



UNIVERSITI PUTRA MALAYSIA

MECHANISM OF THE ANTI-INFLAMMATORY ACTION OF 3-(2-HYDROXY-PHENYL)-1-(5-METHYL-FURAN-2-Y-L) PROPENONE (HMP)

LIEW CHOI YI

FPSK(m) 2010 13

MECHANISM OF THE ANTI-INFLAMMATORY ACTION OF 3-(2-HYDROXY-PHENYL)-1-(5-METHYL-FURAN-2-Y-L) PROPENONE (HMP)

By

LIEW CHOI YI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science**

July 2010

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirement for the degree of Master of Science

MECHANISM OF THE ANTI-INFLAMMATORY ACTION OF 3-(2-HYDROXY-PHENYL)-1-(5-METHYL-FURAN-2-Y-L) PROPENONE (HMP)

By

LIEW CHOI YI

July 2010

Chairman: Professor Daud Ahmad bin Israf Ali, PhD

Faculty: Medical and Health Sciences

Chalcones, a subgroup of flavonoids, are found in many plants and many synthetic analogues have been artificially synthesized. Many natural and synthetic chalcones exhibit varying degrees of anti-inflammatory activity. In an attempt to discover more potent anti-inflammatory compounds, 3-(2-hydroxyphenyl)-1-(5-methyl-furan-2-y-l) propenone (HMP) was evaluated for its ability to inhibit the synthesis of major proinflammatory mediators and cytokines in interferon- γ (IFN- γ)- and lipopolysaccharide (LPS)-induced RAW 264.7 cells and phorbol myristate acetate (PMA)-differentiated/LPS-induced U937 cells in study I, II, III and IV respectively. The 96-well plate assays included cell viability test, griess, chemical scavenging assay and enzyme-linked immunosorbent assays were conducted meanwhile western blotting, reverse transcription-polymerase chain reactions, immunoprecipitation, kinase assay, electrophoretic mobility shift assay and docking experiment were applied for molecular

detection throughout the studies. In study I, II and III, HMP suppressed the production of nitric oxide (NO) at doses as low as 0.78 μ M, prostaglandin E2 (PGE2) and interleukin (IL)-1 β secretion at doses of 12.5 μ M and above meanwhile tumor necrosis factor (TNF)- α and IL-6 secretion at 25 μ M with significant inhibitory effects ($p < 0.05$). HMP did not affect the secretion of chemokines IL-8 and monocyte chemotactic protein-1 (MCP-1) and the anti-inflammatory cytokine IL-10. HMP showed a dose-dependent inhibition of NO synthesis as demonstrated from NO secretion and inducible nitric oxide synthase (iNOS) expression. For study III and IV in which western blotting and kinase assay were conducted, the inhibition of NO synthesis was related to the inhibition of p38 phosphorylation and potent inhibition of p38 kinase activity that led to significant inhibition of phosphorylation of activating transcription factor (ATF)-2. This effect in turn caused significant inhibition of activating protein (AP)-1-DNA binding which partially explains the inhibitory effect upon the synthesis of iNOS. Interestingly, HMP failed to alter phosphorylation of extracellular-signal-related-kinase (ERK) 1/2 and Jun N-terminal kinase (JNK) and did not affect their kinase activity. Furthermore, HMP also failed to inhibit phosphorylation of Inhibitory protein κ B (I- κ B), nuclear translocation of p65 nuclear factor- κ B (NF- κ B) and DNA binding of p65 NF- κ B. Phosphorylation of signal transducers and activators of transcription (STAT)-1 was also unaffected by HMP. Molecular docking experiments confirmed that HMP fits well in the highly conserved hydrophobic pocket of p38 MAP kinase. In conclusion, in contrast to many anti-inflammatory chalcones, HMP shows a higher selectivity toward NO inhibition therefore providing an interesting drug lead that has potentially less side effects.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

