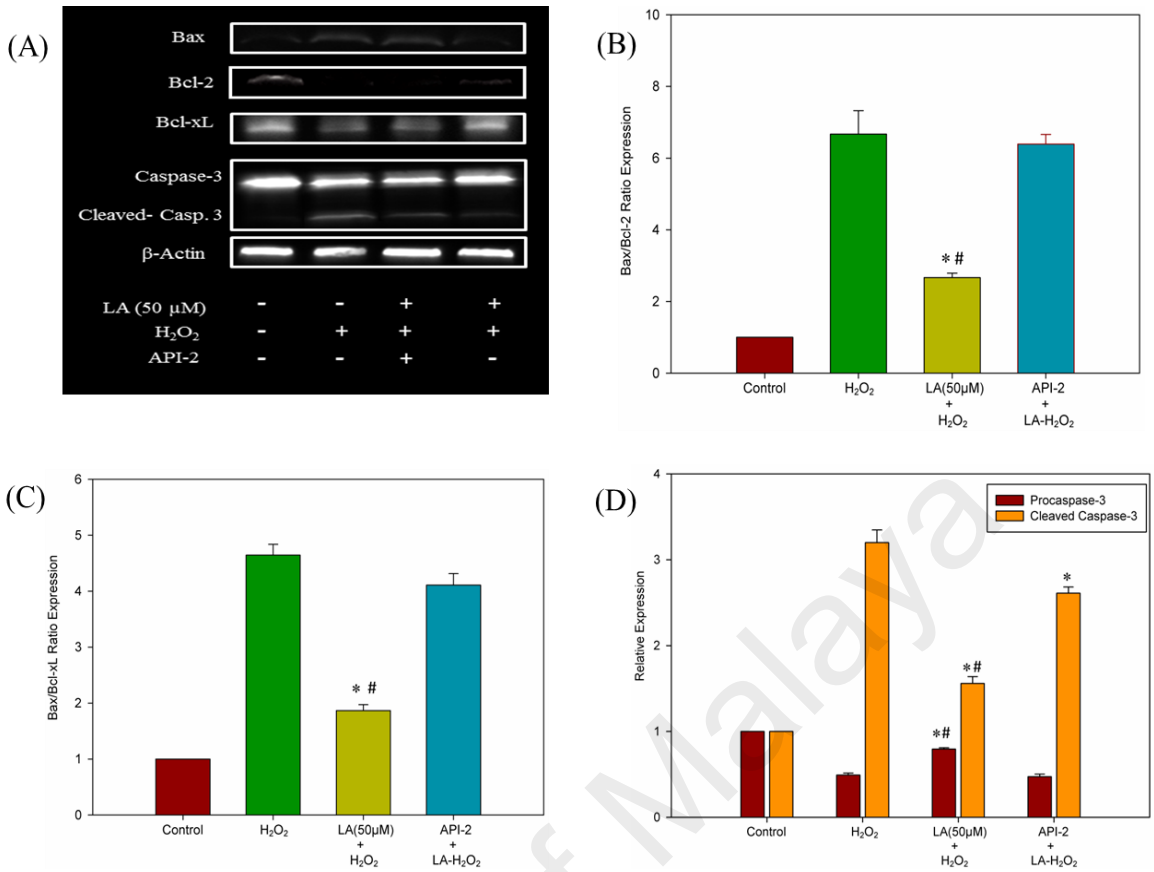

Figure 5.5 (A) Western blot analysis of (R)-(+)-α-lipoic acid (LA) on Bax, Bcl-2, Bcl-xL, procaspase-3 and cleaved caspase-3 expression in NG108-15 cells. The apoptotic protein expression was validated by pretreatment of API-2 (10 µM) or Wortmannin (0.5 nM) for 1 h prior to (R)-(+)-α-lipoic acid (LA) and H_2O_2 exposure. (B) Pretreatment with (R)-(+)-α-lipoic acid reduced Bax/Bcl-2 ratio. (C) Pretreatment with (R)-(+)-α-lipoic acid attenuated Bax/Bcl-xL ratio. (D) Bar chart represents procaspase-3 expression and cleaved caspase-3. β-actin was used as housekeeping protein. Values are means ± S.E. (n=4). *P<0.01 versus H_2O_2, $^{\#}$P<0.01 versus API-2, significantly different as shown.

5.4.6 Pretreatment with (R)-(+)-α-lipoic acid induced PI3K-Akt activation via mTORC1 and mTORC2

PI3K-Akt pathway plays imperative roles in the regulation of neuronal survival. There was a significant reduction in the activation/phosphorylation of Akt (Thr308 and Ser473) in H_2O_2-treated cells when compared to untreated cells, but this change was prevented pretreatment with (R)-(+)-α-lipoic acid (Figure 5.6(a)). The increase in Akt (Thr308 and Ser473) phosphorylation level (Figure 5.6(a)) led to an increase of both pAkt(Thr308)/Akt and pAkt(Ser473)/Akt protein ratios (Figure 5.6(b) and (c)). Full activation of Akt can be achieved via phosphorylation by the mammalian target of rapamycin complex 2 (mTORC2) which contains the regulatory proteins Rictor and mTOR. (R)-(+)-α-lipoic acid treatment was shown to augment phosphorylated mTOR protein expression (Figure 5.6(d)) significantly as compared to H_2O_2-treated cells, resulting in an increase of pmTOR/mTOR protein ratio (Figure 5.6(e)). Furthermore, (R)-(+)-α-lipoic acid treatment upregulated Rictor expression (Figure 5.6(f)) as compared to H_2O_2-treated cells. More interestingly, (R)-(+)-α-lipoic acid also elevated the Raptor expression which implied the activation of mTORC1 complex following Akt activation (Figure 5.6(g)). To validate the PI3K-Akt activation, an inhibitor of PI3K (Wortmannin) and Akt (API-2) was added prior to (R)-(+)-α-lipoic acid addition. Addition of Wortmannin and API-2 significantly attenuated neuroprotective activity of (R)-(+)-α-lipoic acid as shown by the MTT cell viability assay (Figure 5.6(h)) and reduced pAkt protein expression (Figure 5.6(a-c) which suggested the involvement of PI3K-Akt pathway.

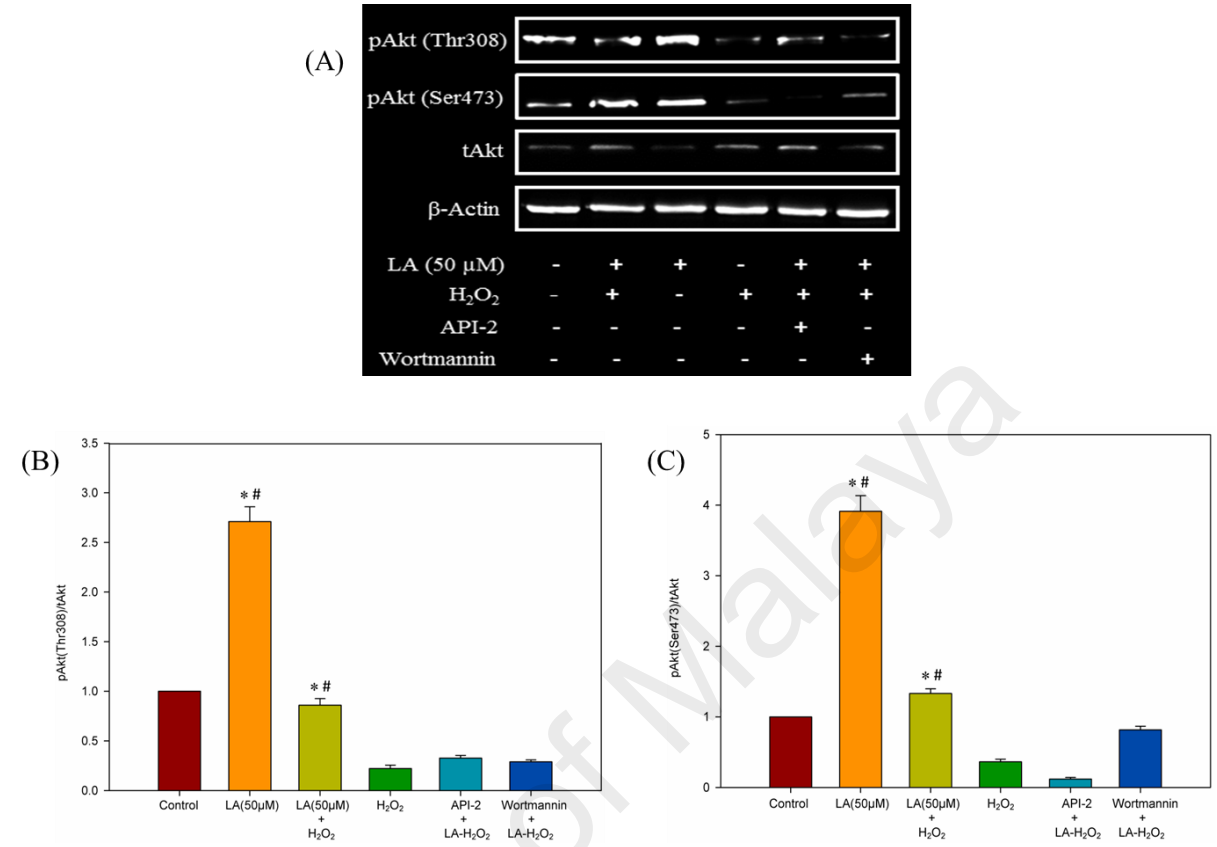

Figure 5.6 (A) Western blot analysis of (R)-(+)-α-Lipoic acid (LA) on pAktTh308, pAktSer473, total Akt in the absence or presence of H_2O_2 after 2 h. The PI3K-Akt activation was validated by pretreatment of API-2 (10 μM) or Wortmannin (0.5 nM) for 1 h prior to (R)-(+)-α-Lipoic acid (LA) and H_2O_2 exposure. β-actin was used as housekeeping protein. Values are means ± S.E. (n=3). *P<0.01 versus H_2O_2, $^{\#}$P<0.05 versus API-2 or Wortmannin, significantly different as shown.

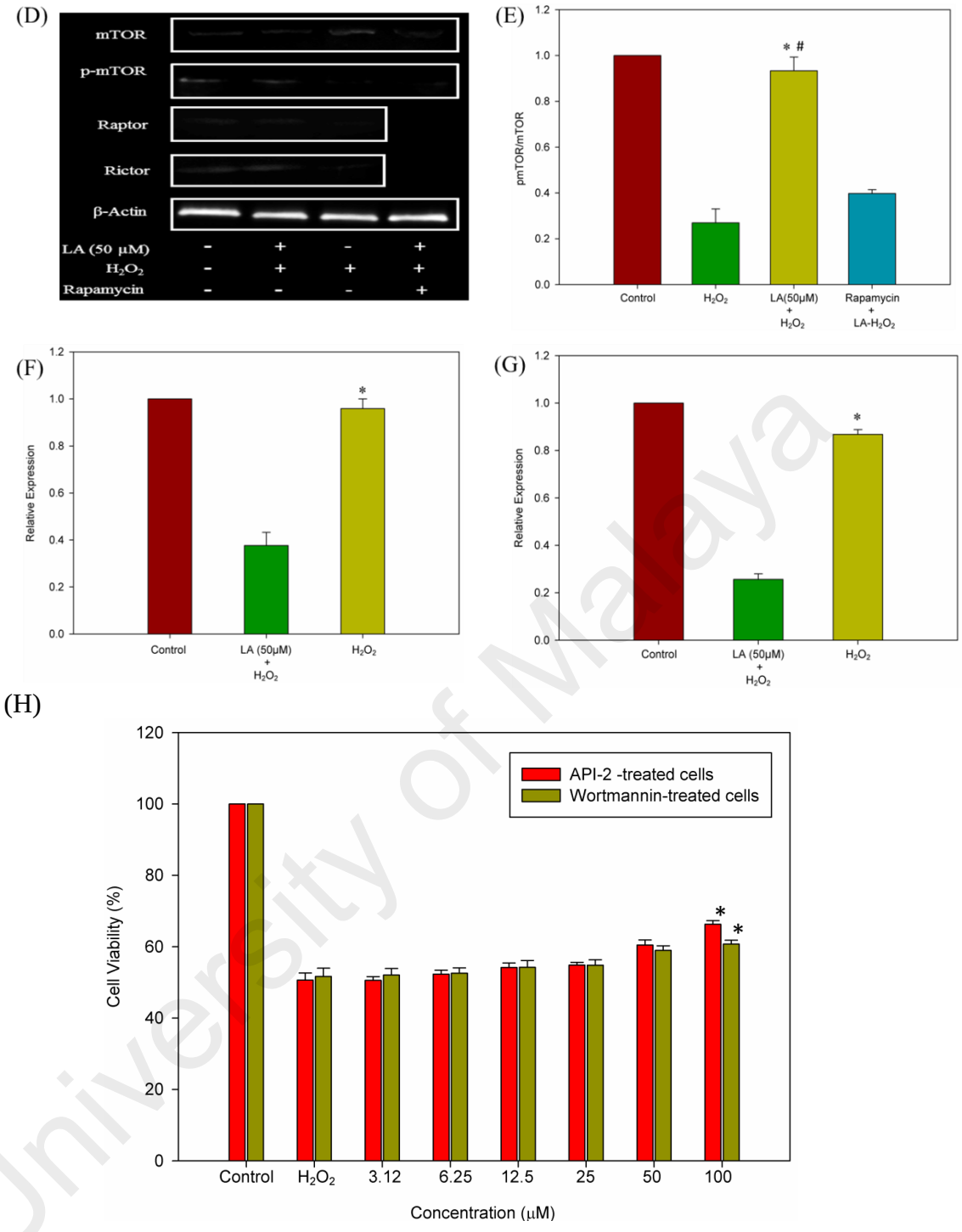

Figure 5.6, continued. (B) pAkt(Ser473)/tAkt ratio (C) pAkt(Th308)/tAkt ratio (D) Western blot analysis of (R)-(+)-α-lipoic acid (LA) on mTOR, rictor and raptor expression in NG108-15 cells. (E) pmTOR/mTOR ratio (F) rictor (G) raptor (H) Viability of NG108-15 cells pretreated with (R)-(+)-α-Lipoic acid (LA) and API-2 or Wortmannin. β-actin was used as housekeeping protein. Values are means ± S.E. (n=3). *P<0.01 versus H2O2, #P<0.05 versus API-2 or Wortmannin, significantly different as shown.

5.4.7 Inactivation of GSK-3β by (R)-(+)-α-lipoic acid inhibited NF-κB p65 translocation and modulated cytokines production

To further corroborate the participation of Akt downstream targets other than mTORC2, the regulation of GSK-3β was then determined. Based on this, the activation of PI3K-Akt by (R)-(+)-α-lipoic acid was postulated to mediate the inhibition of GSK-3β followed by suppression of NF-κB p65 translocation. A significant increase in GSK-3β expression accompanied by a reduction of pGSK-3β(Ser9) was observed in H_2O_2-treated cells (Figure 5.7(a)), but these changes were prevented when the NG108-15 cells were pretreated with (R)-(+)-α-lipoic acid (Figure 5.7(b)). Pretreatment with (R)-(+)-α-lipoic acid suppressed the translocation of NF-κB p65. Similarly, the immunofluorescence data substantiated that treatment with (R)-(+)-α-lipoic acid inhibited the translocation of NF-κB p65 (Figure 5.7(c)). Next, the effects of (R)-(+)-α-lipoic acid on the production of major inflammatory cytokines (TNF-α, IL-6 and IL-10) were analyzed since GSK-3β and NF-κB activation is associated cytokines production. Following H_2O_2 exposure, TNF-α and IL-6 production was augmented significantly as compared to control untreated cells (Figure 5.7(d) and (e)). Moreover, these changes were accompanied by the significant reduction of anti-inflammatory cytokine, IL-10. Intriguingly, upon inhibition of GSK-3β and suppression of NF-κB p65, (R)-(+)-α-lipoic acid aggrandized the production of IL-10 which resulted in further diminution of TNF-α and IL-6 as compared to H_2O_2-treated cells. To further validate this, the NG108-15 cells were pretreated with ethyl 3,4-dihydroxycinnamate, a specific NF-κB inhibitor. It was observed that pretreatment with ethyl 3,4-dihydroxycinnamate suppressed the production of IL-6 and TNF-α as well as IL-10 as compared to the control and H_2O_2-treated groups. Moreover, the production of IL-10 was significantly greater following pretreatment with (R)-(+)-α-lipoic acid which may suggest that (R)-(+)-α-lipoic acid induced neuroprotection against H_2O_2 by modulating both anti- and proinflammatory cytokines through their reciprocal regulation with NF-κB.

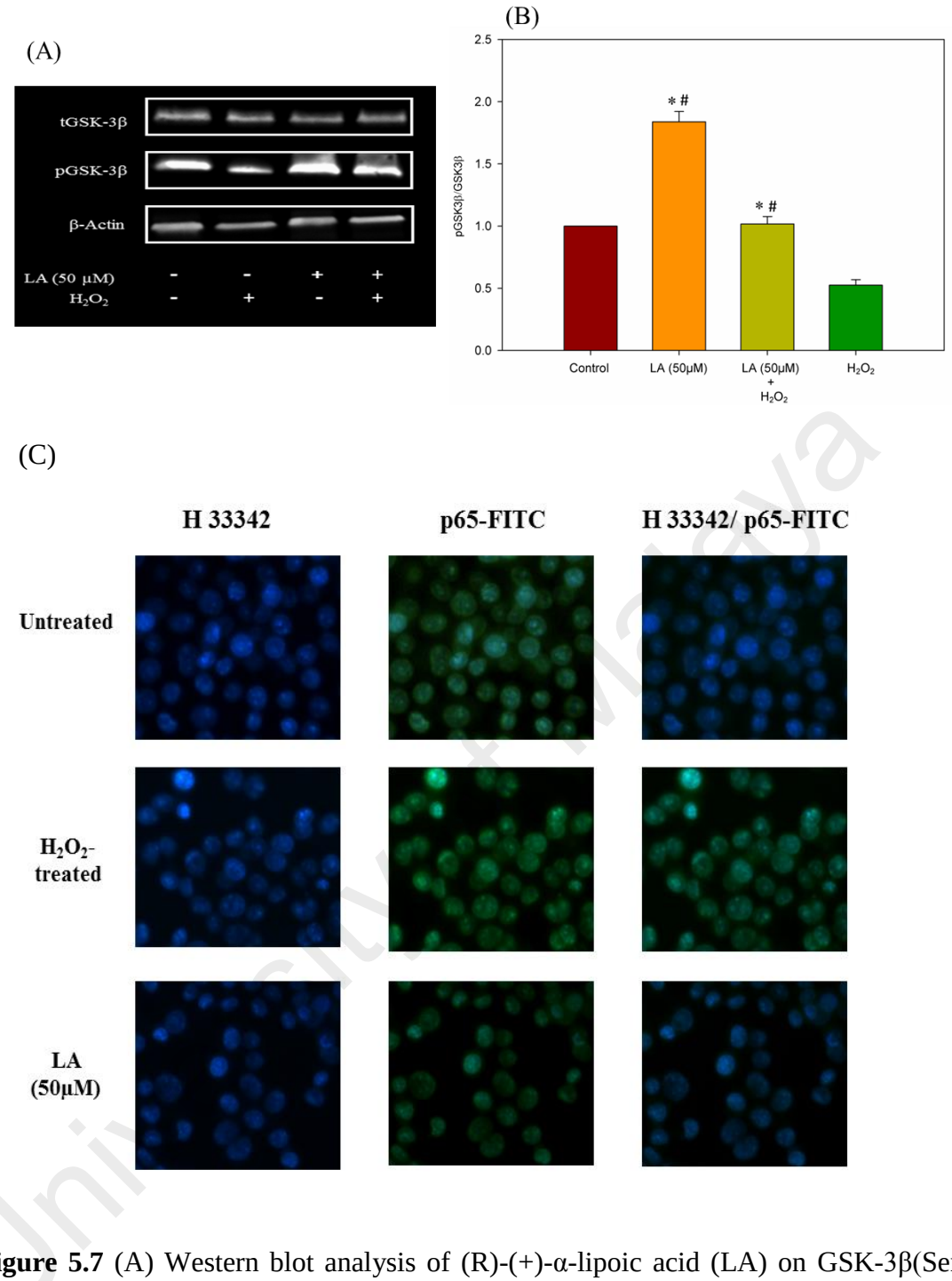

Figure 5.7 (A) Western blot analysis of (R)-(+)-α-lipoic acid (LA) on GSK-3β(Ser9) and NF-κB p65 translocation. (B) GSK-3β(Ser9)/tGSK-3β ratio (C) Cells were pretreated with (R)-(+)-α-lipoic acid (LA; 50 μM) for 2 h before H_2O_2 addition for 4 h. Cells were stained with H33342 for nuclear visualization (blue) and NF-κB p65 translocation was visualised by fluorescence microscopy after immunofluorescence staining with anti-NF-κB p65 antibody and a FITC-labelled anti-rabbit IgG antibody (Magnification: 200x) Values are means ± S.E and the asterisk indicated significantly different values from H_2O_2-treated cells (*P<0.01, n=4).

