UNIVERSITI PUTRA MALAYSIA

EFFECTS OF 2,4,6-TRIHYDROXY-3-GERANYL ACETOPHENONE ON GROWTH FACTOR-INDUCED HUMAN BRONCHIAL SMOOTH MUSCLE CELL PROLIFERATION IN ASTHMA

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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March 2018

Chair : Prof. Daud Ahmad Israf Ali, PhD
Faculty : Medicine and Health Sciences

Asthma is a chronic inflammatory disease of the airways, which can be characterized by airway remodeling and hyperresponsiveness. Increased airway smooth muscle (ASM) mass appears to be a prominent hallmark of airway remodeling attributed to the release of mitogenic factors during the inflammatory process. Corticosteroids and beta-agonists remain the mainstay of current asthma treatment, but do not specifically target airway remodeling. Previous studies have shown that 2,4,6-trihydroxy-3-geranyl acetophenone (tHGA), a non-steroidal synthetic compound, demonstrated anti-inflammatory activity as well as anti-remodeling properties in a chronic murine model of asthma. In this study, the effects of tHGA upon human bronchial smooth muscle cell (hBSMCs) proliferation, apoptosis and migration in response to growth factors was evaluated. hBSMCs were serum-starved overnight prior to induction with growth factor-enriched medium and co-treated with tHGA or forskolin for 48 hours. Cell cycle analysis was carried out to examine the mechanism of action of tHGA upon hBSMCs proliferation. Expression of cell cycle proteins cyclin D1 and $p27^{Kip1}$ was assessed through immunoblotting. The identification of the molecular target of tHGA involved expression studies upon major proliferation-associated signaling pathways involved in ASM proliferation, which include MAPK, PI3K and JAK2/STAT3. Signaling pathways were examined through immunoblotting, immunoprecipitation and kinase assays while the potential molecular target was reconfirmed through transfection. tHGA, at concentration of 20 μM and below, did not cause significant release of LDH, thus was used in the following experiments. tHGA, at concentration of 20 μM and 10 μM, was shown to significantly inhibit hBSMCs proliferation and migration without inducing apoptosis. tHGA, at 20 μM, reduced hBSMCs proliferation to 46.9 ± 5.2% as compared to growth factor-induced cells (100%). This finding was further reconfirmed through Ki-67 expression study. tHGA, at 20 μM, inhibited Ki-67 expression in growth factor-induced cells from a fold change of 1.03 ± 0.03 to 0.43 ± 0.11. The anti-proliferative effect was due to cell cycle arrest at the G_1 phase accompanied by a reduction of cyclin D1 and diminished

degradation of $p27^{Kip1}$ expression. tHGA, at concentration of 20 µM and 10 µM, significantly increased the percentage of hBSMCs at G_1 phase from 39.7 ± 2.1% to 71.6 ± 2.0% and 50.6 ± 1.3% respectively. Besides that, scratch assay revealed that 20 µM of tHGA attenuated cell number of hBSMCs that migrated to the scratch area from 108.6 ± 7.5 cells/mm^2 to 36.2 ± 11.0 cells/mm^2. Analysis of proliferation-related signaling pathways demonstrated tHGA to act as an inhibitor of AKT, JNK and STAT3 phosphorylation. tHGA did not affect the activation of the upstream activators of STAT3 and AKT, thus the potential molecular target was narrowed down to AKT. The major effect upon phosphorylation of AKT was further confirmed following treatment of hBSMCs transfected with constitutively-active AKT (myr-AKT). tHGA was suggested to inhibit the phosphorylation of AKT that leads to cyclin D1 downregulation and growth inhibition. This study highlights the anti-remodeling potential of this drug lead in chronic airway disease.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN 2,4,6-TRIHIDROKSI-3-GERANIL ASETOFENON KEATAS PROLIFERASI SEL OTOT LEMBUT BRONKIAL MANUSIA YANG DIINDUKSI DENGAN FAKTOR PERTUMBHUAN DALAM ASMA