**MEKANISME ANTI-RADANG 3-(2-HYDROXY-PHENYL)-1-
(5-METHYL-FURAN-2-Y-L) PROPENONE (HMP)**

Oleh

LIEW CHOI YI

Julai 2010

Pengerusi: Profesor Daud Ahmad bin Israf Ali, PhD

Fakulti: Perubatan dan Sain Kesihatan

Calkon, sejenis subkelompok flavonoid, diterima dalam banyak tumbuhan telah dihasilkan dalam bentuk analog sintetik. Kebanyakan calkon semula jadi ataupun analog sintetik menunjukkan pelbagai darjah aktiviti anti-radang. Untuk menemui lebih banyak anti-radang *compound*, keupayaan 3-(2-hidroksi-fenil)-1-(5-metil-furan-2-il) propenone (HMP) telah dikaji untuk menghalang mediator pro-inflamasi dan sitokin yang dihasilkan daripada induksi oleh interferon (IFN)- γ dan lipopolisakarida (LPS) dalam sel-sel RAW264.7 dan juga induksi oleh LPS dalam sel-sel U937 yang telah ditransformasi oleh forbol miristat asetat (PMA) dalam kajian I, II, III dan IV masing-masing. Ujian-uji *96-well plate* dimana termasuknya kajian tentang kebolehidupan sel, *griess*, *chemical scavenging* dan asai imunoserapan terangkai enzim telah digunakan. *Western blotting*, tindak balas rantaian *reverse transcription-polymerase*, pengimmunomendakan, ujian kinase, ujian syif pergerakan elektroforesis dan eksperimen

docking juga digunakan dalam keseluruhan projek ini. HMP dapat menghalang pengeluaran oksida nitrik (NO) pada dos serendah 0.78 μ M, prostaglandin E2 (PGE2) and IL-1 pada dos 12.5 μ M dan ke atas serta tumor nekrosis faktor (TNF)- dan IL-6 pada dos 25 μ M dengan $p < 0.05$ dalam ujian-ujian I, II and III masing-masing. HMP tidak dapat mempengaruhi penghasilan IL-8 dan *monocyte chemotactic protein* (MCP)-1 serta sitokin anti-inflamasi (IL-10). Pengurangan NO oleh HMP adalah disebabkan oleh sekatan terhadap rembesan NO dan ekspres iNOS. Melalui *western blotting* dan ujian kinase dalam ujian III and IV, penyekatan terhadap fosforilasi p38 protein dan juga aktiviti enzim-enzim ini yang akan menghasilkan *phospho-ATF2* juga dikurangkan. Kesan-kesan tersebut menyebabkan pula berlakunya halangan terhadap *activating protein* (AP)-1 aktiviti pengikatan di mana halangan inilah yang menyebabkan pengeluaran NO oleh HMP. HMP tidak berkesan terhadap fosforilasi oleh *extracellular-signal-related-kinase* (ERK) 1/2 dan *Jun N-terminal kinase* (JNK) serta aktiviti-aktiviti oleh enzim-enzim ini. Selain itu, HMP juga gagal menyekat fosforilasi oleh *inhibitory protein κ B* (I- κ B) ataupun *signal transducers and activators of transcription* (STAT)-1 dan nuklear translokasi serta *DNA binding* oleh nuklear faktor- κ B (NF- κ B). Pengajian molekul docking telah membuktikan bahawa penyekatan p38 oleh HMP adalah secara selektif. Tambahan pula, HMP menunjukkan persamaan dalam pola pengikatan terhadap halaman aktif p38 *kinase* apabila dibandingkan dengan SB 203580 (p38 inhibitor selektif) tersebut dalam pengajian tersebut. Secara kesimpulannya, keputusan-keputusan ini menunjukkan bahawa tindakan anti-radang oleh HMP adalah lebih selektif, dijangka dapat mengurangkan akibat sampingan jika dibandingkan dengan calkon-calkon yang lain.