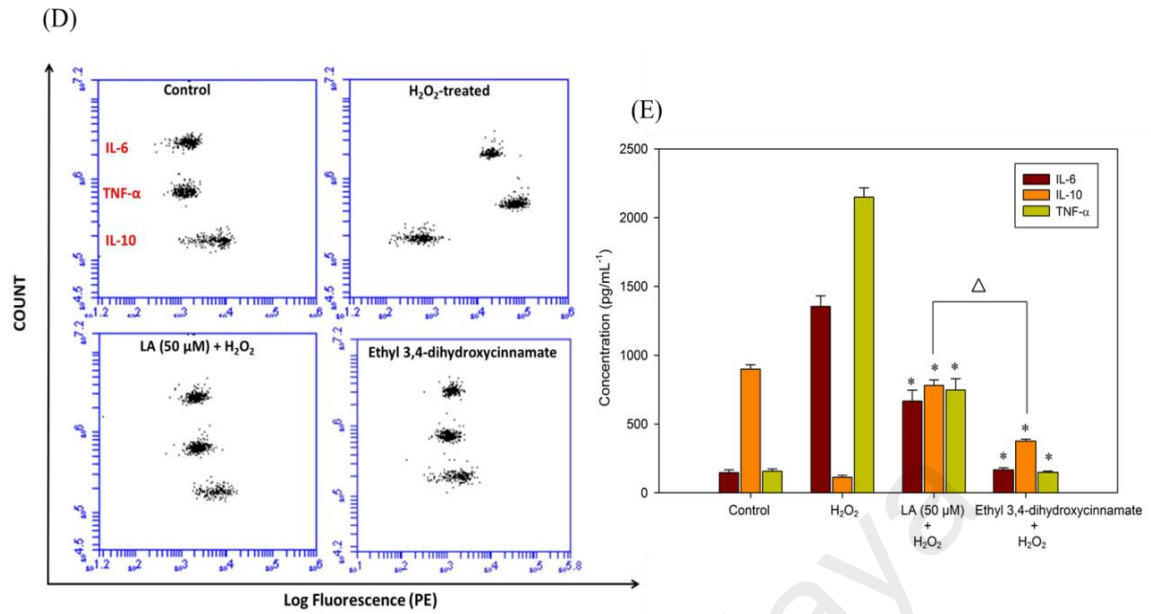

Figure 5.7, continued. (D) Pretreatment with (R)-(+)-α-lipoic acid (LA; 50 µM) and ethyl 3,4-dihydroxycinnamate (10 µM) modulated the cytokines production following H_2O_2 exposure. Representative flow cytometric dot plots (2100 events were collected according to BD Biosciences TH1/TH2/TH17 CBA protocol) of TNF-α, IL-6 and IL-10. (E) Bar chart represents the concentration (pg/mL) of TNF-α, IL-6 and IL-10 modulated by (R)-(+)-α-lipoic acid and ethyl 3,4-dihydroxycinnamate following H_2O_2 exposure after 24 h. (R)-(+)-α-lipoic acid treatment increased the IL-10 significantly as compared to ethyl 3,4-dihydroxycinnamate and H_2O_2-treated cells. Similar to ethyl 3,4-dihydroxycinnamate, treatment with (R)-(+)-α-lipoic acid suppressed the production of TNF-α and IL-6 as compared to H_2O_2-treated cells. Values are means ± S.E and the asterisk indicated significantly different values from H_2O_2-treated cells (*$P<0.05$, n=4). $^{\Delta}P<0.01$, significantly different as shown.

5.5 Discussion

Cogent evidence has supported the multitude use of lipoic acid for metabolic syndrome (Ziegler et al., 2006) and its associated-diseases(Çakatay, Kayalı, Sivas, & Tekeli, 2005), as potential cognitive enhancer(Manda, Ueno, & Anzai, 2008) and an important modulator of various inflammatory signaling pathways(Salinthone et al., 2008) making it a beneficial nutraceutical with multifarious properties. In addition, lipoic acid has been proposed as a potential anti-inflammatory drug since it interferes with the pathogenesis of Alzheimer's disease (Maczurek et al., 2008; Shay et al., 2009). MTT cell viability assay revealed that (R)-(+)-α-lipoic acid exhibited a dose-dependent neuroprotective effect against H_2O_2-induced cell death in NG108-15 cells (Figure 5.1). Morphological changes in H_2O_2-induced apoptosis in NG108-15 cells were characterized by the condensation of chromatin, the shrinkage of cell nuclei and apoptotic bodies appearances stained with intense blue fluorescence. Conversely, these morphological alterations were evidently abrogated following (R)-(+)-α-lipoic acid treatment as compared to the H_2O_2-treated cells. These findings first suggested the evasion of cell death by (R)-(+)-α-lipoic acid through mitigation of apoptosis in NG108-15 cells.

Neurons are considered to be highly susceptible to oxidative stress as they are intrinsically ill-equipped to protect against an increase in ROS due to low levels of antioxidants relative to other mammalian cell types (Uttara, Singh, Zamboni, & Mahajan, 2009). Excessive production of ROS may lead to oxidative stress, loss of cell functions and ultimately cell death (Preiser, 2012). H_2O_2 exposure caused a shift to the right in the histogram (Figure 5.3 (c) and (d)) indicating an elevation in intracellular ROS production. In contrast, pretreatment with (R)-(+)-α-lipoic acid caused a reduction in the intracellular ROS formation which then suggested that (R)-(+)-α-lipoic acid protected the NG108-15 cells through its antioxidative activity. The current paradigm

advocates that a decline in intracellular GSH below a threshold level constitutes an apoptotic signal that will initiate neuronal cell death signaling (Johnson, Wilson-Delfosse, & Mieyal, 2012). H_2O_2 exposure caused a significant reduction in the reduced GSH level and elevated the GSSG level in NG108-15 cells (Figure 5.3 (a) and (b)) leading to a significant diminution in the ratio of GSH/GSSG. The reduction in the ratio of GSH/GSSG indicated oxidative stress in the cellular redox system which eventually led to neuronal cell death. Pretreatment with (R)-(+)-α-lipoic acid dose-dependently markedly increased the ratio of GSH/GSSG (Figure 4.3 (b)) which further corroborated that (R)-(+)-α-lipoic acid protected the NG108-15 by ameloriating the cellular redox state via its antioxidant activity. GSH depletion exacerbates the dissipation of $\Delta\psi m$ which leads to mitochondrial release of apoptogenic factors such as cytochrome *c* and hence, initiating the activation of caspases (Circu & Aw, 2010). However, (R)-(+)-α-lipoic acid treatment significantly mitigated the dissipation of $\Delta\psi m$ (Figure 5.4) which further established the ability of (R)-(+)-α-lipoic acid via its antioxidative property to ameliorate the $\Delta\psi m$ after consequential loss of potential due to H_2O_2 exposure. Collectively, through its capability to preserve the mitochondrial membrane integrity, (R)-(+)-α-lipoic acid directly suppressed the cytochrome *c* release and formation of apoptosome which eventually led to disruption of the apoptotic signaling cascades.

Research in the recent epochs has seen unprecedented advanced understanding of PI3K-Akt signaling that plays a central role in cell survival and proliferation by mediating the antiapoptosis and antiinflammatory mechanisms (Matsuda et al., 2013). This includes the activation of mTOR and its downstream effectors which affect a number of cellular processes including proliferation and motility (Betz & Hall, 2013; Yap et al., 2008). It was observed that treatment with (R)-(+)-α-lipoic acid suppressed H_2O_2-induced inhibition of Akt (Thr308/Ser473) phosphorylation thereby resulting in an increased pAkt/Akt ratio (Figure 5.6 (b) and (c)) suggesting that (R)-(+)-α-lipoic acid

exerted its neuroprotective effect through the activation of PI3K-Akt pathway. The addition of both API-2 and Wortmannin further reduced the (R)-(+)-α-lipoic acid-treated cell viability (Figure 6H) and thereby abrogated the neuroprotective effect of (R)-(+)-α-lipoic acid in NG108-15 cells which further substantiated the involvement of PI3K-Akt. Furthermore, Western blot analysis demonstrated that addition of API-2 and Wortmannin suppressed (R)-(+)-α-lipoic acid-induced Akt (Thr308/Ser473) protein phosphorylation (Figure 5.6 (a-c)), thus indicating the neuroprotective effect of (R)-(+)-α-lipoic acid through activation of PI3K-Akt pathway.

The full activation of Akt requires phosphorylation at Ser473 with the aid of mTORC2 (Sarbassov, Guertin, Ali, & Sabatini, 2005). (R)-(+)-α-lipoic acid-treated cells exhibited an elevation of both mTOR and Rictor expression (Figure 4.6 (e) and (f)), suggesting that administration with (R)-(+)-α-lipoic acid induced full activation of Akt protein. The activated Akt in turn phosphorylates to activate its diverse downstream protein substrates including mTORC1 component, mTOR and Raptor (Figure 5.6 (g)). The suppression of neuronal apoptosis through mTOR mainly depends on Akt activation, which occurs in several stages such as fostering cell survival through preserving $\Delta\psi m$, caspase inactivation and promoting inflammatory cell inactivation (Bhaskar et al., 2009). Moreover, the ability of mTORC1 to inhibit apoptotic neuronal cell death is associated with Akt activation (Guertin et al., 2006) and inflammatory cells have been shown to capitulate apoptosis during oxidative stress following Akt and mTOR inhibition (Maiese, Chong, Shang, & Wang, 2013; Shang et al., 2012). In this context, the data indicated that (R)-(+)-α-lipoic acid induced full activation of Akt through its regulation of mTORC1 and mTORC2 components that confer protection against H_2O_2 insult. In addition, PI3K-Akt protects neurons from damage (Matsuda et al., 2013; Shao et al., 2011) and regulates survival in neurons by regulating the Bcl-2 family of proteins (Malla et al., 2010). H_2O_2 exposure elevated the expression of Bax

protein and attenuated Bcl-2 and Bcl-xL which resulted in the increase of Bax/Bcl-2 and Bax/Bcl-xL ratios (Figure 5.5 (b) and (c)) and thus, led to conditions that favor apoptosis. However, this was prevented by (R)-(+)-α-lipoic acid treatment which increased both Bcl-2 and Bcl-xL protein expression leading to the decrease in the Bax/Bcl-2 and Bax/Bcl-xL ratios which may suppressed the release of cytochrome *c*, formation of apoptosome and caspase activation.

Akt confers neuronal survival by inactivating its other target, GSK-3β through phosphorylation. GSK-3β is a multitasked kinase that plays imperative roles in various signaling pathways since it targets and regulates important metabolic and signaling proteins, structural proteins and transcription factors (Kaidanovich-Beilin & Woodgett, 2011). GSK-3β is tightly regulated by Akt through phosphorylation of its Ser9, rendering it inactive. Inhibition of GSK-3β was reported to be protective against a plethora of neuronal insults (Lei et al., 2011), thus implying that its suppression confers neuronal survival. In accordance with this, activation of Akt was accompanied by an increased phosphorylation of GSK-3β when compared to H_2O_2-treated cells (Figure 5.7 (a) and (b)), suggesting that (R)-(+)-α-lipoic acid prevented cell death through inactivation of GSK-3β. GSK-3β is a well-known upstream mediator of caspase-3 although the precise mechanism by which GSK-3β facilitates neuronal death remains unclear (Jo et al., 2011). Caspase-3 is activated in the apoptotic cell both by extrinsic and intrinsic pathways. Following a decrease in Bax/Bcl-2 and Bax/Bcl-xL ratios and inhibition of GSK-3β, (R)-(+)-α-lipoic acid suppressed the cleavage of procaspase-3, into cleaved caspase-3 (Figure 5. 5 (d)). These findings highlighted that (R)-(+)-α-lipoic acid protected the NG108-15 cells via activation of PI3K-Akt which led to subsequent inhibition of proapoptotic Bax and GSK-3β accompanied by activation of antiapoptotic Bcl-2 and Bcl-xL which concomitantly suppressed caspase-3 activation.

Suppression of GSK-3β can result in both activation and suppression of an array of transcription factors that are critical in regulating the production of pro- and anti-inflammatory cytokines by enhancing the binding of cAMP response element binding protein (CREB) while inhibiting the binding of NF-κB p65 to the nuclear coactivator CREB binding protein (CBP) (Martin et al., 2005). Inhibition of GSK-3β was reported to suppress TLR-mediated inflammatory responses. For instance, GSK-3β inactivation inhibited the translocation of NF-κB p65 into cell nuclei and the production of inflammatory factors in various neuronal models (Huang et al., 2009b; Wang et al., 2010a). NF-κB is an important regulator of DNA transcription that regulates the expression of genes involved in innate and adaptive immune responses, cell survival and cell proliferation (Ghosh & Hayden, 2012; Hayden & Ghosh, 2011). Furthermore, NF-κB and pro-inflammatory cytokines can regulate each other and play a major role in inflammatory processes (Tourniaire et al., 2013; Wang et al., 2010a). However, the aberrant activation of NF-κB can result in excessive neuroinflammation that may lead to neuronal cell death (Minami et al., 2006; Starossom et al., 2012).

Hydrogen peroxide escalated the intracellular ROS level, thereby induced oxidative stress which was followed by NF-κB p65 activation in NG108-15 cells. Moreover, oxidative stress was reported to promote NF-κB p65 activation through phosphorylation of IκB (Kabe, Ando, Hirao, Yoshida, & Handa, 2005; Morgan & Liu, 2011) and addition of ROS scavenger compound subsequently abated the transcriptional activity and translocation of NF-κB p65 (Fang, Yang, Yang, & Chen, 2013) which concomitantly suppressed the production of TNF-α and IL-6. The data demonstrated that H_2O_2 exposure significantly induced NF-κB p65 nuclear translocation (Figure 5.7 (c)) as well as production of pro-inflammatory cytokines (Figure 5.7 (d) and (e)) as compared to untreated cells. However, the increase in the production of pro-inflammatory cytokines was suppressed following pretreatment with NF-κB inhibitor,

ethyl 3,4-dihydroxycinnamate as compared to both control untreated and H_2O_2-treated cells. Following GSK-3β inhibition, (R)-(+)-α-lipoic acid suppressed the NF-κB p65 nuclear translocation and subsequently decreased the production of pro-inflammatory cytokines. Similar to ethyl 3,4-dihydroxycinnamate, (R)-(+)-α-lipoic acid inhibited H_2O_2-induced production of TNF-α and IL-6 which are NF-κB-regulated cytokines (Figure 5.7 (d) and (e)). Cytokines are well-known major regulators of the inflammatory responses in the brain since they constitute a cardinal role in the nerve cell response towards brain infection or injury (Kriz, 2006b). Although neuronal release of cytokines aims to eradicate threats, nevertheless, when they are secreted in imbalanced fashion, the homeostatic balance will be broken and the damage will persist leading to excessive production of pro-inflammatory cytokines that promote progressive neuronal cell death. TNF-α and IL-6 are usually among the first pro-inflammatory cytokines to be expressed following neuronal injury and their overproduction is highly detrimental on neuronal cells (Correale & Villa, 2004). TNF-α and IL-6 are major components of neuroinflammation in Alzheimer's and Parkinson's diseases since direct intra-parenchymal injection of TNF-α induced dopaminergic neuron degeneration and inhibition of both TNF-α and IL-6 prevented preplaque amyloid aggregation, thereby prevented the acceleration of Alzheimer's-like pathology (Chen et al., 2012c; McAlpine et al., 2009). Furthermore, chronic inhibition of soluble TNF ameliorated dopaminergic neurons death by interfering with the PI3K-Akt signaling and activation of caspase-3 (Martinez, Chen, Bandyopadhyay, Merrill, & Tansey, 2012). Moreover, TNF-α was shown to promote neuronal cell death in neuroblastoma cells through the activation of Fas Ligand/Fas receptor (FasL/Fas) in the absence of glial cells (Álvarez, Blanco, Fresno, & Munoz-Fernandez, 2011).

Alzheimer's disease (AD) is the most common cause of dementia, is pathologically characterized by senile plaques (SPs) and neurofibrillary tangles (NFTs)

in the brain. In particular, the senile plaques are extracellular aggregates of amyloid beta-peptide (Aβ) that are cleaved from the amyloid precursor protein (APP) (Mattson et al., 2004). Moreover, studies with transgenic animals have revealed that neuroinflammation accelerates amyloidogenesis (the formation of Aβ) in the process of cerebral amyloid deposition (Fang et al., 2010; Nichol et al., 2008). Studies found that various cytokines (TNF-α, IFN-γ IL-1β), chemokines (MCP-1, MIP-α), free radicals and eicosanoids such as prostaglandins are important mediators of neuroinflammatory responses in Alzheimer's disease. Furthermore, intraperitoneal administration of LPS induces an immediate, strong and persistent up-regulation of proinflammatory cytokines and chemokines primarily from activated microglia which can affect the neurobiological condition. Systemic administration of a single dose of LPS through IP injections induces neuroinflammation that can persist for 10 months resulting in the progressive loss of neurons in the hippocampal region. Single and three repeated IP injections of LPS increased the production of cytokines and chemokines granulocyte colony-stimulating factor, IL- 1α, IL-6, MCP-1, MIP-1α and TNF α, which are responsible to accelerate amyloidogenesis via up-regulation of the β-secretase level and activity in the mouse brain (Mouton et al., 2012). The present findings in combination with the anti-inflammatory effects of (R)-(+)-α-lipoic acid and mesuagenin c in Chapter 3, further accentuate the neuroprotection strategy by suppressing the aberrant inflammation that produces toxic factors and oxidative stress which can induce neuronal cell death. Therefore, the data in this thesis (Chapter 3 – 5), could provide the missing link between Aβ, amyloidogenesis and Alzheimer's pathogenesis. However, further studies of this work should evaluate the formation and clearance of Aβ in the neuro-glia system with CCL21 knockdown as an attempt to gauge this missing link.