Oleh

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Asma adalah penyakit keradangan kronik saluran pernafasan yang bercirikan pemodelan semula dan kehipergerakbalasan. Penambahan jisim sel otot lembut saluran pernafasan merupakan antara ciri utama dalam pemodelan semula saluran pernafasan yang disebabkan oleh pembebasan faktor mitogenik semasa proses keradangan. Walaupun kortikosteroid dan agonis adrenoseptor beta merupakan rawatan asma yang utama, namun drug ini tidak menyasarkan pemodelan semula secara spesifik. Kajian sebelum ini telah menunjukkan bahawa 2,4,6-trihidroksi-3-geranil asetofenon (tHGA), satu sebatian sintetik bukan-steroid, mempunyai aktiviti anti-keradangan dan anti-pemodelan semula dalam model asma mencit kronik. Kajian ini menentukan kesan rawatan tHGA ke atas proliferasi, migrasi dan apoptosis sel otot lembut saluran pernafasan (hBSMCs) yang dirangsangkan oleh faktor pertumbuhan. Sel hBSMCs dibiarkan semalaman tanpa serum sebelum induksi dengan faktor pertumbuhan dan dirawat serentak dengan tHGA ataupun forskolin selama 48 jam. Analisis kitaran sel dijalankan untuk mengkaji mekanisma tindakan tHGA ke atas proliferasi sel hBSMCs. Ekspresi protin yang berkait rapat dengan kitaran sel iaitu cyclin D1 dan $p27^{Kip1}$ dikaji dengan menggunakan kaedah *immunoblotting*. Pengenalpastian molekul sasaran tindakan tHGA melibatkan kajian ekspresi ke atas tapakjalan pengisyaratan utama proliferasi sel hBSMCs iaitu MAPK, PI3K dan JAK2/STAT3. Tapakjalan pengisyaratan dikaji dengan kaedah *immunoblotting*, *immunoprecipitation* dan ujikaji kinase sementara molekul sasaran yang berpotensi dikenalpasti dengan kaedah transfeksi. tHGA, pada konsentrasi 20 μM dan 10 μM, didapati merencatkan proliferasi dan migrasi sel hBSMCs secara bermakna tanpa menginduksikan apoptosis. tHGA, pada konsentrasi 20 μM, menurunkan proliferasi hBSMCs kepada 46.9 ± 5.2% jika dibandingkan dengan sel yang diinduksikan dengan faktor pertumbuhan (100%). Hasil kajian ini dikenalpastikan dengan kajian ekpresi Ki-67. tHGA, pada konsentrasi 20 μM, merencatkan ekspresi Ki-67 dalam sel yang diinduksikan dengan faktor pertumbuhan daripada 1.03 ± 0.03 kadar perubahan kepada 0.43 ± 0.11. Kesan anti-proliferasi ini dikaitkan dengan penyekatan kitaran sel pada fasa G_1 yang diiringi dengan penurunan ekspresi cyclin D1 dan pengurangan degradasi

$p27^{Kip1}$. tHGA, pada konsentrasi 20 µM dan 10 µM, meningkatkan peratus hBSMCs yang berada pada fasa G_1 daripada 39.7 ± 2.1% kepada 71.6 ± 2.0% dan 50.6 ± 1.3% masing-masing. Selain daripada itu, *scratch assay* menunjukkan bahawa tHGA pada kepekatan 20 µM mengurangkan bilangan sel hBSMCs yang bermigrasi ke kawasan goresan daripada 108.6 ± 7.5 cells/mm^2 kepada 36.2 ± 11.0 cells/mm^2. Analisis tapakjalan pengisyaratan berkait-proliferasi menunjukkan tHGA bertindak sebagai perencat fosforilasi AKT, JNK dan STAT3. tHGA tidak mempengaruhi pengaktifan *upstream activators* kepada STAT3 dan AKT, oleh itu molekul sasaran yang berpotensi dikhususkan kepada AKT. Kesan utama keatas fosforilasi AKT disahkan melalui rawatan sel hBSMCs yang ditransfeksi dengan AKT yang aktif-juzuk (myr-AKT). tHGA dicadangkan merencatkan fosforilasi AKT lalu menyebabkan penurunan ekspresi cyclin D1 dan rencatan pertumbuhan. Kajian ini menonjolkan potensi anti-pemodelan semula oleh tHGA dalam penyakit saluran pernafasan kronik.

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I certify that a Thesis Examination Committee has met on 6 March 2018 to conduct the final examination of **YAP HUI MIN** on her thesis entitled "**EFFECTS OF 2,4,6-TRIHYDROXY-3-GERANYL ACETOPHENONE (tHGA) UPON GROWTH FACTOR-INDUCED HUMAN BRONCHIAL SMOOTH MUSCLE CELL PROLIFERATION IN ASTHMA"** 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 the degree of Doctor of Philosophy.