ACKNOWLEDGEMENTS

First, I would like to express my full gratitude to my project supervisor, Professor Dr. Daud Ahmad Israf Ali, the Chairman of my Supervisory Committee, for his invaluable advice, guidance, constant support and encouragement.

I would like to extend my appreciation to Dr. Kim Min Kyu and my co-supervisors, Professor Dr. Mohd Roslan bin Sulaiman and Associate Professor Dr. Cheah Yoke Kqueen, from the Department of Biomedical Sciences, Faculty of Medicine and Health Sciences for their generous input, constructive criticism, advice and support throughout the course of this study. In addition, I would like thank to my colleagues, all the staff and friends of the cell signaling and molecular biology laboratories, Faculty of Medicine and Health Sciences for their kind, excellent and constant technical assistance.

Subsequently, I would like to extend my thanks and appreciation to Professor Dr Md Nordin Hj Lajis for providing compounds for this research project as well as his student, Lam Kok Wai for conducting the chemical synthesis and docking experiment of the compounds. Last but not least, I would like to express my greatest and innumerable blessing and gratitude to my dearest parents and family members for their sacrifice, moral support and constant love all this time.

I certify that a Thesis Examination Committee has met on date of viva voce to conduct the final examination of Liew Choi Yi on her Master of Science thesis entitled 'Mechanism of anti-inflammatory action of 3-(2-hydroxy-phenyl)-1-(5-methyl-furan-2-y-l) propenone (HMP)' in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded degree of Master of Science.

Members of the Examination Committee were as follows:

Mohd Aziz Dollah, PhD

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Sharida Fakurazi, PhD

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Zainul Amirudin Zakaria, PhD

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Ahmad Rohi Ghazali, PhD

Associate Professor
Faculty of Allied Health Sciences
Universiti Kebangsaan Malaysia
(External Examiner)

BUJANG KIM HUAT, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 19 July 2010

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Daud Ahmad bin Israf Ali, PhD

Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Mohd Roslan bin Sulaiman, PhD

Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Cheah Yoke Kqueen, PhD

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

HASANAH MOHD GHAZALI, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 19 July 2010

DECLARATION

I declare that the thesis is my original work except for the synthesis of tested compounds, docking experiment, quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

LIEW CHOI YI

Date: 19 July 2010

TABLE OF CONTENTS

	Page
ABSTRACT	I
ABSTRAK	III
ACKNOWLEDGEMENTS	V
APPROVAL	VI
DECLARATION	VIII
LIST OF TABLES	XII
LIST OF FIGURES	XIII
LIST OF ABBREVIATIONS/ ANNOTATIONS	XVI
 CHAPTER	
 1 INTRODUCTION	 1
2 LITERATURE REVIEW	6
2.1 Inflammation	6
2.2 Nitric oxide and inducible nitric oxide synthase	8
2.3 Prostaglandin and cyclooxygenase-2	9
2.4 Cytokines	10
2.5 Proinflammatory Activation Pathways	11
2.5.1 Janus kinases/ signal transducers and activators of transcription (JAK/STAT)	11
2.5.2 Mitogen-activated protein kinases (MAPKs)	12
2.5.3 Nuclear factor- κ B (NF- κ B)	16
2.6 Anti-inflammatory drugs	18
2.6.1 Corticosteroids	18
2.6.2 Non-steroidal anti-inflammatory drugs (NSAIDs)	19
2.6.3 Natural anti-inflammatory compounds	22
2.7 Chalcone	23
3 MATERIALS AND METHODS	26
3.1 Media preparation	26
3.2 Cell culture and viability	26
3.3 Cell viability assay	27
3.4 Cell treatment	28
3.5 Reverse transcription-polymerase chain reaction (RT-PCR) analysis	29
3.5.1 Ribonucleic acid (RNA) preparation	29
3.5.2 Quantitation of RNA	30
3.5.3 RNA integrity check	30