In a more intricate inflammatory network, various anti- and pro-inflammatory cytokines such as TNF-α, IL-1β, IL-2, IL-6 and IL-10 are produced simultaneously and

may stimulate or suppress each other during inflammation (Guyon et al., 2008). Inhibition of GSK-3(α/β) mediated by mTORC1 through PI3K-Akt was reported to increase the production of a potent anti-inflammatory cytokine, IL-10 that is capable of inhibiting the production of pro-inflammatory cytokines (TNF-α , IL-1, IL-6, IL-12) (Huang et al., 2009b; Wang et al., 2011a). Furthermore, IL-10 has been reported to suppress neuroinflammation and production of pro-inflammatory cytokines and inflammatory mediators through its inhibitory activity on the NF-κB pathway activation in various cellular model (Dhingra, Sharma, Arora, Slezak, & Singal, 2009; Heyen et al., 2000). Likewise, in the present study (R)-(+)-α-lipoic acid demonstrated an increase in IL-10 production, thereby decreased the production of TNF-α and IL-6 resulting in the suppression of caspase-dependent neuronal apoptosis. Moreover, pretreatment with (R)-(+)-α-lipoic acid was shown to increase the production of IL-10 as compared to ethyl 3,4-dihydroxycinnamate. Even though both ethyl 3,4-dihydroxycinnamate and (R)-(+)-α-lipoic acid suppressed H_2O_2-induced neuroinflammation in NG108-15 cells through suppression of NF-κB p65 translocation, however, (R)-(+)-α-lipoic acid was observed to significantly increase the IL-10 level significantly higher than both H_2O_2 and ethyl 3,4-dihydroxycinnamate-treated cells. This observation may suggest that (R)-(+)-α-lipoic acid induced the production of IL-10 which exerted its antiinflammatory effect by inhibiting the NF-κB activation with concomitant IL-6 and TNF-suppression. The current study clearly demonstrates that (R)-(+)-α-Lipoic acid significantly attenuated H_2O_2-induced overproduction of proinflammatory cytokines by reciprocally stimulating the production of anti-inflammatory cytokine by modulating NF-κβ-cytokines regulation.

5.6 Conclusion

(R)-(+)-α-lipoic acid inhibition of GSK-3β resulted in the suppression of pro-inflammatory cytokines and mitigation of mitochondrial-mediated caspase-dependent neuronal apoptosis. Targeting GSK-3β through PI3K-Akt confers neuronal survival in NG108-15 cells owing to GSK-3β diverse mechanisms which include NF-κB-cytokines regulation. (R)-(+)-α-lipoic acid mitigated the H_2O_2-induced neuronal cell death in NG108-15 cells by suppressing both mitochondrial-mediated caspase-dependent apoptosis and production of pro-inflammatorry cytokines via PI3K-Akt/GSK-3β pathway. These findings exhibited (R)-(+)-α-lipoic acid ability to modulate NF-κB-cytokines expression in neuronal model and highlighted the rational use of (R)-(+)-α-lipoic acid in the intervention of neurodegenerative diseases (Figure 5.8).

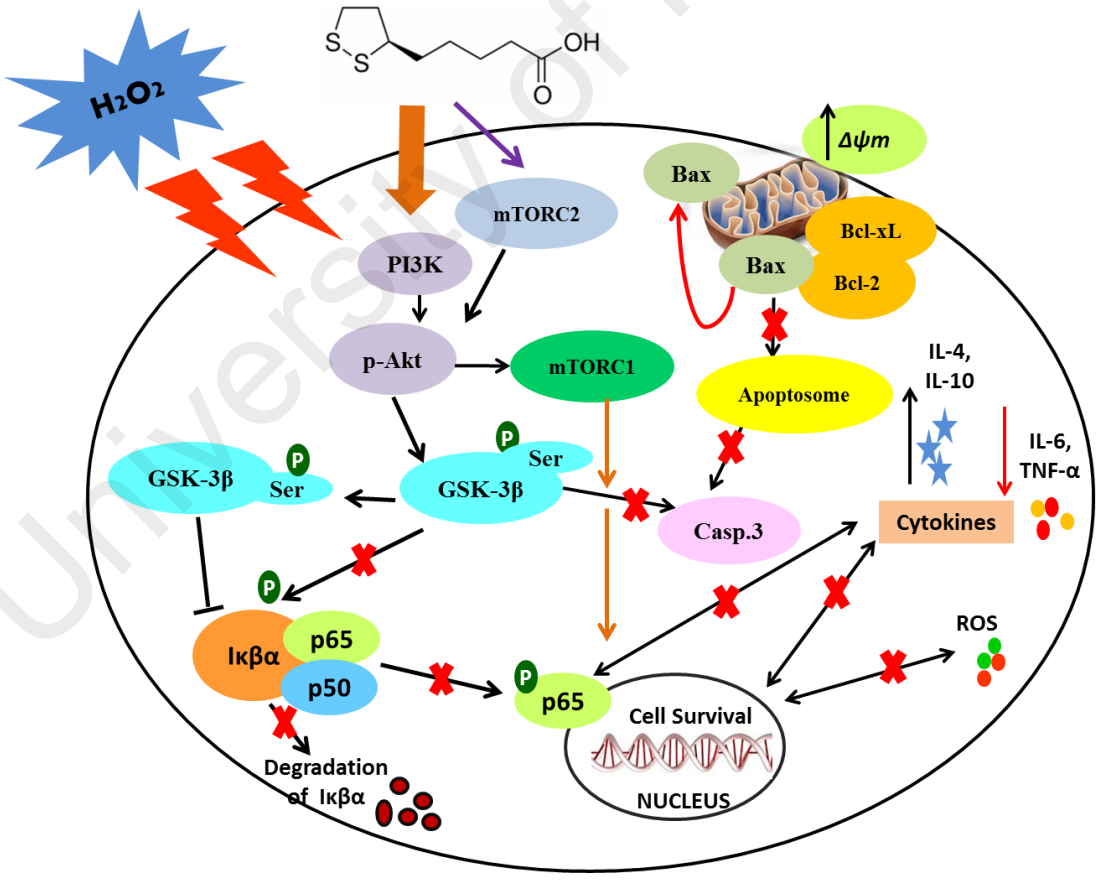

Figure 5.8 Schematic figure of the neuroprotective mechanisms of (R)-(+)-α-Lipoic acid in NG108-15 cells through actiavation of PI3K-Akt/GSK-3β and NF-κB-cytokines suppression.

CHAPTER 6:

Mesuagenin c induced neuritogenesis by modulating cytokines, chemokines and Galectin-1 via PI3K-Akt and ERK1/2 pathways in NG108-15 cells.

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In preparation

6.1 Introduction

Neurons form functional circuits by extending axons and dendrites (collectively called as neurites) that are responsible to connect neurons to other cells via functional synapses. Promotion of neuroregeneration that in central nervous system (CNS) is thought to contribute to neuroprotection leading to neuritogenesis and ultimately, neuroplasticity (Enciu et al., 2011; Short, 2015). Furthermore, a common feature in various neurological disorders is characterized by the extreme neuronal cell loss characterized by massive synapse and neurite damage (Di Giovanni et al., 2005). It was once believed that nerve regeneration in the mammalian CNS was irreversible, once they are loss, nothing will be regenerated. However, research in the last decades has reported that the damaged neurons can regenerate in an active process under the stimulation of neurotrophic factors such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) (Allen, Watson, Shoemark, Barua, & Patel, 2013; Fournier & Strittmatter, 2001). Therefore, the concept of neuroregeneration through the reconstruction of the neuronal and synaptic networks is found to be necessary for the recovery of injured CNS (Enciu et al., 2011).

Various neurotrophic factors possess the ability to prevent synapse loss and neurite damage that are beneficial for neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Nevertheless, the administration of neurotrophic factors has met various challenging issues such as their negligible and limited entry through the blood brain barrier (BBB) and destabilization by peripheral peptidases (Dakas et al., 2013). In some instances, neurotrophic substances were used in combination with natural products that elicit neuritogenic activity to promote neuroregeneration and neuritogenesis (Guo et al., 2006). Despite this combination approach, it only delays the deterioration of the nervous system by reversing the damages. However, when the CNS is under the exposure of insults that promote massive neuronal damage, the

neuroregenerative activity of the neutrophic factors is not sufficient in protecting the system. Therefore, the use of natural products that possess neuroprotective, immunomodulatory and neuritogenic properties would be extremely beneficial in the treatment of a various neuronal disorders of the human nervous system.

6.2 Literature Review

The neuritogenesis can be characterized by the sprouting and growth of axons or dendrites and synapse formation. It is responsible in strengthening of the synapses, to promote neuronal survival as well as for neuronal path finding and the formation of synaptic connections during development (Min et al., 2006). Neuronal cell survival and neuritogenesis can be regulated by two major growth factor receptor downstream cascades, the phosphatidylinositol-3-kinase-Akt (PI3K-Akt) pathway and Ras-mitogen-activated protein kinase (Ras-MAPK) pathway. The serine/threonine kinase Akt or protein kinase B (PKB) has been well documented to promote neuronal survival and to mediate several key aspects of neuritogenesis, including neurite elongation, branching and calibre (Brazil & Hemming., 2001). It is well known that Akt is the major downstream effector of the phosphatidylinositol 3-kinase (PI3K) signaling pathway and rapidly recruited to the plasma membrane where it binds to the Trk or GPCRs following its activation by PI3K. Extracellular stimuli such as neurotophofic factors (NGF, BDNF, IGF) can induce the activation of Akt by binding to the Trk (Huang & Reichardt., 2003) and hence allowing Akt to mediate its diverse cellular signaling by targeting its various downstream susbstrates such as ranscription factors forkhead/FOXO (Brunet et al, 1999), NF-κB (Kane et al., 1999), GSK-3β, mTOR, peripherin and girdin. mTOR activation by Akt has been demonstrated to induce promotes popaminergic neuronal ifferentiation on human neural stem cells (Lee et al., 2015). NF-κB has been proposed to be involved in the synaptic plasticity where it is found to be present in synaptic terminals and can be activated locally at those sites in

response to synaptic transmission (Sestan et al., 1999). NF-κB is activated in association with long-term potentiation of synaptic transmission (LTP), a process believed to be a cellular mechanism of learning and memory (Salama-Cohen et al., 2005).

Other than PI3K-Akt pathway, neuritogenesis is also regulated by the extracellular signal regulated kinases 1 and 2 (ERK1/2) in response to a variety of extracellular stimuli. In the nervous system, ERK1/2 is crucial for neuronal differentiation, plasticity and may also modulate neuronal survival (Adams & Sweatt, 2002). Contrary to this, ERK1/2 activation may act as a defensive mechanism that helps to compensate for the deleterious effects of a damaging insult in damaged cells (Hetman & Gozdz, 2004). ERK1 or p44 mitogen activated protein kinase (MAP kinase) and ERK2 (p42 MAP kinase) are closely related protein kinases of the MAP kinase family (Pearson et al., 2001). ERK1/2 is regulated by dual phosphorylation at the Thr/Tyr residues of the ERK1/2 activation domain that is carried out by MAP kinase kinase 1/2 (MKK1/2) (Pearson et al., 2001). The activation of ERK activation also enhances the production of several neurotrophic factors such as basic fibroblast growth factor, NGF and BDNF that are responsible in promoting neuritogenesis (Chen, Lin, Cimprich, & Meyer, 2012a; Cortese, Barrientos, Maier, & Patterson, 2011; Zhang et al., 2012). For example, BDNF stimulates the neuronal differentiation in primary hippocampal neurons and human umnbilical cord through the activation of ERK (Lim et al., 2011; Luo et al., 2015).

6.3 Materials and Method

6.3.1 Cell culture and materials

The NG108-15 was obtained from the American Type Culture Collection (ATCC). The cells were cultured in DMEM medium and conditioned at 5% CO_2 moist atmosphere at

37°C. The media were supplemented with 10% (v/v) heat inactivated FBS, 2% penicillin/streptomycin, 1% amphotericin B. In addition, the media for NG108-15 cells were supplemented with HAT media supplement (50x) Hybri-Max and the cells were primed for 3-4 weeks prior to its use. The media were filter sterilized by using µm filter membrane. Mesuagenin c was isolated via a neuroprotective-assay guided and fractionation method. Morphological analysis was performed with a fluorescence microscope (Leica Inverted Fluorescence Microscope, DM16000B) and flow cytometric analysis was acquired with BD Accuri C6 Flow Cytometry and BD CFlow® Software. Mesuagenin c was dissolved in DMSO to produce a stock solution of 5 mM. The cells were then pretreated with various concentrations of mesuagenin c and cells exposed to vehicle alone (1% FBS DMEM, DMSO ≤ 0.5% v/v) were used as the control group.

6.3.2 Cell viability assay

The cytotoxicity of mesuagenin c was evaluated by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mossman, 1983). The NG108-15 cells were raised to confluency, harvested and plated at a total density of 2 x 10^4 cells/well into a 96-well plate. The cells were left to adhere for 24 h and then treated with various concentration of mesuagenin c for 48 h. Following this, MTT solution (20 µL, 5 mg/mL) was added into each well and incubated at 37°C for another 4 h. The media were then removed by gentle aspiration and replenished with DMSO (150 µL) to dissolve the formazan crystals. The amount of formazan product was measured by using a microplate reader (ASYS UVM340) at 570 nm (with a reference wavelength of 650 nm). The percentage of cell viability was calculated based on the formula below:

Viability = [absorbance of treated cells/absorbance of control cells] x 100%

6.3.3 Neuritogenesis assessment of mesuagenin c in NG108-15 Cells

The neurite outgrowth analysis was conducted based on previous protocols (Lozano, Schmidt, & Roach, 1995; Mitchell, Hanson, Quets-Nguyen, Bergeron, & Smith, 2007). The cells were primed by changing the medium to 1% FBS for 4-6 days prior to the neurite outgrowth assay. Cells were plated into at cell density of 2×10^4 cells and were left to adhere overnight. Cells were then treated with different range of concentration of mesuagenin c at 48 h in order to determine the best concentration of the compound that would induce neurite outgrowth without toxicity effect. For comparison purpose, cells were also treated with different concentration of NGF-2.5S from murine submaxillary gland (Promega) which served as positive control. Cells with 1% medium were used as negative control. Cell bearing neurite was scored positive if it possesses polygonal morphology with a thin neurite extension with length greater than twofold the cell body diameter and possessing a terminal growth cone. Cells were photographed with inverted microscope (Leica Inverted Fluorescence Microscope, DM16000B) using phase-contrast objectives. The percentage of neurite-bearing cells were quantified by scoring total number of neurite-bearing cells and total number of viable cells in 10 microscopic fields.

6.3.4 Immunofluorescence staining and microscopy analysis

NG108-15 cells were plated onto 60 mm culture dishes and left to adhere whole night. Cells were then subjected to the designated treatment with mesuagenin c (1 μM). Following treatment, cells were rinsed and fixed in 4% paraformaldehyde in PBS (50 mm, pH 7.4) for 20 min at room temperature. Cell membranes were permeabilized with 0.1% Triton X-100 in PBS (1% BSA, 1% sodium azide, 50 mM, pH 7.4). After washing carefully with PBS, the cell samples were blocked with bloking buffer (3% BSA) and incubated with 1:200 dilution of monoclonal rabbit anti-neurofilament-200 kDa (NF-H) primary antibody for 1 hour (Abcam, USA)). Cells were rinsed and incubated with

FITC-conjugated anti-rabbit secondary antibody (1:500) for another hour at room temperature. Cells were then washed carefully and counter stained with H33342 (1 μM) dye for 1 minute. Cells were then analysed under fluorescence microscopy and images were taken using Leica Inverted Fluorescence Microscope, DM16000B.

6.3.5 Western blot analysis

NG108-15 cells were plated on 60 mm culture and subjected to the designated treatment. Following this, the cells were then collected, rinsed with PBS and centrifuged. Protein extracts were prepared by lysing the cells RIPA buffer (Thermo Fisher Scientific, Rockford, IL, USA). The protein concentration was determined using the Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA). 20 μg of each protein sample was electrophoresed on 7 - 15% SDS–PAGE. Proteins on the gel were transferred onto a nitrocellulose membrane and blocked with 5% BSA. Membrane was then probed with the following primary antibodies: anti-pAkt, anti-Akt, anti-ERK1/2, anti-pERK1/2, anti-IκBα, anti-pIκBα, anti-lamin B, anti-mTOR, anti-pmTOR, anti-NF-κB p65, anti-pNF-κB p6, anti-murine exodus-2(CCL21) and anti-rictor at 4°C overnight. All of the antibodies with exception for anti-murine exodus-2 CCL21 (Peprotech) were purchased from Cell Signaling and diluted at 1:1000. Protein bands were visualized by enhanced chemiluminescence (ECL) substrate solution kit (Bio-Rad) for 1 minute. Proteins were quantified with Bio-1D software as a proportion of the signal of the housekeeping protein band (β-actin and lamin B).

6.3.6 CCL21 knockdown in NG108-15 cells

CCL21/Exodus knockdown was generated by using siRNAs commercially obtained from Life Technology. The siRNAs are available at three different locations, 170, 261 and 373. The cells were transfected with the three different siRNAs at concentration 20 – 30 nM by using Lipofectamine® RNAiMAX in DMEM-serum and antibiotic free

medium. In the control experiments, the cells were transfected with 30 nM of scrambled siRNA. The cells were left to incubate for 4 h at 37°C in a 5% CO_2. Following this, the transfection complexes were removed, cell were washed with PBS twice and incubated with complete DMEM medium (24 - 48 h) for the knockdown analysis.