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AHR	: Airway hyperresponsiveness
ASK	: Apoptosis signal-regulating kinase
α-SMA	: Alpha smooth muscle actin
ASM	: Airway smooth muscle
ATF	:Activating transcription factor
AP-1	: Activator protein-1
BAD	: Bcl-2-associated death promoter
BCA	: Bicinchoninic acid
bFGF	: Basic fibroblast growth factor
BrdU	: Bromodeoxyuridine
BSA	: Bovine serum albumin
BT	: Bronchial thermoplasty
CAK	: CDK-activating kinase
cAMP	: Cyclic adenosine monophosphate
CAMs	: Cell adhesion molecules
CCL3/5	: Chemokine (C-C motif) ligand 3/5
CDK(s)	: Cyclin-dependent kinase(s)
C/EBP-α	: CCAAT-enhancer binding protein-α
CHAPS	: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CKI	: Cyclin-dependent kinase inhibitor
CKS1	: Cyclin-dependent kinases regulatory subunit 1
COX	: Cyclo-oxygenase
CREB	: cAMP-response element binding protein
CRM1	: Chromosome region maintanence 1

CXCL8 : C-X-C motif chemokine ligand 8

CysLTs : Cysteinyl leukotrienes

DSS : Disuccinimidyl suberate

ECM : Extracellular matrix

EGF : Epidermal growth factor

EMT : Epithelial-mesenchymal transition

ERK : Extracellular receptor kinase

FITC : Fluorescein isothiocyanate

FoxO : Forkhead box O

GAPDH : Glyceraldehyde-3-phosphate dehydrogenase

GDP : Guanosine diphosphate

GFP : Green fluorescent protein

GM-CSF : Granulocyte/macrophage colony-stimulating factor

GPCRs : G protein-coupled receptors

GR : Glucocorticoid receptor

GSK3β : Glycogen synthase kinase 3 beta

GTP : Guanosine triphosphate

HA : Hemagglutinin

hBSMCs : Human bronchial smooth muscle cells

hFGF : Human fibroblast growth factor

hEGF : Human epidermal growth factor

HUVECs : Human umbilical vein endothelial cells

ICS : Inhaled corticosteroids

IGF : Insulin-like growth factor

IgE : Immunoglobulin E

IgG : Immunoglobulin G

IL-4/5/8/9 : Interleukin-4/5/8/9

IP : Immunoprecipitation

JAKs : Janus kinases

JAK2 : Janus kinase 2

JNK : c-Jun N-terminal kinase

LABA : Long acting beta agonists

LB : Luria Bertani

LDH : Lactate dehydrogenase

LOX : Lipoxygenase

LPS : Lipopolysaccharide

MAPKK : MAPK kinase

MAPKKK : MAPKK kinase

MAPK(s) : Mitogen-activated protein kinase(s)

Mdm2 : Murine double minute 2

MEF2A : Myocyte enchancer factor 2A

MEK : MAPK/ERK kinase

MEKK : MEK kinase

MLCK : Myosin light chain kinase

MLK : Mixed lineage kinase

mLST8 : Mammalian lethal with SEC13 protein 8

mRNA : Messenger ribonucleic acid

mTOR : Mammalian target of rapamycin

mTORC1 : Mammalian target of rapamycin complex 1

mTORC2 : Mammalian target of rapamycin complex 2

MTS : 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-
-(4-sulfophenyl)-2H-tetrazolium)

NFATC3 : Nuclear factor of activated T-cells 3

NF-κB : Nuclear factor kappa B

OVA : Ovalbumin

PCNA : Proliferating cell nuclear antigen

PDGF : Platelet-derived growth factor

PDK1 : 3-phosphoinositide-dependent protein kinase-1

PFA : Paraformaldehyde

PH : Pleckstrin homology

PI : Propidium iodide

PI3K : Phosphoinositide 3-kinase

PIP_2 : Phosphatidylinositol 4,5-biphosphate

PIP_3 : Phosphatidylinositol 3,4,5-triphosphate

PKB : Protein kinase B

pRb : Retinoblastoma protein

PS : Phosphatidylserine

RICTOR : Rapamycin-insensitive companion of mammalian target of rapamycin

RIPA : Radioimmunoprecipitation assay

Rsk : Ribosomal S6 kinase

RTKs : Tyrosine kinase receptors

RT-PCR : Reverse-transcriptase polymerase chain reaction

SAPK(s) : Stress activated protein kinase(s)