3.5.4	RT-PCR	31
3.5.5	Electrophoresis	31
3.6	Western Blot Analysis	32
3.6.1	Protein preparation	32
3.6.2	Preparation of the whole cell extract	33
3.6.3	Preparation of nuclear and cytoplasmic fractions	34
3.6.4	Determination of protein concentration	34
3.6.5	Preparation for sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)	35
3.6.6	Gel electrophoresis	35
3.6.7	Gel staining and destaining	36
3.6.8	Semi-dry protein transfer	36
3.6.9	Membrane staining	37
3.6.10	Immunoblotting	37
3.6.11	Visualisation	38
3.7	Immunoprecipitation and kinase assay	38
3.7.1	Preparation of cell lysates	39
3.7.2	Immunoprecipitation with immobilized antibodies	39
3.7.3	Kinase assay	39
3.7.4	Immunoblotting	40
3.8	Electrophoretic mobility shift assay (EMSA)	40
3.8.1	Preparation of cell lysates	41
3.8.2	Oligonucleotide labeling	41
3.8.3	Gel shift Assay	42
3.9	Study I: Nitrite secretion assay	43
3.10	Study I: iNOS activity assay	44
3.11	Study I: Nitrite scavenging activity assay	45
3.12	Study I: iNOS mRNA expression analysis	46
3.13	Study I: iNOS protein expression analysis	46
3.14	Study II: PGE2 secretion assay	47
3.15	Study II: COX-2 activity assay	48
3.16	Study II: COX-2 mRNA expression analysis	48
3.17	Study II: COX-2 protein expression analysis	49
3.18	Study III: Cytokine secretion determination	49
3.19	Study III: Proinflammatory cytokines (TNF- α and IL-1 β) mRNA expression analysis	50
3.20	Study IV: MAPKs protein expression analysis	50
3.21	Study IV: MAPKs activity assay	51
3.22	Study IV: I- κ B expression analysis	52
3.23	Study IV: p65 NF- κ B expression analysis	53

3.24	Study IV: STAT1 expression analysis	53
3.25	Study IV: NF- κ B and AP-1 DNA binding activity assay	54
3.26	Study IV: Docking experiment	54
3.27	Statistical analysis	55
4	RESULTS	56
4.1	Effect of HMP on cell viability of RAW264.7 and U937 cells	56
4.2	Study I: Effect of HMP on NO secretion	58
4.3	Study I: Effect of HMP on iNOS enzyme activity	60
4.4	Study I: Scavenging activity of HMP	61
4.5	Study I: Effect of HMP on mRNA level of iNOS gene	62
4.6	Study I: Effect of HMP on iNOS protein expression	63
4.7	Study II: Effect of HMP on PGE2 secretion	64
4.8	Study II: Effect of HMP on COX-2 enzymatic activity	65
4.9	Study II: Effect of HMP on COX-2 gene expression	66
4.10	Study II: Effect of HMP on COX-2 protein expression	67
4.11	Study III: Effect of HMP on proinflammatory cytokine and chemokines secretion	68
4.12	Study III: Effect of HMP on proinflammatory cytokines mRNA (TNF- α and IL-1 β)	71
4.13	Study IV: Effect of HMP on activation of MAPKs in RAW 264.7 cells	72
4.14	Study IV: Effect of HMP on p38 activity	76
4.15	Study IV: Effect of HMP on phosphorylation and degradation of I- κ B α	78
4.16	Study IV: Effect of HMP upon the IFN- γ /LPS-induced levels of p65 into the nucleus	79
4.17	Study IV: Effect of HMP on phosphorylation of STAT	81
4.18	Study IV: Effect of HMP on NF- κ B and AP-1 DNA binding activity	82
4.19	Study IV: Docking Studies of HMP upon p38 kinase	83
5	DISCUSSION	86
6	CONCLUSION	96
	REFERENCES	97
	APPENDICES	113
	BIODATA OF STUDENT	140
	LIST OF PUBLICATIONS	142