6.3.7 Real-time quantitative polymerase chain reaction (qPCR) analysis

Total RNA was isolated using RNAqueous-4PCR kit (Applied Biosystem) based on the manufacturer's protocol. CCL21 genes expression were evaluated by one-step SYBR Green relative Q-PCR (RotorGene-6000 System, Qiagen) and normalized to HMBS as reference gene. The reactions were carried out in a total volume of 25 µL using the SensiMix One-Step Kit (Quantace). The PCR amplification conditions for Bax was 40 cycles of 10 seconds at 95 ^{o}C, 45 seconds at 60 ^{o}C and 10 seconds at 72 ^{o}C, whereas Bcl-2 was 40 cycles of 10 seconds at 95 ^{o}C, 45 seconds at 51 ^{o}C and 10 seconds at 72 ^{o}C while for CCL21 was 40 cycles of 10 seconds at 95 ^{o}C, 45 seconds at 45 ^{o}C and 10 seconds at 72 ^{o}C. The fluorescence threshold Ct values were calculated and the ΔCt values were determined using the formula $\Delta Ct = Ct_{CCL21} - Ct_{HMBS}$. The ΔΔCt values were then calculated based on formula ΔΔCt = ΔCt treated - ΔCt untreated. The expression level of CCL21 in the treated cells was measured relative to the level observed in the H_2O_2-treated cells and was quantitated using formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The primer sequences were shown in the Table 6.1 below:

Table 6.1 The primer sequence for CCL21 and HMBS

Genes	Function	Sequence
CCL21	Forward	5'-TGC CTT AAG TAC AGC CAG AAG-3'
	Reverse	5'-TTC CTC AGG GTT TGC ACA TAG-3'
HMBS	Forward	5'-CCG AGC CAA GGA CCA GGA TA-3'
	Reverse	5'-CTC CTT CCA GGT GCC TCA GA-3'

6.3.8 Cytokines and chemokines antibody array

NG108-15 cells were plated in a 6-well plate and left to adhere overnight. The cells were subjected to the designated treatment and the culture media were collected for cytokines and chemokines analysis by using RayBio® Custom Mouse Antibody Array (RayBiotech Inc., Norcross, GA, USA). The total protein concentration for each sample was determined and standardized by using Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). The glass chip was assembled and the sub-array slides were blocked with 100 µL Blocking Buffer for 30 min. The Blocking Buffer was aspirated and 100 µL of sample was added into each sub-array. The arrays with samples were incubated at room temperature for 2 h. The arrays were washed carefully and 70 µL Biotin-conjugated Anti-cytokines was added into each sub-array and left to incubate for 2 h with gentle shaking. Following this, glass chip was washed and 70 µL Streptavidin-Fluor was added into each sub-array for 2 h. The glass chip was then washed, rinsed with deionized water and centrifuged at 1000 rpm for 3 min to remove water droplets. The sub-array slide was sent to the manufacturer for scanning and data extraction. The extracted data was then analyzed by using RayBio® Analysis Tool.

6.3.9 Data analysis

All the experimental data are expressed in mean ± standard error (S.E.). Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) and followed by Dunnett's test. P-values below 0.05 were considered to be statistically significant.

6.4 Results

6.4.1 Toxicity and cell proliferative effects of mesuagenin c

The toxicity and cell proliferative effects of mesuagenin c in NG108-15 cells were assessed by MTT assay after treatment for 48 h. The NG108-15 cell viability increased when treated with mesuagenin c at a low range of concentration (0.1 - 0.75 µM) with a significant increment of cell viability at 0.75 µM. This first indicated that mesuagenin c possesses neuronal proliferative activity at low concentration. Moreover, the NG108-15 cell viability was around 95% when treated with mesuagenin c from 0.1 – 6.25 µM. The initial toxicity of mesuagenin c was detected at 12.5 µM and the IC_{50} level was detected at mesuagenin concentration of 70.4 ± 3.42 µM. This observation indicated that range of concentration of mesuagenin c used for neuritogenic analysis (0.1 - 5 µM) is safe and non-toxic comparing to the IC_{50} value. When comparing this value to Chapter 3 and 4, the exposure to mesuagenin c alone without the presence of toxic insults such as LPS and H_2O_2, the range of mesuagenin c from 0.1 – 5 µM is suggested to be safe since no induction of toxicity and reduction of cell viablity were observed. Moreover, the longer exposure time, up to 48 h limits the safety range of mesuagenin c as compared to the higher dose used against LPS and H_2O_2 in chapter 3 and 4.

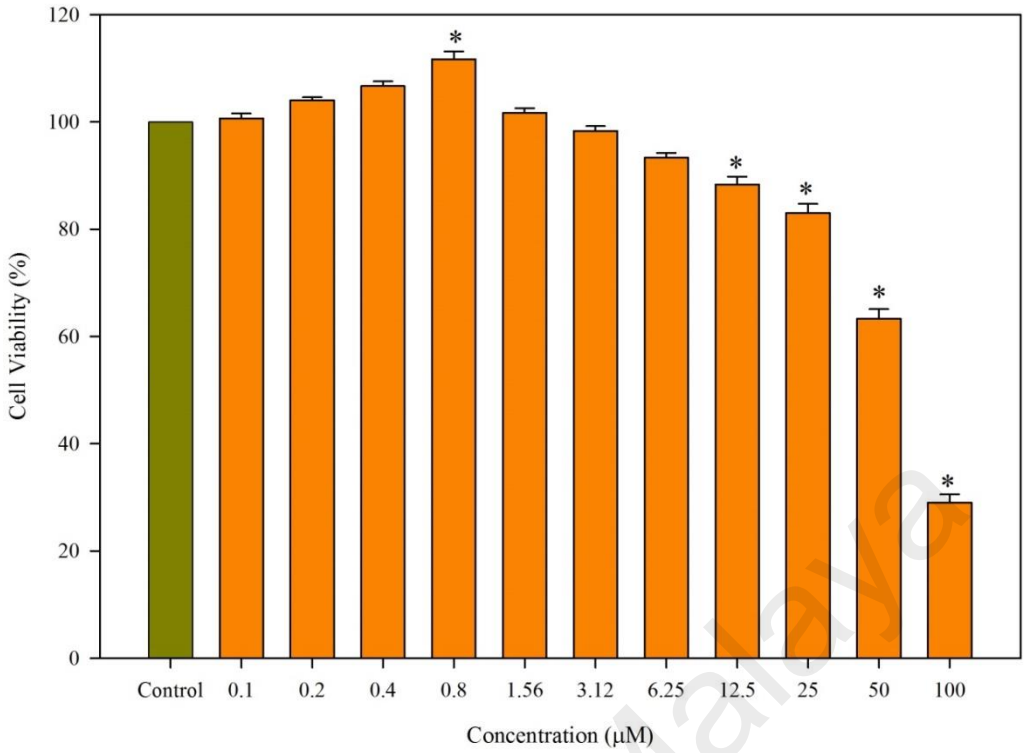


Figure 6.1 The cytotoxicity evaluation mesuagenin c (0.1 – 100 µM) in NG108-15 cells for 48 h. Mesuagenin c at 0.75 µM increased in NG108-15 cell viability. The toxicity level, IC_{50} was detected at concentration of 62.5 µM. Values are means ± S.E. from (n=6). *P < 0.05 indicates significantly different values from untreated group.

6.4.2 Mesuagenin c induced neuritogenesis in NG108-15 cells

Since mesuagenin c was shown to induce cell proliferation at the low range of concentrations, therefore concentrations ranging from 0.125 - 5 µM were selected for the neuritogenic evaluation at 12 - 48 h. As shown in Figure 6.2(a), the NG108-15 cells demonstrated a dose-dependent (0.125 - 5 µM) neurite outgrowth activity when treated for 12 h. The similar observation was obtained at 24 h with a significant rapid neurite outgrowth activity when the cells were treated with mesuagenin c at 5 µM. Treatment with mesuagenin c for 36 h significantly increased the neurite outgrowth activity where mesuagenin c at 2.5 µM exhibited the highest percentage of neurite bearing cells (57.17 %). However, the percentage of neurite bearing cells was significantly reduced when the cells were treated with mesuagenin c at 5 µM. At 48 h of treatment, treatment mesuagenin c (0.125 – 1 µM) demonstrated a higher neurite outgrowth activity while treatment with mesuagenin c at 2.5 and 5 µM significantly decreased the percentage of neurite bearing cells. In addition, treatment with mesuagenin c at 1 µM induced a time-dependent neurite outgrowth activity (12 - 48 h) with the highest percentage of neurite bearing cells (54.13%) at 48. The loss of neurite outgrowth at higher concentration and time could be due to the rapid induction of neuritogensis that occur at higher concentration but at shorter time incubation. This might have cause complete neuronal differentiation of the NG108-15 cells rather than induction of neuritogenesis as early characteristic of neuronal differentiation when the cells were left with higher concentration at longer time incubation. Therefore, this concentration was used for the subsequent neuritogenic evaluation and mechanistic studies.

The NG108-15 cells were treated with mesuagenin c (1 µM) for 1 - 48 h and compared with NGF-2.5S. Mesuagenin c induced the initial polygonal-like structure in NG108-15 cells as early as 1- 6 h (indicated by yellow circle, 1,2 and 3) significantly as compared to NGF-2.5S-treated cells and untreated cells (Figure 6.2(b)). Moreover, mesuagenin c-

treated group displayed elongation and branching of neurite (shown by red arrows) that progressed from 1 until 6 h of treatment. At 12 and 24 h of treatment, approximately 80% of the NG108-15 cells exhibited polygonal morphology with long neurites (red arrows 4 and 5) that were connected to other cells and formed multiple complex neurite extensions (Figure 6.2(c)). In contrast, when treated with NGF, approximately 50% majority of the NG1081-5 cells formed polygonal structure with complex neurite extensions (orange arrows 4 and 5). More importantly, almost all of the cells that were treated with mesuagenin c (48 h) exhibited polygonal morphology which indicated that most of the NG108-15 cells have fully differentiated (red circle 6). Moreover, the cells possessed longer neurites that formed multiple and complex neurite branches as compared to 12 and 24 h (yellow arrows, Figure 6.2(c)). In addition, NGF-treated cells also displayed similar observations (orange cirle 6) but the complexity of neurite braching was more significant in mesuagenin c-treated cells (Figure 6.2(c)).

(A)

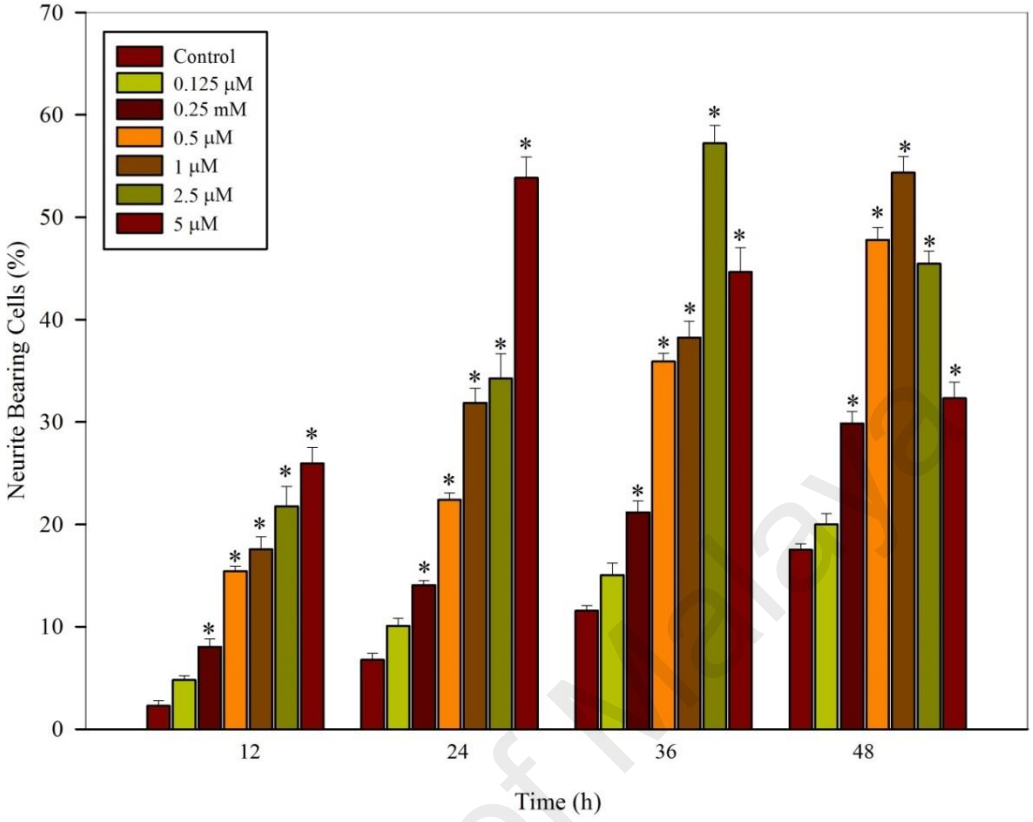
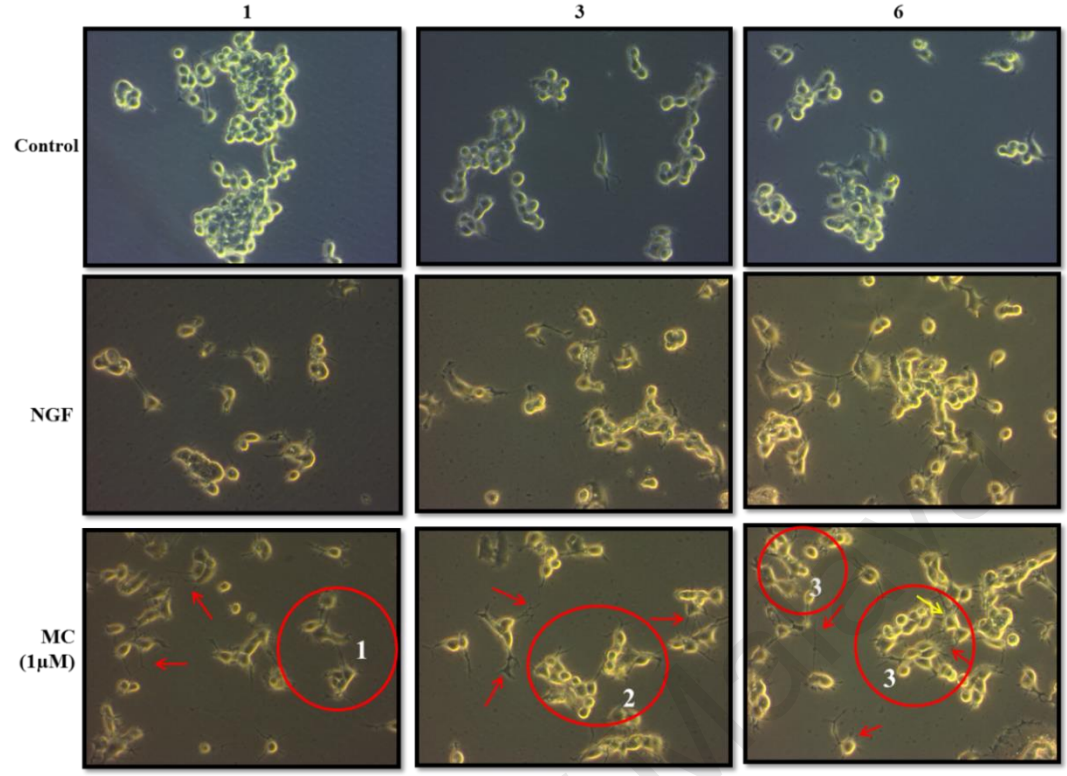


Figure 6.2 (A)Neuritogenesis evaluation of mesuagenin c at different dose and time in NG108-15 cells. Bar charts represent the percentage of positive neurite outgrowth observation at different dose and time. Values are means ± S.E. from (n=4). *P < 0.05 indicates significantly different values from untreated group.

(B)



(C)

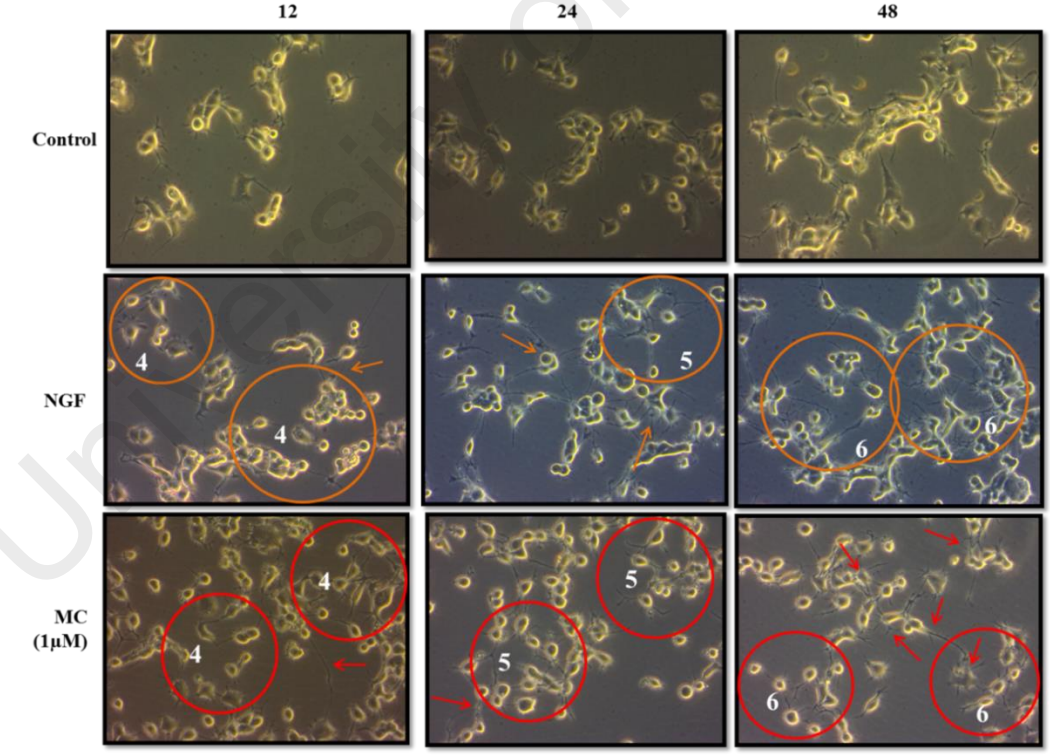


Figure 6.2, continued. (B) The phase-contrast neurite outgrowth observation induced by mesuagenin c (MC; 1 µM) for 1 – 6 h. (C) The phase-contrast neutite outgrowth analysis from 12 – 48 h following treatment with mesuagenin c. The circles represent the morphology of the NG108-15 cells whereas the arrows display the neurites branching, (Magnification: 100x).

6.4.3 Mesuagenin c enhanced the expression of neurofilament protein

Following the neuritogenic analysis, the NG108-15 cells that were first treated with mesuagenin c (1 μM) from 6 - 24 h and subjected to immunofluorescence neurofilament heavy (200 kDa) analysis. The immunofluorescence staining first revealed that treatment with mesuagenin c induced the expression of neurofilament heavy 200 kDa that led to the morphological changes in the NG108-15 cells accompanied with the elongation of neurites from 6 - 24 h (Figure 6.3 (a)). Following this observation, the NG108-15 cells were treated with mesuagenin c (1 μM) for 1 - 48 h and subjected to Western blot analysis. As shown in Figure 6.3 (b), treatment with mesuagenin c significantly increased the expression of neurofilament light and heavy (70 and 200 kDa) from 1 - 6 h. Moreover, it was demonstrated that the increased expression of neurofilament light and heavy was maintained high throughout the experiment as compared to untreated cells. In addition, mesuagenin c also increased the expression of neurofilament medium (150 kDa) significantly higher than untreated cells from 6 to 48 h. More importantly, mesuagenin c was shown to induce nerutogenesis by increasing the expression of all the neurofilament proteins which led to neuronal differentiation in NG108-15 cells.