SDS-PAGE : Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Ser : Serine

SIN1 : Stress-activated protein kinase-interaction protein 1

Skp2 : S-phase kinase-associated protein 2

SmBM	: Smooth muscle basal medium
SmGM	: Smooth muscle growth medium
STATs	: Signal transducer and activator transcriptions
STAT3	: Signal transducer and activator transcription 3
TAK1	: Transforming growth factor beta-activated kinase 1
TAO	: Target of avirulence protein B operation
TCN	: Triciribine
TEMED	: Tetramethylethylenediamine
TGF-β	: Transforming growth factor beta
tHGA	: 2,4,6-trihydroxy-3-geranyl acetophenone
T_H2	: T helper 2
Thr	: Threonine
TMB	: 3,3'5,5'-tetramethylbenzidene
TNS	: Trypsin neutralizing solution
Tyr	: Tyrosine
UPS	: Ubiquitin proteasome pathway
YAP	: Yes-associated protein

CHAPTER 1

INTRODUCTION

1.1 Background of study

Asthma is a chronic inflammatory disease of the airways which can be characterized by airway hyperresponsiveness (AHR), inflammation and remodeling (Carr and Bleecker, 2016; Kim et al., 2010; Busse and Lemanske, 2001; Postma and Kerstjens, 1998). It affects more than 300 million people worldwide with the prevalence higher in early childhood, declining during late adolescence and peaking in the elderly (Boulet, 2016; Global Asthma Report, 2014; Yanez et al., 2014; Hanania et al., 2011). New onset of asthma is usually detected in children and is found to be atopy-related (Braman, 2010). Asthma was previously labelled as a childhood disease, however elderly asthmatic patients were found to demonstrate higher asthma-related mortality and morbidity, which could be due to the declining lung function as they aged (Boulet, 2016; Yanez et al., 2014).

Symptoms of asthma attacks include coughing, chest tightness, wheezing and shortness of breath (Kim and Mazza, 2011). During an asthma attack, the airways undergo bronchoconstriction and inflammation which involves the activation of inflammatory cells and the release of pro-inflammatory cytokines and growth factors. Prolonged inflammation will lead to airway structural changes, collectively termed airway remodeling, that contributes to airway narrowing, AHR and airway obstruction (Murdoch and Lloyd, 2010). Increased airway smooth muscle (ASM) mass, the principal factor in airway remodeling, has been recognized as one of the most prominent hallmarks related to AHR and corresponds to the severity of asthma (Munakata, 2006). A thickened layer of ASM reduces the diameter of the airways as they contract and causes significant airflow limitation and AHR (Berair et al., 2013; Camoretti-Mercado, 2009). Numerous studies have linked patients with fatal asthma with increased ASM mass (Saetta et al., 1991; James et al., 1989; Sobonya,1984). Thus, therapy targeting ASM remodeling constitutes another option in the management of asthma.

ASM mass increases primarily through ASM hyperplasia, which can be defined as the increase in number of ASM cells (Woodruff et al., 2004). ASM hyperplasia can arise from increased ASM proliferation, reduced ASM apoptosis and/or increased migration of ASM towards the lumen of the asthmatic airway in response to the inflammatory mediators released (Camoretti-Mercado, 2009). These inflammatory mediators, include growth factors such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and insulin-like growth factor (IGF), will bind to tyrosine kinase receptors (RTKs) and G protein-coupled receptors (GPCRs), resulting in the activation of signal transduction pathways. These signal transduction pathways, which include mitogen-activated protein kinases (MAPKs), Janus kinase 2/signal transducers and

activators of transcription 3 (JAK2/STAT3) and phosphoinositide-3-kinase (PI3K) signaling pathways, are known to regulate cell proliferation, cell motility as well as cell survival (Stamatiou et al., 2012; Gosens et al., 2008; Simon et al., 2002; Ravenhall et al., 2000; Page et al., 2000; Ediger and Toews, 2000).

Asthma treatments have been developed primarily targeting the management of airway contraction and inflammation. Combination therapy with anti-inflammatory drugs, such as inhaled corticosteroids (ICS), and bronchodilators, such as long acting beta agonists (LABA) remains the mainstay of asthma treatment (Barnes, 2011; Barnes, 1995). However, the response of patients towards current treatment is highly variable, with up to 40% of asthmatic patients having minimal or no response at all to therapy (Tantisira et al., 2011; Szefler et al., 2002; Drazen et al., 2000). Furthermore, these treatments are effective in treating allergic inflammation, but do not specifically target airway remodeling (Royce and Tang, 2016). Animal models have demonstrated that steroid treatment inhibits structural airway changes as well as AHR induced by aerosolized ovalbumin (OVA) exposure however these treatments fail to reverse established airway remodeling (Vanacker et al., 2001). These findings suggest that airway remodeling may not be reversible but may be prevented by steroid treatment. In addition, several studies have been carried out to elucidate the direct effects of glucocorticoids on ASM proliferation. However, the effects of corticosteroids on ASM growth remains controversial (Panettieri, 2004).