6.4.4 Mesuagenin c induced neuritogenesis through the rapid activation of Akt and ERK1/2 in NG108-15 cells

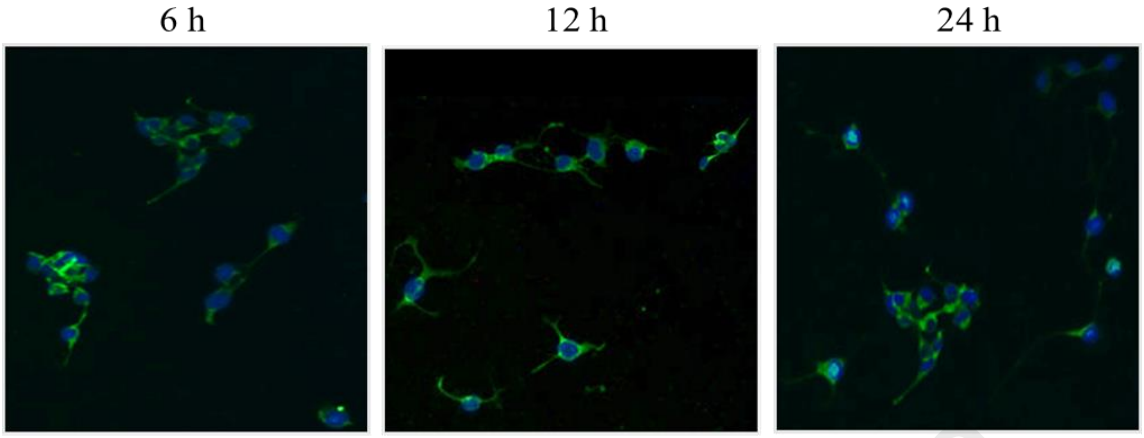
To determine the involvement of PI3K-Akt and/or ERK1/2 signaling pathways in mesuagenin c-mediated neuritogenesis in NG108-15 cells, the activation of Akt and ERK1/2 was examined. As shown in Figure 6.4, mesuagenin c treatment induced rapid phosphorylation of both Akt (Ser473) and ERK1/2 but not p38 MAPK and JNK from 1 to 3 h. Moreover, mesuagenin c was shown to increase the expression of total Akt protein but not total ERK1/2 protein. Treatment with mesuagenin c increased the phosphorylation of both Akt and ERK1/2 significantly higher than untreated cells

leading to an increase in both pAkt/Akt and pERK1/2/ERK1/2 ratios (Figure 6.4 (b) and (c)). As an attempt to validate the involvement of Akt and ERK1/2 pathways, the the NG108-15 cells were pretreated with either API-2 or UO126 for 1 h prior to addition of mesuagenin c (1 μM) for 24 h. Interestingly, immunofluorescence staining of neurofilament heavy (200 kDa) showed that the pretreatment with both API-2 and UO126 significantly diminished mesuagenin c-induced neuritogenesis in NG108-15 cells (Figure 6.4 (d). These observations further indicated that mesuagenin c-induced neuritogenesis in NG108-15 cells was mediated through the activation of PI3K-Akt and ERK1/2 signaling pathways.

6.4.5 Mesuagenin c induced full activation of Akt via mTORC2

The activation of Akt can be achieved via phosphorylation of its Thr308 and Ser 473 residues However, the maximum activity of Akt requires the phosphorylation of its Ser473 residue in the regulatory domain by the mTOR-rictor complex (mTORC2). Treatment with mesuagenin c (1 μM) was shown to significantly induce the phosphorylation of mTOR which led to the significant increase in the ratio of pMTOR/mTOR (Figure 6.5 (a) and (b)). Moreover, the increased phosphorylation of mTOR was accompanied with the significant increase expression of rictor protein as compared to untreated cells as shown in Figure 6.5 ((a) and (c)). These results suggested that induction of neuritogenesis by mesuagenin c could be mediated by the full activation of Akt via the activation of mTORC2 complex.

(A)



(B)

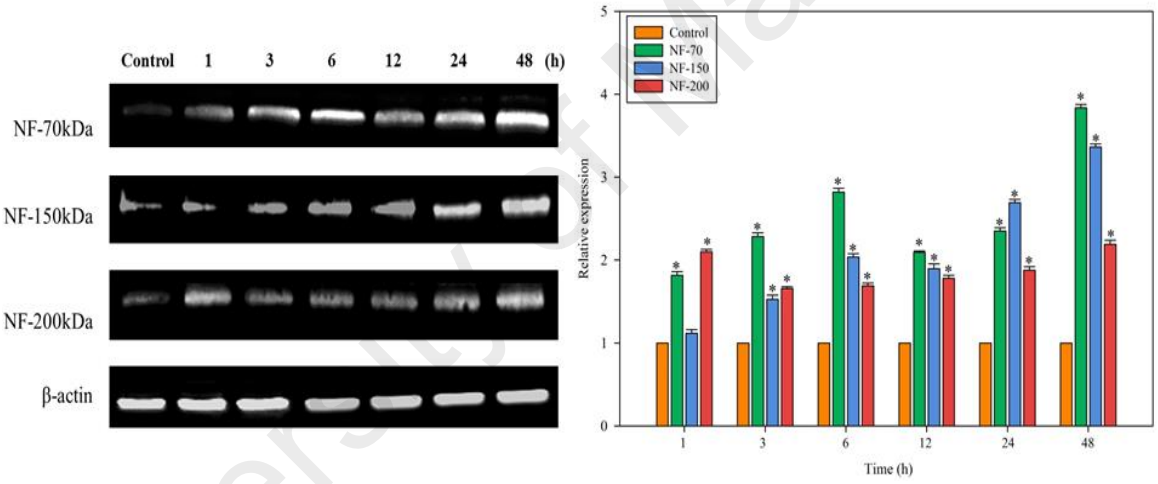


Figure 6.3 (A) Immunofluroscence staining of neurofilament heavy (200 kDa) of cesuagenin c-treated cells for 6, 12 and 24 h. (B) Western blot analysis of neurofilament light, medium and heavy (70, 150 and 200 kDa) following treatment with mesuagenin c (1 µM) from 1 – 48 h. Values are means ± S.E. (n=4). The asterisk indicated significantly different values from untreated cells (*P<0.05).

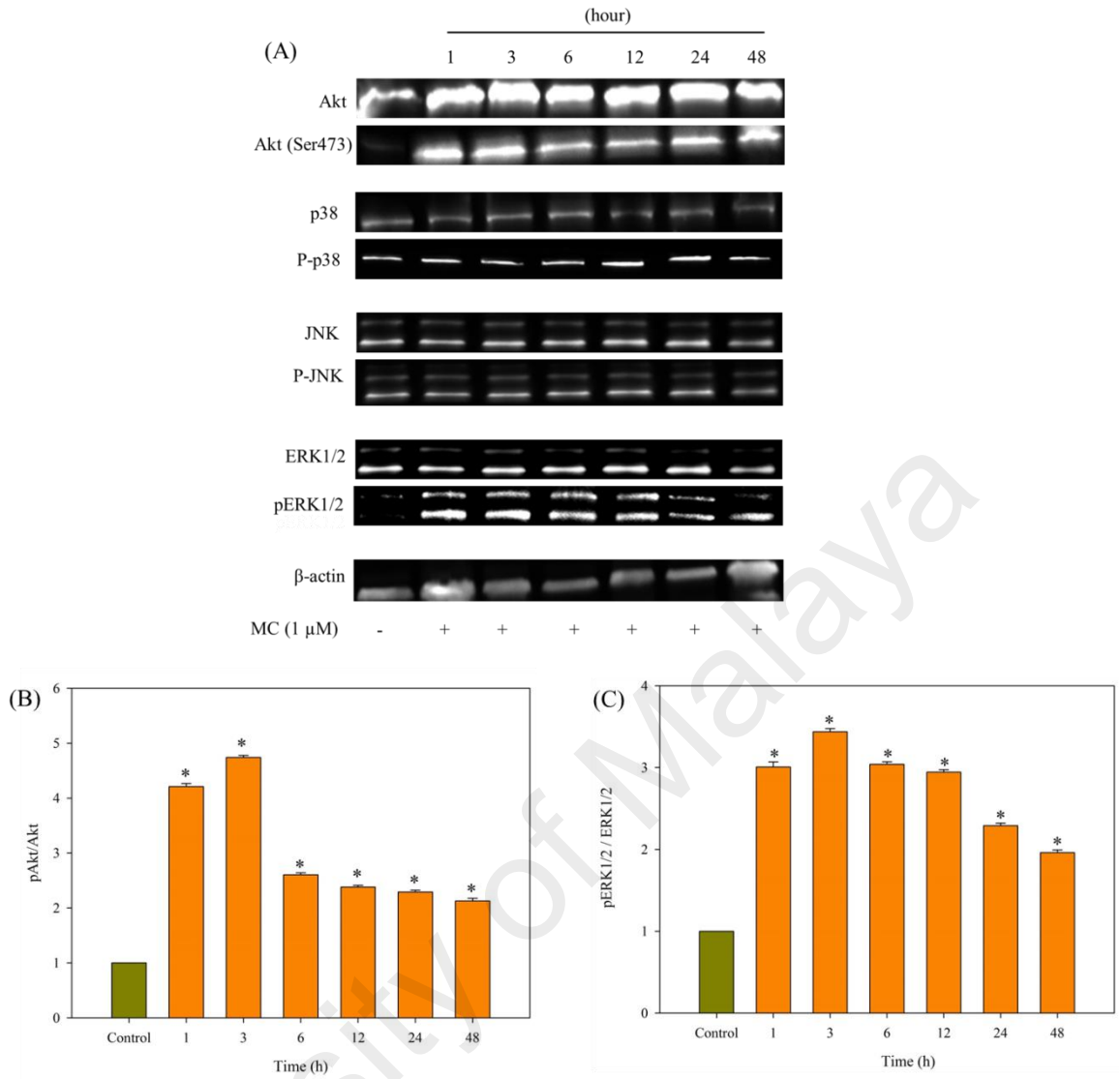


Figure 6.4 Mesuagenin c (MC; 1 μM) induced rapid activation of Akt and ERK1/2 in NG108-15 cells. (A) Western blot analysis of Akt, ERK1/2, JNK and p38 following treatment with mesuagenin c. (B) Bar chart represents the ratio of pAkt/Akt. (C) Bar chart represents the ratio of pERK1/2/ERK1/2. Values are means ± S.E. (n=4). The asterisk indicated significantly different values from untreated cells (*P<0.05).

(D)

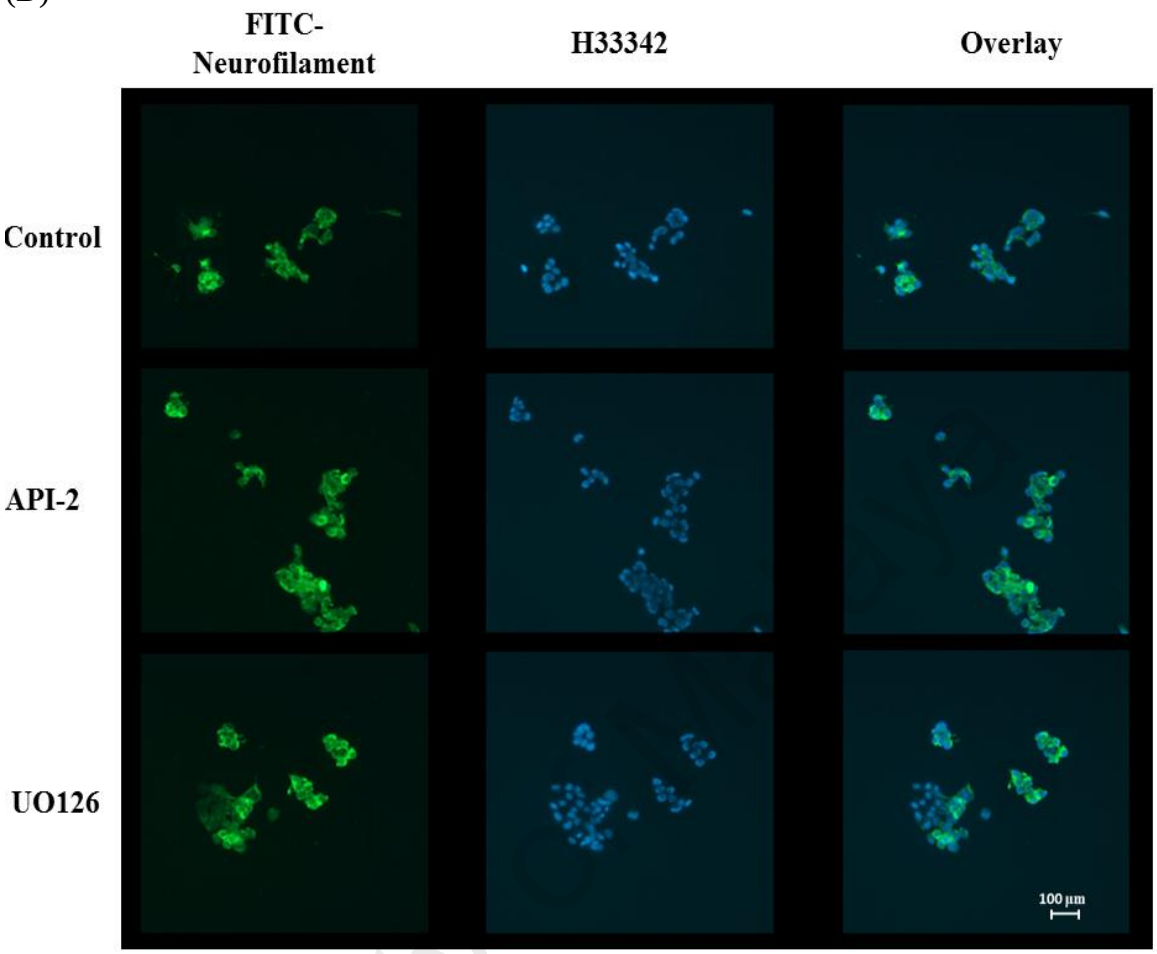


Figure 6.4, continued. (D) Addition with API-2 and UO126 abolished the expression of of neurofilament heavy (200 kDa) in mesuagenin c-treated cells for 24 h. β-actin was used as housekeeping protein. Values are means ± S.E. (n=4). The asterisk indicated significantly different values from untreated cells (*P<0.05).

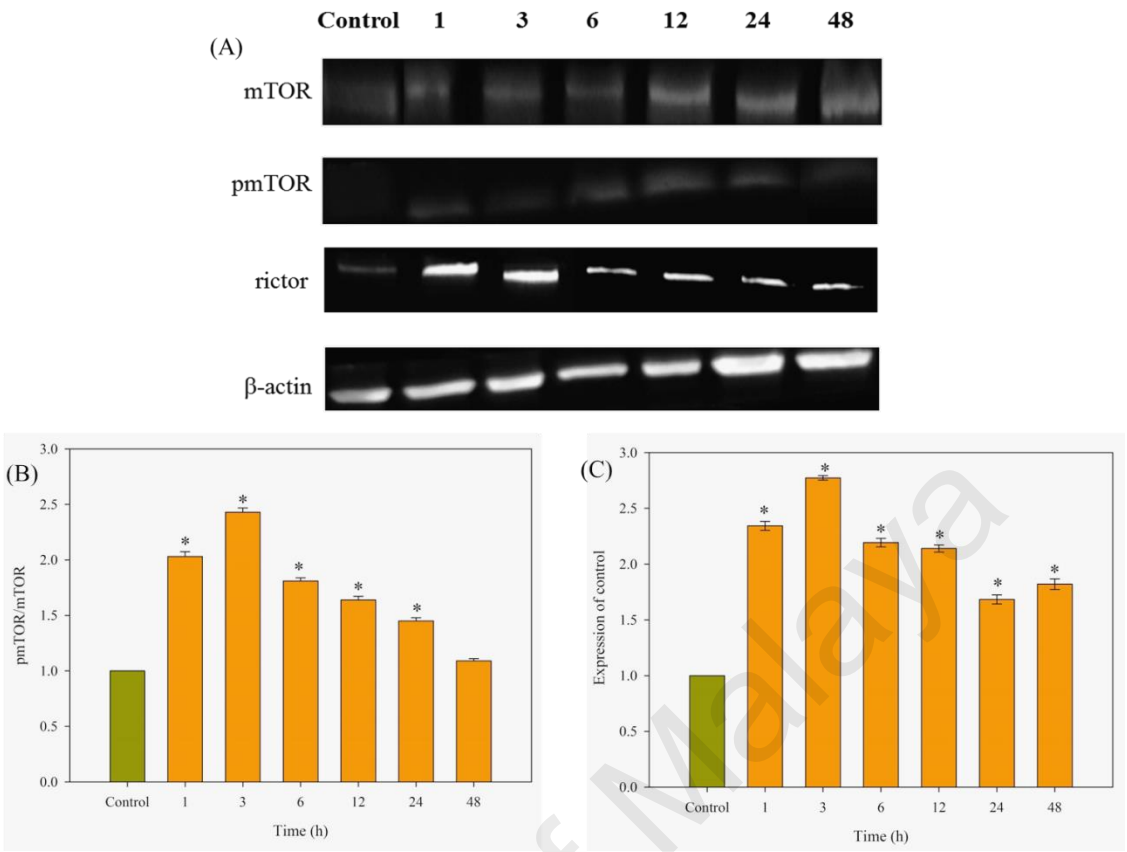


Figure 6.5 Mesuagenin c (1 µM) induced the activation of mTORC2. (A) Western blot analysis of mTOR, pmTOR and rictor by mesuagenin c. (B) Bar chart represents the ratio of pmTOR/mTOR. (C) Bar chart represents the protein expression of rictor. β-actin was used as housekeeping protein. Values are means ± S.E. (n=4). The asterisk indicated significantly different values from untreated cells (*P<0.05).

6.4.6 Activation PI3K-Akt, ERK1/2 and chemokines by mesuagenin c induced activation of NF-κB p65

NF-κB is a transcription factor that regulates various cellular processes. Moreover, the activation of NF-κB induces the expression of various inflammatory cytokines and chemokines which in turn induce its activation. The activation of PI3K-Akt and ERK1/2 by mesuagenin c was postulated to mediate the NF-κB p65 nuclear translocation via degradation of IκBα. Treatment with mesuagenin c induced the phosphorylation of IκBα in the cytosolic fraction resulting in nuclear translocation of NF-κB p65 subunit (Figure 6.6(a)) and a significant increase of pIκBα/IκBα and p65(nuclear)/p65(cytosolic) ratios (Figure 6.6(a)). Conversely, when the cells were pretreated with API-2 and Wortmanin (WM), the IκBα degradation and NF-κB p65 nuclear translocation were inhibited. Interestingly, the addition of UO126 and PTx also suppressed mesuagenin c-induced IκBα degradation and NF-κB p65 nuclear translocation (Figure 6.6(a)) which further substantiated that mesuagenin c induced NF-κB p65 nuclear translocation through activation of PI3K-AKT and ERK1/2. Moreover, this observation also indicated that chemokines could also induce neuritogenesis by influencing the NF-κB transcriptional activity. The involvement of chemokines in neuritogenesis was first evaluated by pretreating the NG108-15 cells with Pertussis Toxin (PTx). Addition of PTx suppressed the expression of neurofilament heavy (200 kDa) (Figure 6.6 (b)) and abolished the mesuagenin c-induced neuritogenesis in NG108-15 cells. This indicated that treatment with mesuagenin c induced neuritogenesis in NG108-15 cells was also modulated by chemokines. As an attempt to investigate the novel role of CCL21 in neuritogenesis, the level of CCL21 was determined following treatment with mesuagenin c. Interestingly, the expression of CCL21 was increased higher against untreated cells throughout the experiment and this may suggest a potential role of CCL21 in neuritogenesis (Figure 6.6(c)).