A geranyl acetophenone namely 2,4,6-trihydroxy-3-geranyl acetophenone (tHGA), was found to be effective in attenuating AHR in response to methacholine challenge as well as reducing the inflammatory cell infiltration and goblet cell metaplasia in both acute and chronic murine models of asthma (Lee et al., 2017; Ismail et al., 2012). Further investigation has demonstrated that tHGA inhibited the structural alteration of the airway in a chronic murine model of asthma (Lee et al., 2017). tHGA is a compound containing a phloroglucinol core structure with a hydrophilic acyl group and a hydrophobic geranyl group (Ng et al., 2014). Acylphloroglucinols, including tHGA, are gaining attention due to their broad range of pharmacological properties, such as anti-bacterial, anti-oxidative, anti-proliferative and anti-depressant effects (Sun et al., 2014). Cysteinyl leukotrienes (CysLTs) are leukotrienes which are known to play pivotal roles in the pathophysiology of asthma, such as increased airway mucus production, bronchoconstriction as well as AHR in chronic asthma (Montuschi, 2010). tHGA was reported to act as cysteinyl leukotriene synthesis blocker via a dual lipoxygenase/cyclo-oxygenase (LOX/COX) inhibitory mechanism (Shaari et al., 2011). This finding further reinforced the anti-asthmatic properties of tHGA. Furthermore, tHGA also reduces the expression of alpha-smooth muscle actin (α-SMA) surrounding the airway lumen hence suggesting potential anti-remodeling properties of tHGA in reducing ASM mass although the direct effect of tHGA upon airway remodeling remains unknown (Lee et al., 2017). In this study, the potential anti-remodeling effects of tHGA upon human ASM proliferation, was assessed and the exact molecular target(s) was determined.

1.2 Problem statement

Current asthma treatment is only effective in relieving symptoms. Furthermore, up to 40% of asthmatic patients have minimal or no response to current treatments. Poor compliance of asthmatic patients to ICS also affects the drug delivery and efficacy (Rifaat et al., 2013; Khassawneh et al., 2008). Long term usage of high doses of ICS has been reported to cause side effects such as cataract, decreased bone mineral density and impaired growth in children (Dahl, 2006). Airway remodeling caused by the persistent airway inflammation has been linked to the development of AHR and the severity of asthma (Oliver et al., 2007). Current treatments do not specifically target the remodeling process. Hence, treatments that target single or multiple components of airway remodeling as well as airway inflammation may be useful in the management of asthma. tHGA, an in-house synthetic non-steroidal compound, inhibits the release of inflammatory cytokines, expression of α-SMA, fibronectin, vimentin and tenascin-C as well as AHR in animal models of asthma, is indicative of an anti-remodeling agent. The effects upon human airways and the mechanism and molecular target of tHGA remain unknown.

1.3 Research objectives

1.3.1 General objective

The general objective of this study is to determine the effects of 2,4,6-trihydroxy-3-geranyl acetophenone (tHGA) upon growth factor-induced human bronchial smooth muscle proliferation, the *in vitro* model of airway remodeling in asthma.

1.3.2 Specific objectives

- To determine the effect of tHGA upon growth factor-induced hBSMCs proliferation and migration.
- To determine the mode of cell death of growth factor-induced hBSMCs upon tHGA treatment.
- To determine the effect of tHGA upon the cell cycle regulation in growth factor-induced hBSMCs.
- To determine the involvement of MAPKs, JAK2/STAT3 and PI3K signaling pathways in growth factor-induced human bronchial smooth muscle cell treated with tHGA
- To identify the molecular target of tHGA in growth-factor induced hBSMCs.

1.4 Hypotheses

- tHGA attenuates growth factor-induced hBSMCs proliferation and migration.
- tHGA induces apoptosis in growth factor-induced hBSMCs.
- tHGA inhibits hBSMCs proliferation through cell cycle arrest.
- tHGA exerts its anti-remodeling effects through inhibition of a/several signaling molecules within the MAPKs, PI3K and/or JAK2/STAT3 signal transduction pathways.

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PUBLICATIONS

Yap Hui Min, Daud Ahmad Israf, Hanis Hazeera Harith, Chau Ling Tham, Mohd Roslan Sulaiman (2019). Crosstalk between signalling pathways involved in the regulation of airway smooth muscle cell hyperplasia. Frontiers in Pharmacology, 10: 1-16.

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