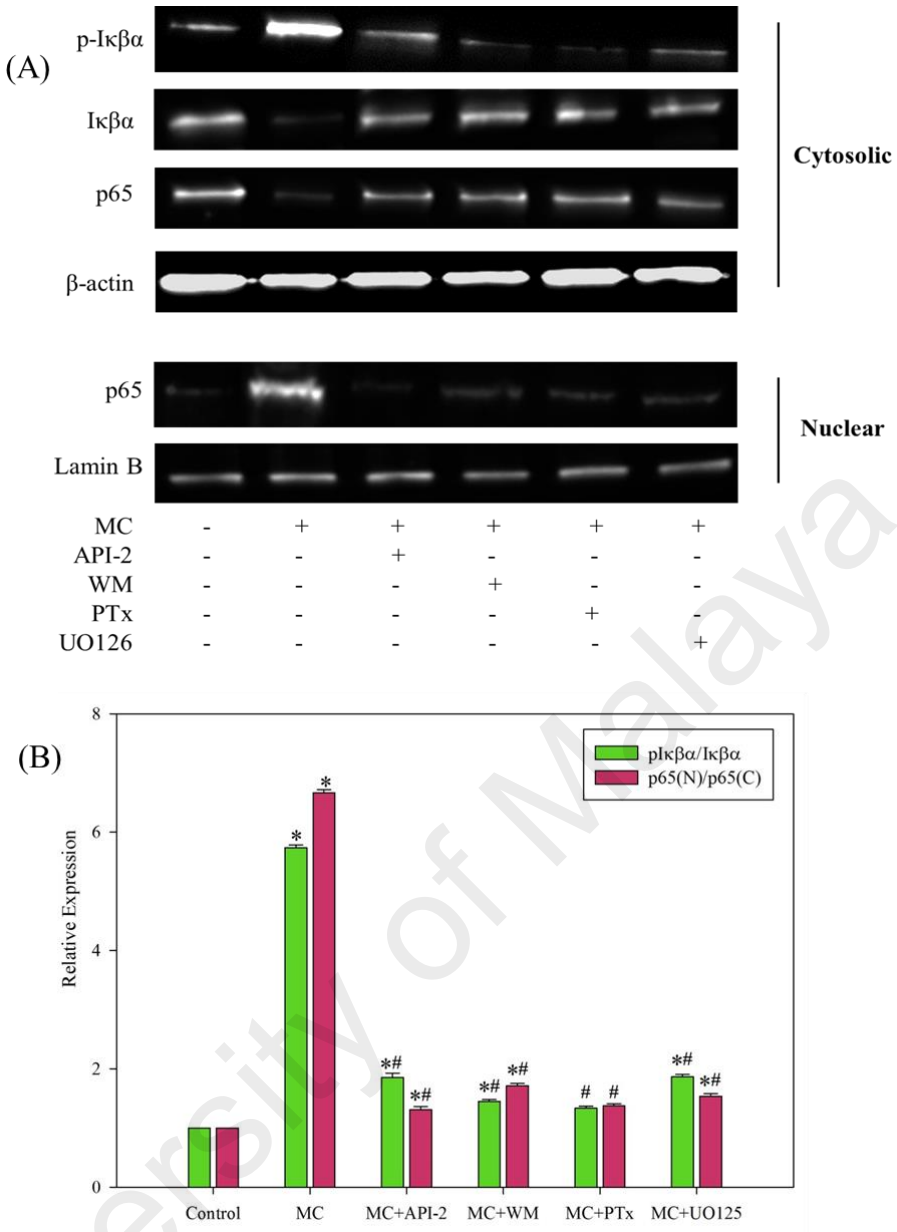


Figure 6.6 (A) Activation of PI3K-Akt by mesuagenin c (MC; 1 µM) induced the NF-κB p65 nuclear translocation in NG108-15 cells.Western blot analysis and bar chart shows the phosphorylation of IκBα which led to NF-κB p65 nuclear translocation in NG108-15 cells (API-2-triciribine hydrate; WM-wortmannin; PTx-pertussis toxin; UO126-monoethanolate). (Values are means ± S.E. (n=4). The asterisk indicated significantly different values from untreated cells (*P<0.05).

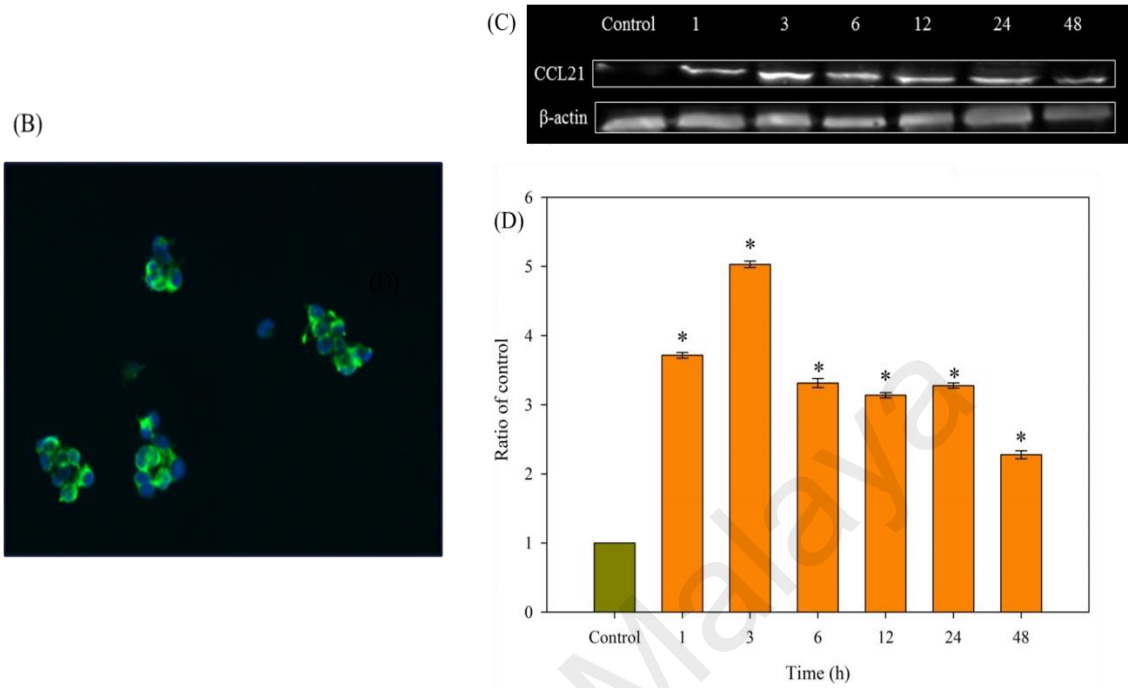


Figure 6.6, continued. (B) Addition of PTx significantly abolished the neuritogenic activity of mesuagenin c in NG108-15 cells. Mesuagenin c (1 µM) induced the expression of CCL21 and chemokines during neuritogenesis in NG108-15 cells. (C) Western blot analysis and (D) bar chart shows the consistent production of CCL21 following treatment with mesuagenin c for 1- 48 h. (Values are means ± S.E. (n=4). The asterisk indicated significantly different values from untreated cells (*P<0.05).

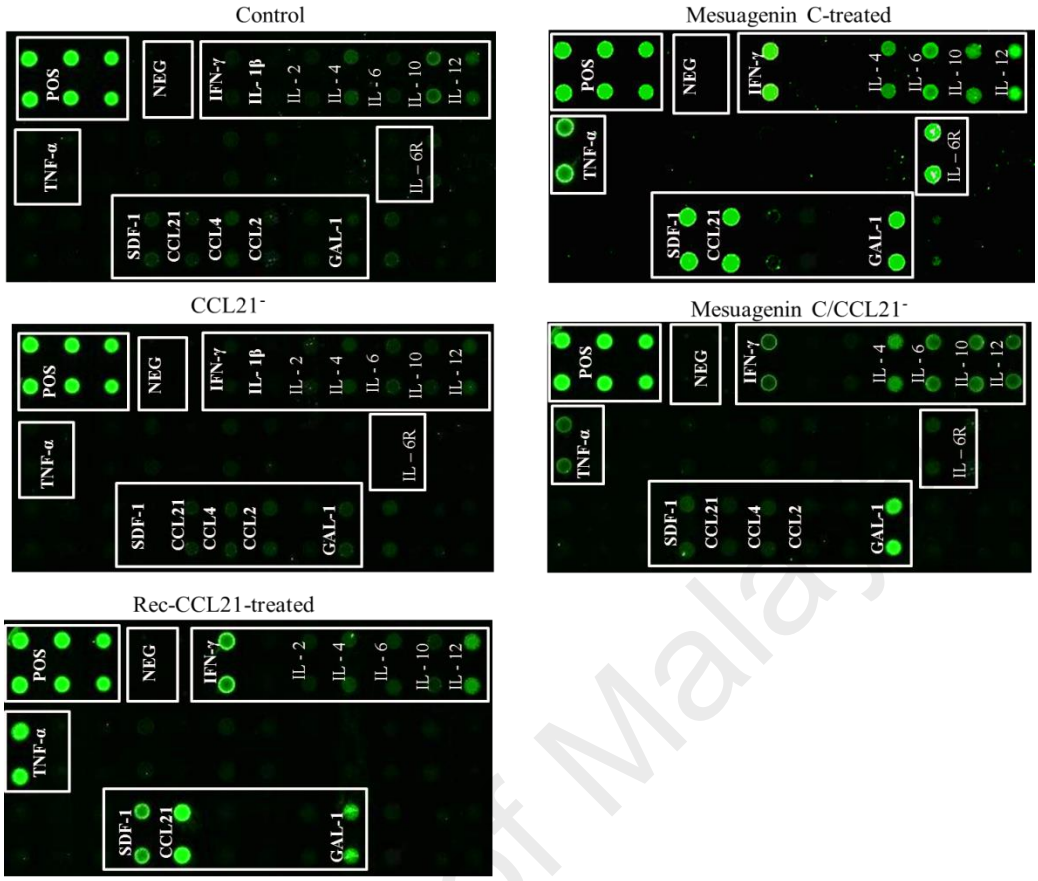
6.4.7 Mesuagenin c modulated galectin-1, pro-inflammatory cytokines, chemokines and CCL21 through PI3K-Akt and ERK1/2 signaling pathways

The NG108-15 cells that were treated with mesuagenin c demonstrated a significant increase in the production of pro-inflammatory cytokines (IFN-γ, IL-6, IL-12 and TNF-α) and chemokines (CXCL12 and CCL21). Additionally, mesuagenin c also increased production of anti-inflammatory cytokines, IL-4 and IL-10 (Figure 6.7 (a) and (b)). Interestingly, treatment with mesuagenin c showed an increase in the production of galectin-1 (Gal1) as compared to untreated cells. More importantly, consistent with the previous Western blot analysis (Figure 6.7 (b)), CCL21 production was significantly elevated following mesuagenin c treatment. To further investigate this event, the CCL21 gene in NG108-15 cells was knocked down and further subjected to cytokines/chemokines protein array and phase-contrast neuritogenesis analysis. The data revealed that the knocked down CCL21/mesuagenin c ($CCL21^-$/mesuagenin c)-treated group recorded significant reduction of all aforementioned cytokines, chemokines as well as Gal1 production (Figure 6.7 (a) and (b)). Moreover, the phase contrast analysis demonstrated that most of NG108-15 cells remained undifferentiated with reduced neurite extension (yellow circle) as compared to mesuagenin c-treated group (Figure 6.7 (e)).

Treatment of NG108-15 cells with recombinant CCL21 significantly increased the production of some of the cytokines and chemokines (IFN-γ, TNF-α, CXCL-12 and CCL21). The production of Gal-1 was also increased as compared to $CCL21^-$ /mesuagenin c-treated group. Furthermore, phase contrast observation showed that most of the cells displayed early polygonal morphological changes with some elongated neurites as compared to $CCL21^-$/mesuagenin c-treated group (Figure 6.7 (e)). Additionally, the NG108-15 cells were pretreated with pharmacological inhibitors (API-2, PTx and UO126) and subjected to cytokines/chemokines protein array. The addition

of API-2 and UO16 significantly reduced the level of all cytokines, chemokines and as well as Gal-1 as compared to mesuagenin c-treated group (Figure 6.7 (c) and (d)). In addition, when compared with PTx, the level of all cytokines, chemokines and Gal1 were found to be similar to untreated cells (Figure 6.7 (c) and (d)). More importantly, the addition of PTx also induced the colonial growth of undifferentiated NG108-15 cells (Figure 6.7 (e)). Collectively, these observations further substantiated that mesuagenin c induced neuritogenesis through the modulation of galectin-1, pro-inflammatory cytokines and chemokines, specifically, CCL21 through PI3K-Akt and ERK1/2.

(A)



(B)

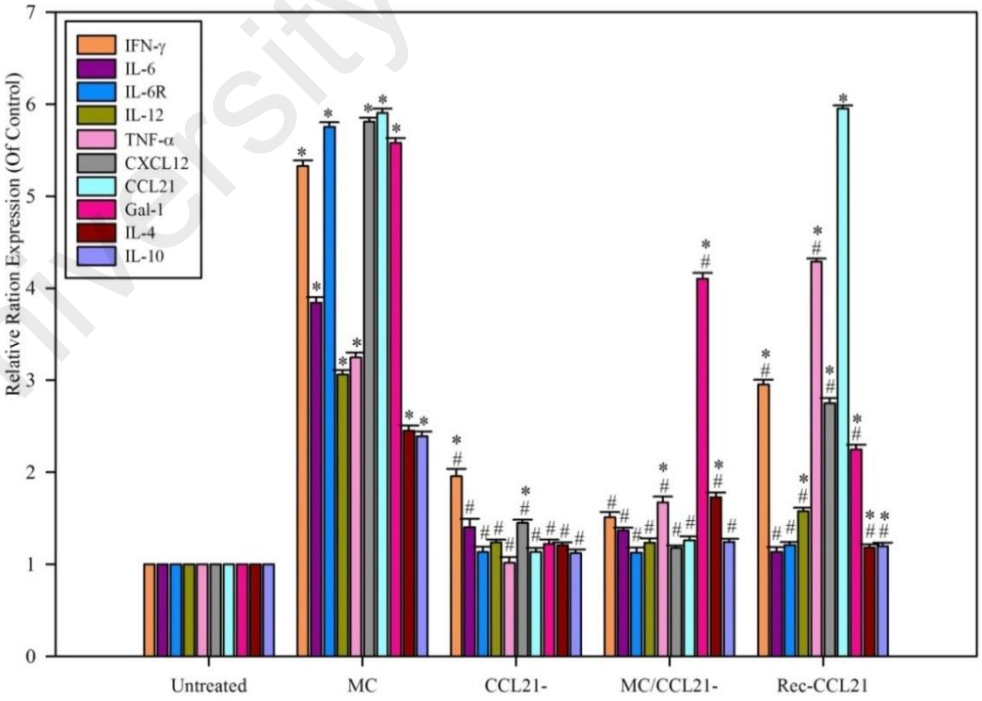
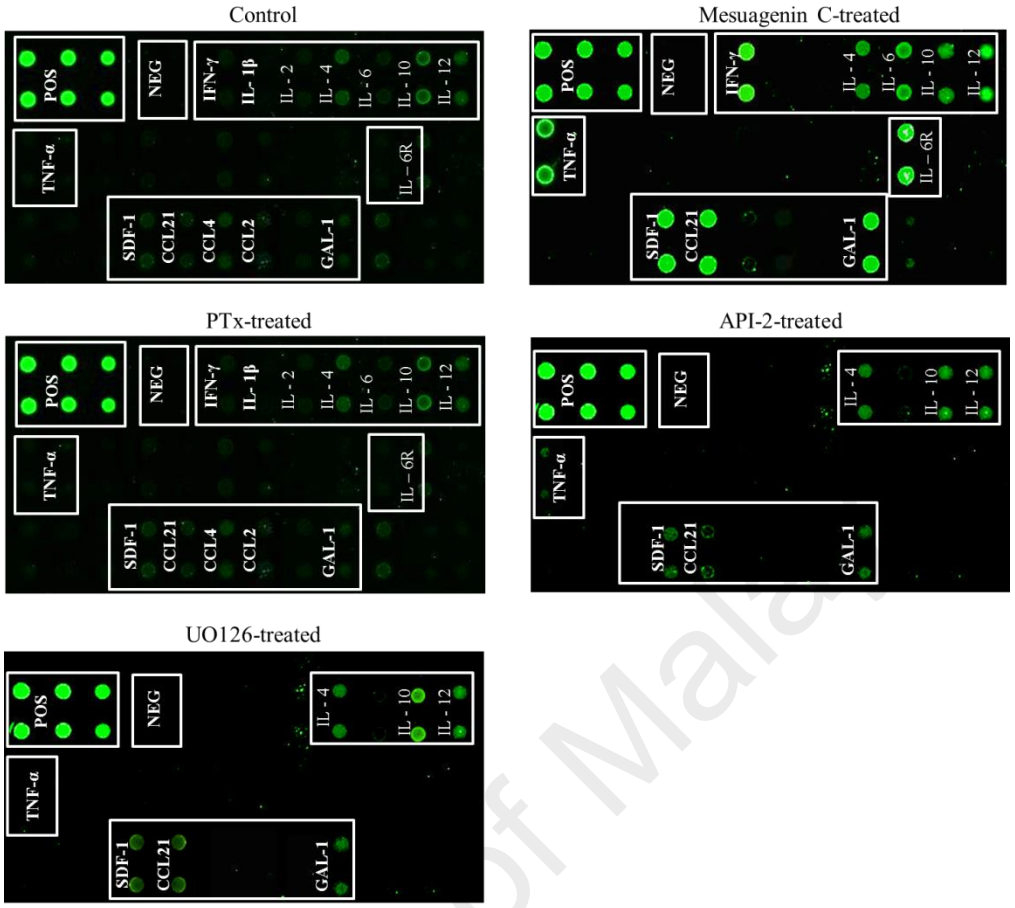


Figure 6.7 Modulatory effects of mesuagenin c (MC) on Gal1, inflammatory cytokines and chemokines production (A) Protein array slide image analysis of inflammatory cytokines, chemokines and Gal-1 by mesuagenin c and recombinant CCL21 with or without CCL21 knockdown. (B) Bar charts represent the significant increase of IL-4 and IL-10 which led to reduction of pro-inflammatory cytokines and chemokines.

(C)



(D)

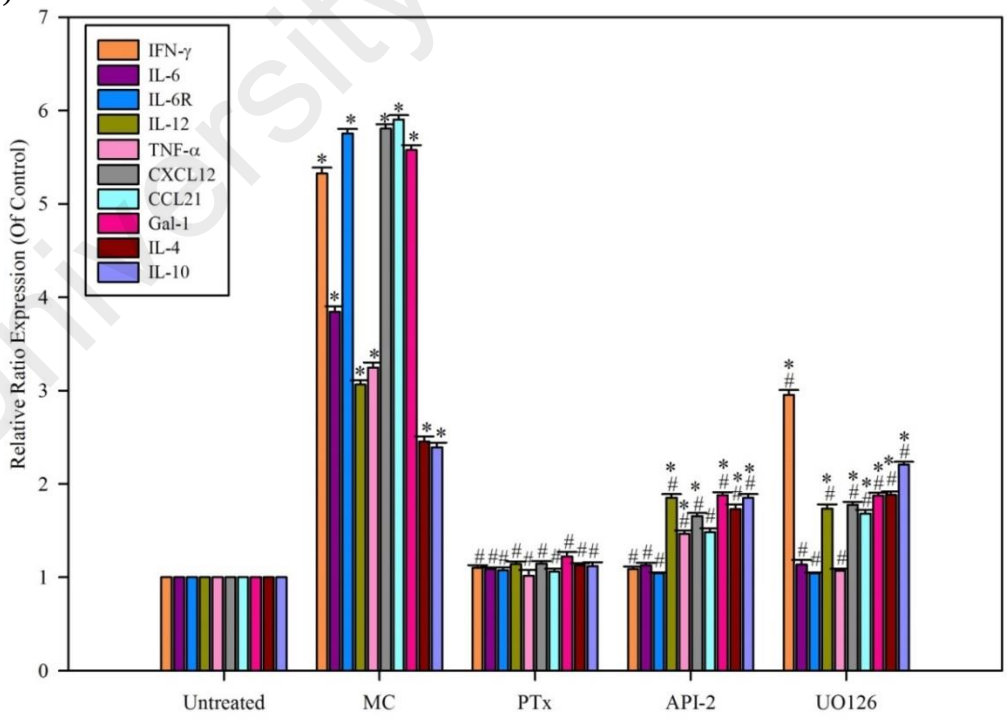


Figure 6.7, continued. (C) Protein array slide image analysis of inflammatory cytokines, chemokines and Gal-1 by following the addition of inhibitors; PTx, API-2 and UO126. (D) Bar charts represent the level of inflammatory cytokines, chemokines and Gal-1 by following the addition of PTx, API-2 and UO126.

(E)

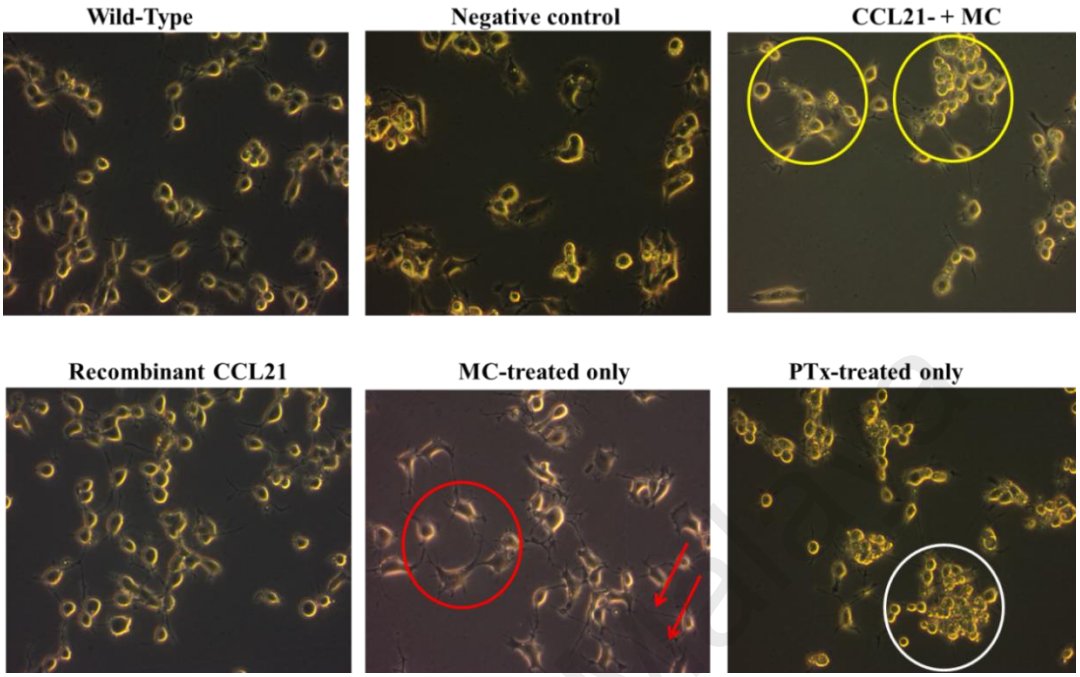


Figure 6.7, continued. (E) The phase-contrast neutite outgrowth following CCL21 knockdown, addition of recombinant CCL21, PTx or mesuagenin c (MC). (Magnification: 100x). The circles represent the NG108-15 cells morphology whereas the arrows represent the elongated neurites. Values are means ± S.E. (n=3). *P<0.05 versus untreated, $^{\#}$P<0.01 versus mesuagenin c, significantly different as shown.

6.5 Discussion

During the neuroprotective evaluation of mesuagenin c (chapter 3), it was observed that mesuagenin c significantly induced rapid neuritogenesis in NG108-15 cells during the pretreatment period. Therefore, the neuritogenic activity of mesuagenin c was evaluated and its molecular mechanisms involved were investigated. Neuritogenesis is one of the important aspects of neuroplasticity that stimulates neuroregeneration following neuronal injury as well as in various neuropathological conditions (Di Giovanni et al., 2005; Read & Gorman, 2009b). Neuritogenesis also plays an important role in neuronal pathfinding and the establishment of functional synaptic connections during nervous system development (Franze, 2013). This normally occurs via the formation of neurofilaments that are found to be present in dendrites and axons. The current results demonstrated that mesuagenin c induced neuritogenesis that was first characterized by the long extension of neurites with dendritic arborization in a time-dependent manner. The immunocyctochemistry of neurofilament heavy (200 kDa) first indicated that mesuagenin c induced neuronal differentiation and neuritogenesis in NG108-15 by upregulating the expression of neurofilament protein (Figure 6.3 (a)). Further analysis revealed that mesuagenin c induced neuritogenesis by increasing the expression of neurofilament light, medium and heavy (Figure 6.3 (b)) which led to the rapid differentiation of NG108-15 cells with complex neurite branches. Neurofilaments are important in maintaining the axon caliber as well as electrical impulses transmission along the axons (Hoffman, Griffin, & Price, 1984; Križ, Zhu, Julien, & Padjen, 2000; Marszalek et al., 1996). In addition, neurofilaments have remarkably long half-lives (Millecamps, Gowing, Corti, Mallet, & Julien, 2007) and they possess elastic fibrous properties that are important in maintaining the asymmetrical shape of neurons (Wagner et al., 2004; Yuan, Rao, & Nixon, 2012).

Other than its essential role in promoting neuronal survival, Akt can also induce neuronal differentiation and neuritogenesis (Jaworski et al., 2005; Jin et al., 2012). Therefore, the involvement of Akt in regulationg neuritogenesis by mesuagenin c was investigated. Mesuagenin c treatment significantly induced the rapid phosphrorylation and activation of Akt (Ser473) which led to significant increase of pAkt/Akt ratio within 1 – 3 h of treatment (Figure 6.4 (a) and (b)). In addition, the activation of Akt was sustained throughout the experiment and significantly higher than untreated cells. The activation of Akt regulates neuritogenesis and cytoskeletal rearrangement by targetng its downstream targets such as of peripherin (Konishi et al., 2007), girdin (Enomoto et al., 2005; Kitamura et al., 2008), mTOR (Kumar, Zhang, Swank, Kunz, & Wu, 2005) and GSK-3β (Li et al., 2013; Mercado-Gómez et al., 2008). Mitogen-activated protein kinase (MAPK) activation has been implicated in various cell signalings such as in inflammation, cell proliferation, apoptosis and neuritogenesis depending on the cell types and nature of stimuli. It was reported that a group of second-generation antipsychotic drugs such as olanzapine, dozapin and artepillin C induced neuritogenesis in PC12 cells by activating the ERK signaling pathway (Lu & Dwyer, 2005). Moreover, 4-Omethylhonokiol promotes neuritogenesis by activating ERK in embryonic neuronal cells model (Lee et al., 2009). Sustained ERK activation has been reported to be responsible in NGF-induced neuronal differentiation in PC12 cells (Chen et al., 2012a; Mullenbrock, Shah, & Cooper, 2011; Qiu & Green, 1992). In addition, DHA was shown to induce sustained ERK1/2 phosphorylation that was shown to be essential in DHA-mediated neuritogenesis in SH-SY5Y cells (Wu et al., 2009a). In support of these findings, treatment with mesuagenin c induced the rapid activation and phosphorylation of ERK1/2 in the NG108-15 cells (Figure 6.4 (a) and (b)). Moreover, when the NG108-15 cells were pretreated with Wortmannin and UO126, the

ability of mesuagenin c to induce neuritogenesis was suppressed and further highlighted the importance of Akt and ERK1/2 activation in promoting neuritogenesis.

A full activation of Akt requires phosphorylation at its Ser473 by the mTORC2 complex that consists of mTOR and rictor (Frias et al., 2006). Furthermore, Akt possesses the ability to phosphorylate and interact with various proteins that may positively regulate neuronal differentiation. Treatment with mesuagenin c was shown to induce the phosphorylation of mTOR and accompanied with significant increase of rictor expression. These findings suggested that mesuagenin c may induce neuritogenesis in NG108-15 cells through the full activation of Akt via mTORC2. Apart from inducing a full the activation of Akt, mTOR signaling has also been reported to promote the growth and branching of neurites in various neuronal models (Jaworski & Sheng, 2006; Kumar et al., 2005). Moreover, mTOR also induces axonal regeneration in the adult CNS and stimulates the axonal growth of injured peripheral nerves (Kim et al., 2011; Leibinger, Andreadaki, & Fischer, 2012). In another report, the RNAi knockdown of mTOR was shown to reduce the dendritic complexity thus suggesting that the Akt-mTOR pathway is the primary mediator of PI3K regulated dendritic branching (Jaworski et al., 2005). NF-κB activation has been reported to promote the neuritogenesis and enhance the complexity level and size of neuronal processes in the developing nervous system and in cultured neurons (Gallagher et al., 2007; Gutierrez & Davies, 2011). Moreover, the activation of NF-κB induces the production of inflammatory cytokines and chemokines which in turn induce its activation. Following the activation of PI3K-Akt and ERK1/2, mesuagenin c induced NF-κB p65 nuclear translocation by promoting the degradation of IκBα in the cytosol. This observation was reversed following the addition of Wortmannin, API-2, UO126 and Pertussis Toxin indicating that the activation of Akt and ERK1/2 is essential in promoting the NF-κB transcriptional activity and in some part, through modulation of GPCRs. Since it was

previously described that mesuagenin c modulated the inflammation in neuron-glia co-culture system through the regulation of NF-κB transcriptional activity of pro-inflammatory cytokines and chemokines, hence, one of its potential mechanisms in inducing neuritogenesis through inflammation is postulated to be mediated via GPCRs and NF-κB. Increasing evidences have reported that GPCRs hold a cardinal role in the transcriptional regulation (Lappano & Maggiolini, 2011; Millar & Newton, 2010). Moreover, the local expression and production of cytokines, chemokines and growth factor are dependent on GPCR-induced NF-κB activation (Fraser, 2008; Richard, 2001; Turner et al., 2014). For instance, the G inhibitory subunit of GPCR, Gi has been shown to be involved in coupling chemokine and chemoattractant receptors to leukocyte functions such as chemotaxis, superoxide generation, and degranulation (Bokoch, 1995; Karimian, Buist-Homan, Faber, & Moshage, 2012; Neptune & Bourne, 1997). Furthermore, a number of those receptors can mediate the activation of transcription factors such as NF-κB (Richard, 2001). Moreover, the activation of NF-κB and the expression of transcripted genes induced by some of GPCRs and NF-κB regulation is sensitive to Pertussis Toxin (PTx). GPCRs activation can to some extent regulate the activation of Akt and ERK1/2 signalling pathways. For instance, conjugated bile acids was reported to promote ERK1/2 and AKT activation via a pertussis toxin-sensitive mechanism which primarily activate receptor tyrosine kinases and intracellular signaling pathways in a GPCR (G(iα))-dependent and ROS-dependent manner (Dent et al., 2005). Additionally, several studies have argued that GPCRs and its components have the capacity to promote activation of receptor tyrosine kinases in various neuritogenic and cancer models (Lu & Dwyer, 2005; Wang et al., 2014; Wu & Wong, 2005). Hence, the addition of PTx prior to exposure of mesuagenin c could have led to the inactivation of GPCRs that subsequently induced the suppression of Akt and ERK1/2 activation which ultimately abolished neuritogenesis. Therefore, the current

data suggest the regulation of cytokines and chemokine through GPCRs mediated NF-κB activity that is associated with the activation of PI3K-Akt and ERK1/2-NF-κB dependent signaling pathways were largely responsible for induction of neuritogenesis in NG108-15 cells (Cassatella et al., 1992; Del Corno et al., 2001; Kang et al., 2014).

The NG108-15 cells treated with mesuagenin c significantly increased the production of pro-inflammatory cytokines (IFN-γ, IL-6, IL-12 and TNF-α), chemokines (CXCL12 and CCL21), Gal-1 as well as anti-inflammatory cytokines (IL-4 and IL-10) (Figure 6.7 (a) and (b)). However, the addition of API-2 and UO126 significantly reversed these observations. Gal-1 is a small carbohydrate-binding protein (Camby et al., 2006) that is expressed in various normal and pathological tissues that possesses an array of biological activity (Sakaguchi et al., 2007; Sasaki et al., 2004; Starossom et al., 2012). Gal-1 also plays an important role in the regulation of neural progenitor cell proliferation in two neurogenic regions, the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus (Ishibashi et al., 2007). In addition, Gal-1 was upregulated through the RET/PI3K signaling by glial-derived nerve factor (GDNF) which promoted neuritogensesis in cultured adult rat dorsal root ganglion neurons (Takaku et al., 2013). The current study is in agreement with the neurogenic/neuritogenic property of Gal-1 in other neuronal models and therefore, suggests that induction of neuritogenesis by mesuagenin c is also regulated by Gal-1 through Akt and ERK1/2.

Although inflammatory cytokines have been shown to affect growth and survival of neurons, little is known about its involvement in neuronal differentiation. More importantly, inflammation is an intricate regulatory network that is essential for the efficient repair of neuronal loss and can result in various neurological diseases (Chen et al., 2012b; Kriz, 2006a; McAlpine et al., 2009). For example, TNF-α, IFN -γ and IL-6

was shown to induce the *in vitro* differentiation of the neuroblastoma cell line N103 in a dose-dependent manner (Muñoz-Fernández et al., 1994). Another study demonstrated that IL-6 enhanced neuritogenesis in neurons *in vitro* where it upregulated the expression of GAP-43, SPRR1A and Arginase I while promoting axonal sprouting and synapse formation (Shuto et al., 2001). In addition, IL-6 also increased the expression of neuronal mTOR protein as well as the expression of pSTAT3 in neurons around lesion site *in vivo* model (Yang et al., 2012). The current findings supported these previous studies where treatment with mesuagenin c augmented the production of pro-inflammatory cytokines (TNF-α, IFN-γ and IL-6). By doing so, mesuagenin c also increased the level of anti-inflammatory cytokines, IL-4 and IL-10. Generally known as anti-inflammatory cytokine, IL-10 was reported to provide neuroprotective effects by protecting the formed neurites in primary cortical neurons through the activation of PI3K/AKT signaling pathway (Lin et al., 2015). In another report, IFN-γ dependent secretion of IL-10 from Th1 cells and microglia/macrophages significantly contributed to the functional recovery after spinal cord injury (Ishii et al., 2013). Therefore, it is suggested that the increase of IL-4 and IL-10 could be beneficial to protect the formed neurites throughout the neuritogenesis and to control the production of pro-inflammatory cytokines and chemokines from inducing aberrant inflammation.

Mesuagenin c induced the sustained production of CCL21 and CXCL-12 (SDF-1α) in NG108-15 cells and the inhibition of GPCRs by PTx abolished the neuritogenic activity of mesuagenin c. The chemokine stromal cell-derived factor (SDF-1α) or CXCL12 possesses dual roles in both neurodegeneration and/or neuroprotection (Rostène et al., 2007). SDF-1α is generally produced by glia and neurons to promote neurogenesis within the neurogenic zones (neurovascular niches) following cerebral ischemia (Li et al., 2010b). Furthermore, simvastatine increased SDF-1α level following stroke and initiated the neurogenic as well as angiogenic responses within the affected

and damaged area in the ischemic rats (Cui et al., 2009; Liu et al., 2008). The present study demonstrated that mesuagenin c induced the augmentation of SDF-1α in NG108-15 cells and could suggest its role in neuronal differentiation. In comparison to SDF-1α, CCL21 is a chemokine that is specifically produced in endangered neurons that activates distant migration and activation of microglia following excitotoxicity. Moreover, recent study reported that CCL21 specifically enhanced and upregulated the microglia P2X4 receptor expression *in vitro* and *in vivo* and initiated neuropathic pain development (Biber et al., 2011). The knockdown of CCL21 was shown to reduce the neuritogenic activity of mesuagenin c in NG108-15 cells when compared with mesuagenin c-treated cells (Figure 6.7 (e)). Moreover, this observation was accompanied with the significant reduction of cytokines, chemokines and Gal-1 production in $CCL21^{-}$/mesuagenin c-treated group (Figure 6.7 (a) and (b)). Interestingly, following the addition of recombinant CCL21, the production of IFN-γ, TNF-α, CXCL-12 and CCL21 were found to be increased. These interesting observations suggest that CCL21, other than being a chemotactic chemokine, could influence neuritogenesis in neuronal model. The current findings highlight the ability of CCL21 to enhance neuritogenesis following the activation of PI3K-Akt and ERK1/2 that induced NF-κB, cytokines and chemokines regulation. Nevertheless, further investigations are required to validate these observations. It is suggested for the role of CCL21 in neuronal differentiation to be investigated in primary neurons and neural stem cell model. CCL21 can induce distant microglia activation which can either enhance inflammatory responses or provide neutrophic effects on neurons. Therefore, CCL21 effects on microglial activation that could render the microglia to induce neurotrophic effects such as neuritogenesis on neurons should also be investigated.

6.6 Conclusion

The current findings highlighted the intricate inflammatory network between anti- and pro-inflammatory cytokines and chemokine by mesuagenin c in inducing its neuritogenic effects in NG108-15 cells. The inhibition of CCL21 reduced the neuritogenic activity of mesuagenin c and suggests its role in neuritogenesis. Collectively, mesuagenin c induced neuritogenesis through the activation of PI3K-Akt and ERK1/2 that modulate the transcriptional activity of NF-κB, cytokines and chemokines and galectin-1 regulation (Figure 6.8). These findings could pave the way for the integration of neuroprotective and neuritogenic activities of mesuagenin c in its development as therapeutic agent for neurodegenerative diseases through the regulation of inflammation via PI3K-Akt and ERK1/2.

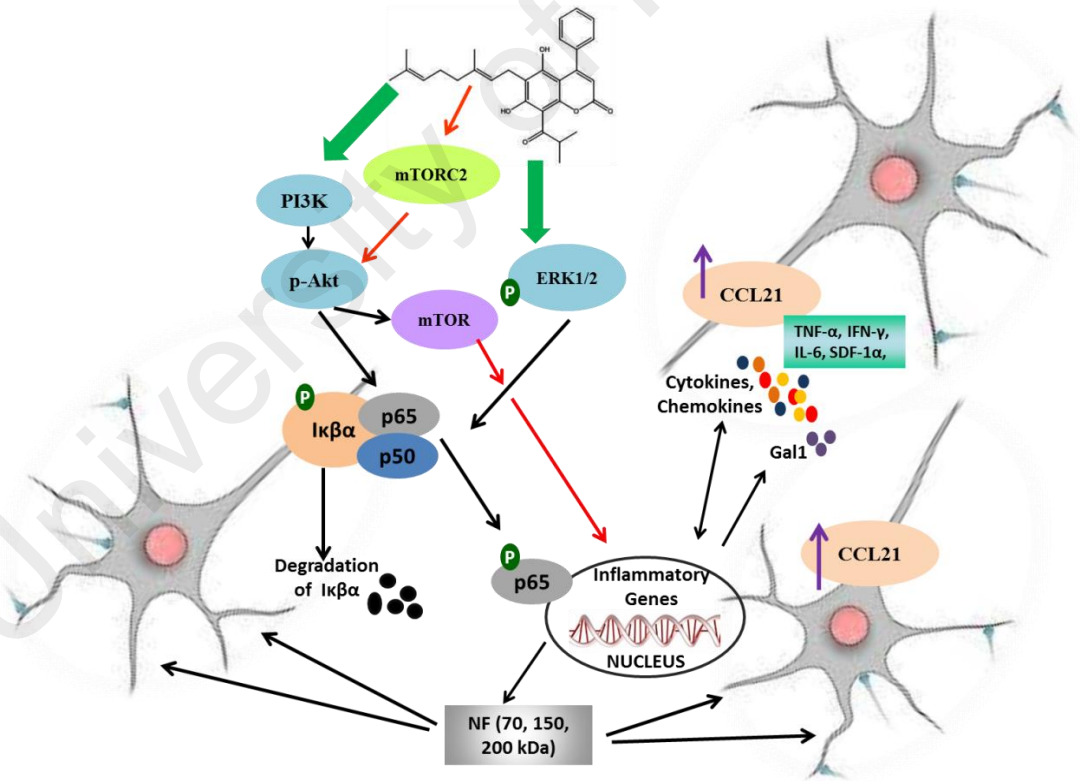


Figure 6.8 Schematic illustration of neuritogenic mechanisms of mesuagenin c in NG108-15 cells.

CHAPTER 7: CONCLUSION

(R)-(+)-α-lipoic acid and mesuagenin c modulated the NF-κB, cytokines, chemokines and galectin-3 regulation via PI3K-Akt activation and GSK-3β inactivation that induced a self-limiting feedback mechanism against LPS-stimulated BV-2 cells. CCL21 inibition enhanced the suppression of pro-inflammatory mediators which reduced the inflammatory responses between NG108-15 and BV-2 cells. Additionally, (R)-(+)-α-lipoic acid and mesuagenin c protected the NG108-15 cells against H_2O_2 by mitigating the mitochondrial-mediated caspase-dependent neuronal apoptosis through the activation of PI3K-Akt/GSK-β pathway via mTORC2. Furthermore, (R)-(+)-α-lipoic acid also induced the activation of mTORC1. The activation of PI3K-Akt also induced GSK-3β inhibition that modulated NF-κB, anti- and pro-cytokines regulation in NG108-15 cells. R-(+)-α-lipoic acid and mesuagenin c protected the neuronal models through PI3K-Akt activation and GSK-3β inactivation that inhibited oxidative stress and aberrant inflammatory responses between microglia and neurons which suppressed neuronal death (Figure 7.1). Additionally, mesuagenin c induced neuritogenesis by modulating NF-κB, cytokines, chemokines and galectin-1 via PI3K-Akt and ERK1/2 in NG108-15 cells (Figure 7.1). Moreover, CCL21 inhibition reduced mesuagenin c-induced neuritogenesis and highlighted its potential role in neuritogenesis. Moreover, a well regulated anti- and pro-inflammatory regulation through PI3K-Akt/GSK-β and ERK1/2 pathways is beneficial in protecting NG108-15 cells against aberrant inflammation during neuritogenesis. The findings suggest that both (R)-(+)-α-lipoic acid and mesuagenin c could be developed as potential therapeutic agents targeting multiple target sites via PI3K-Akt/GSK-β and ERK1/2 pathways in the intervention of neurodegenerative diseases. Although the mechanistic part of (R)-(+)-α-lipoic acid and mesuagenin c justify their role as potential therapeutics for neurodegenerative diseases, the current findings still need further investigation particularly, animal studies and *in*

vitro and *in vivo* BBB modeling. Moreover, computational modelling that investigate the (R)-(+)-α-lipoic acid and mesuagenin c interaction with receptors and proteins invoved in PI3K-Akt/GSK-3β and ERK1/2 pathways would provide better understanding on their mechanism of actions. Additionally, animal studies that include toxicity and pharmacokinetics studies are needed to justify the safety, bioavailability and metabolism of (R)-(+)-α-lipoic acid and mesuagenin c in the body. These suggested studies would be helpful in developing (R)-(+)-α-lipoic acid and mesuagenin c as multitarget therapeutics for the intervention of neurodegenerative diseases.

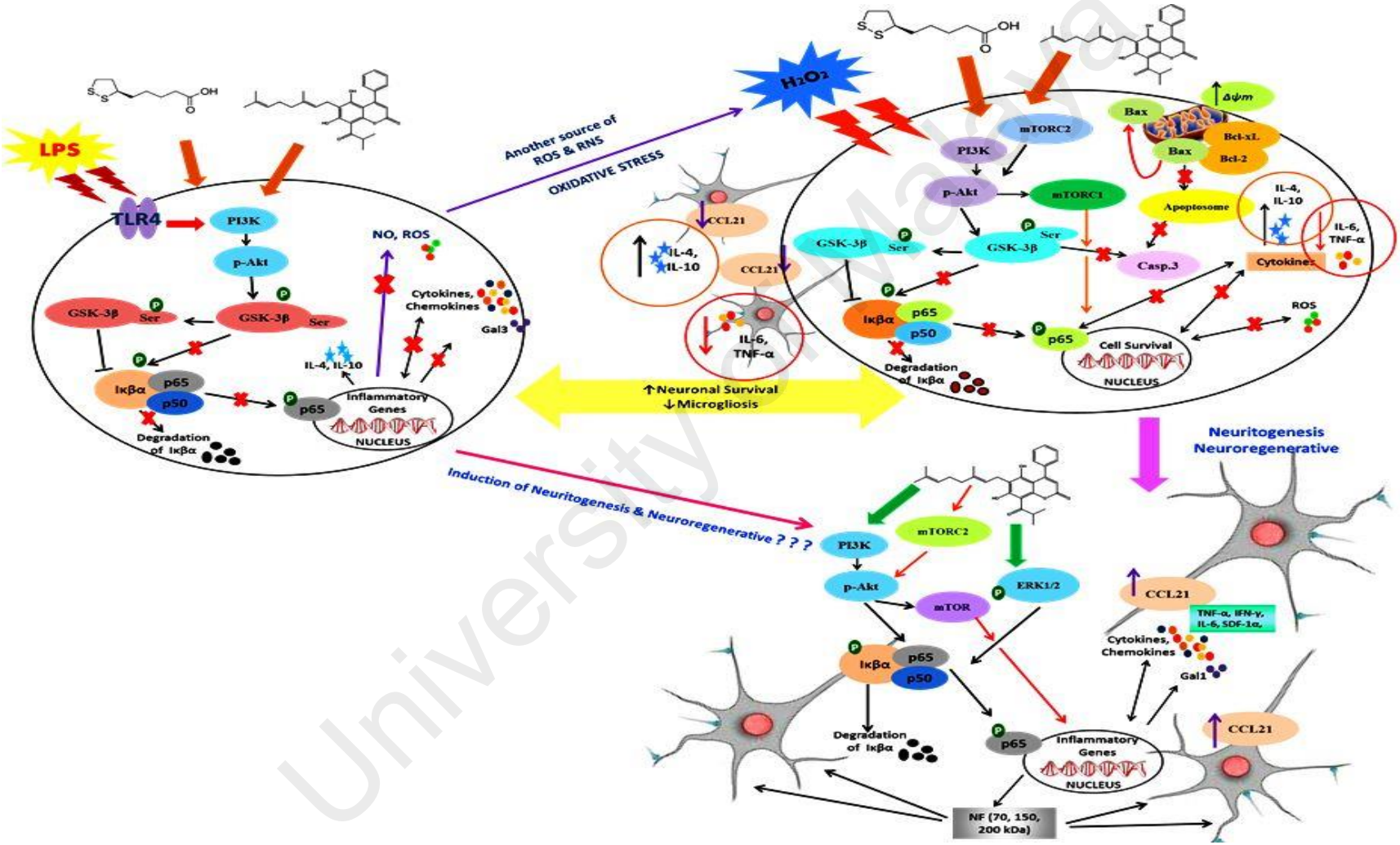


Figure 7.1 Activation of the PI3K-Akt/GSK-β and ERK1/2 pathways that modulate the regulation of NF-κB, cytokines, chemokines and Galectins.

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APPENDIX A- PAPERS PRESENTED AND AWARDS

1. Kamarudin, M. N. A., Kadir, H.A, Chan, G., & Awang, K. Mesuagenin C suppresses microglia inflammation and improves neuronal survival by modulating NF-κβ-cytokines and chemokines through PI3K-Akt/GSK-3β. International Post-Graduate Seminar University of Malaya 2014. (Oral Presentation, 1st Prize Presenter, International)

2. Kamarudin, M. N. A., Kadir, H.A, Chan, G., & Awang, K. Neuroprotection and neuritogenesis effects of Mesuagenin C from *Mesua kunstleri* in NG108-15 cells. The 1st World Congress on Healthy Ageing 2012, 19th to 22nd March 2012 at Kuala Lumpur Convention Centre. (Poster Presentation, 1st Prize Award, International)

3. Kamarudin, M. N. A., Kadir, H.A, Chan, G., & Awang, K. Neuroprotective and neuritogenic molecular mechanisms of novel mesuagenin C from Mesua kunstleri (King) Kosterm in NG108-15 cells. Symposium on Natural Products and Medicinal Chemistry (NPMC), 17th Malaysian Chemical Congress (17MCC), October 15- October 17, 2012, Putra World Trade Centre, Kuala Lumpur, Malaysia. (Oral Presentation, 1st Prize Presenter, International)

4. Kamarudin, M. N. A., Kadir, H.A, Chan, G., & Awang, K. Neuroprotective and neuritogenic molecular mechanisms of mesuagenin C from *Mesua kunstleri* through CCL21 regulation in NG108-15 cells. International Conference on Molecular Biology and Biochemistry, WASET. May 28 – Jun 1, Chiba, Tokyo, Japan. (Oral Presentation, International)

5. Mohd Raflee, N. A., Kamarudin, M. N. A. & Kadir, H.A. α-Lipoic acid mitigated H_2O_2-induced mitochondrial-mediated apoptosis in NG108-15 cells through PI3K-AKT pathway. 24th Intervarsity Biochemistry Seminar, 11 May 2013 to 11 May 2013, Taylor's University College & Malaysian Society of Biochemistry and Molecular Biology (MSBMB). (Poster Presentation, Best Poster Award, National)

6. Hadi Supriady, Kamarudin, M. N. A. & Kadir, H.A. Swietenia macrophylla ethyl acetate fraction suppressed LPS-induced neuroinflammation in BV-2 cells through PI3K/Akt and MAPK pathways. 39th Annual Conference of The Malaysian Society for Biochemistry & Molecular Biology, 25th-26th June 2014, Sama-Sama

Hotel, KLIA, Sepang, Malaysia. (Poster Presentation, Best Poster Award, National)

7. Kamarudin, M. N. A., Kadir, H.A, Chan, G., & Awang, K. (2014). Mesuagenin C attenuated LPS-stimulated BV-2 and NG108-15 cells by modulating NF-κβ, CCL21 and cytokines through PI3K- Akt/GSK-3β. Special Issue: FEBS EMBO 2014 Conference, *FEBS Journal*, 281(s1), 1(823). Proceeding of the FEBS EMBO 2014 Conference held at Palais des Congrès in Paris, France on August 30-September 4, 2014. (Poster Presentation, International)

8. Kamarudin, M. N. A., & Kadir, H.A. Protective effects of (R)-(+)-α-Lipoic Acid against MPP+-stimulated microglia cells and toxicity in dopaminergic SH-SY5Y cells through PI3K-Akt/GSK- 3β pathway. Special Issue: FEBS EMBO 2014 Conference, *FEBS Journal*, 281(s1), 1(823). Proceeding of the FEBS EMBO 2014 Conference held at Palais des Congrès in Paris, France on August 30-September 4, 2014. (Poster Presentation, International)

9. Kamarudin, M. N. A., Kadir, H.A, & N. A. M. (2013). Catechin supressed LPS-induced neuroinflammation in BV-2, C6 and NG108-15 cells by modulating CCL21 through PI3K-Akt. *Journal of the Neurological Sciences*, 333, e337. Proceeding of the XXI World Congress of Neurology held at Reed Messe Wien GmbH Congress Center, Vienna, Austria on September 21-26, 2013. (Poster Presentation, International)

10. Kadir, H.A, Kamarudin, M. N. A., Raflee, N. A. M. & Shabab, T. (2013). α-Lipoic acid protects against LPS-induced BV-2 activation and MPTP- induced toxicity in SH-SY5Y neuronal cells. *Journal of the Neurological Sciences*, 333, e338. Proceeding of the XXI World Congress of Neurology held at Reed Messe Wien GmbH Congress Center, Vienna, Austria on September 21-26, 2013. (Poster Presentation, International)

11. Kamarudin, M. N. A., Kadir, H.A, Chan, G., & Awang, K. (2013). Mesuagenin C mitigated LPS-induced neuroinflammation in BV-2 and NG108-15 cells through pMTOR-PI3K-Akt and CCL21 downregulation. *Journal of the Neurological Sciences*, 333, e349. Proceeding of the XXI World Congress of Neurology held at Reed Messe Wien GmbH Congress Center, Vienna, Austria on September 21-26, 2013. (Poster Presentation, International)

12. Lo Jia Ye, Muhamad Noor Alfarizal Kamarudin, Habsah Abdul Kadir. Curcumenol from curcuma zedoaria suppresses neuroinflammation induced by LPS in BV-2 neuronal cell model. 18th Biological Sciences Graduate Congress, 06 Jan 2014 to 08 Jan 2014, Faculty of Science, University of Malaya, (International). (Poster Presentation, 2nd Prize Best Poster, International)

13. Sharifah Salwa Syed Hussein, Muhamad Noor Alfarizal Kamarudin, Habsah Abdul Kadir. Catechin from Loranthus parasiticus (L.) Merr attenuated LPS-induced neuroinflammation in BV-2 cells. 38th The Malaysian Society for Biochemistry & Molecular Biology Annual Conference, 28 Aug 2013 to 29 Aug 2013, The Malaysian Society for Biochemistry & Molecular Biology (MSBMB), (National). (Poster Presentation, National)

14. Sharifah Salwa Syed Hussein, Muhamad Noor Alfarizal Kamarudin, Habsah Abdul Kadir. (+)-Catechin Attenuates NF-κB Activation Through Regulation of Akt, MAPK, and AMPK Signaling Pathways in LPS-Induced BV-2 Microglial Cells. 19th Biological Sciences Graduate Congress, 12 Dec 2014 to 14 Dec 2014, Department of Biological Sciences in National University of Singapore (International). (Poster Presentation, 2nd Prize Poster, International)

APPENDIX B- LIST OF PUBLICATION

I. Chan, G., Kamarudin, M. N. A., Wong, D. Z. H., Ismail, N. H., Abdul Latif, F., Hasan, A., . . . Abdul Kadir, H. (2012). Mitigation of H_2O_2-induced mitochondrial-mediated apoptosis in NG108-15 cells by novel mesuagenin C from Mesua kunstleri (King) Kosterm. *Evidence-Based Complementary and Alternative Medicine*, 2012.

Special Issue: Therapeutic Approaches to Neuroprotective Activity by Complementary and Alternative Medicines

II. Kamarudin, M. N. A., Raflee, N. A. M., Hussein, S. S. S., Lo, J. Y., Supriady, H., & Kadir, H. A. (2014). (R)-(+)-α-Lipoic acid protected NG108-15 cells against H_2O_2-induced cell death through PI3K-Akt/GSK-3β pathway and suppression of NF-κβ-cytokines. *Drug design, development and therapy, 8*, 1765.

III. Syed Hussein, S. S., Alfarizal Kamarudin, M. N., & Kadir, H. A. (+)-Catechin Attenuates NF-κB Activation Through Regulation of Akt, MAPK, and AMPK Signaling Pathways in LPS-Induced BV-2 Microglial Cells. The American Journal of Chinese Medicine, 0(0), 1-26.

IV. Ye, L. J., Kamarudin, M. N. A., Hamdi, O. A. A., Awang, K. & Kadir, H. A. (2015). Curcumenol isolated from Curcuma zedoaria suppresses Akt-mediated NF-κB activation and p38 MAPK signaling pathway in LPS-stimulated BV-2 microglial cells. Food and Function.

V. Supriady, H., Kamarudin, M. N. A., Chan, C. K., Goh, B. H., & Kadir, H. A. (2015). SMEAF attenuates the production of pro-inflammatory mediators through the inactivation of Akt-dependent NF-κB, p38 and ERK1/2 pathways in LPS-stimulated BV-2 microglial cells. Journal of Functional Foods, 17, 434-448.

VI. Hamdi, O. A. A., Ye, L. J., & Alfarizal, M. N. (2015). Neuroprotective and Antioxidant Constituents from Curcuma zedoaria Rhizomes. Records of Natural Products, 9(3).

VII. Chan, C. K., Goh, B. H., Kamarudin, M. N. A., & Kadir, H. A. (2012). Aqueous Fraction of Nephelium ramboutan-ake Rind Induces Mitochondrial-Mediated Apoptosis in HT-29 Human Colorectal Adenocarcinoma Cells. Molecules, 17(6), 6633.

VIII. Goh, B. H., Chan, C. K., Kamarudin, M. N. A., & Abdul Kadir, H. (2014). Swietenia macrophylla King induces mitochondrial-mediated apoptosis through p53 upregulation in HCT116 colorectal carcinoma cells. Journal of Ethnopharmacology, 153(2), 375-385.

IX. Moghadamtousi, S. Z., Kamarudin, M. N. A., Chan, C. K., Goh, B. H., & Kadir, H. A. (2014). Phytochemistry and Biology of Loranthus parasiticus Merr, a Commonly Used Herbal Medicine. The American Journal of Chinese Medicine, 42(01), 23-35.

X. Kamarudin, M. N. A., Chan, G., Awang, K. & Kadir, H. A. (2015). Novel Mesuagenin C modulates NF-κB-cytokines, chemokines and Galectin-3 by activating PI3K-Akt/GSK-3β in LPS-stimulated BV-2 co-cultured with NG108-15 cells. Scientific Reports (SUBMITTED)