

UNIVERSITI PUTRA MALAYSIA

EFFECTS OF SYNTHETIC 2, 4, 6-TRIHYDROXY-3-GERANYLACETOPHENONE UPON TNF-α-INDUCED EPITHELIAL DYSFUNCTION

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

February 2016

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

EFFECTS OF SYNTHETIC 2, 4, 6-TRIHYDROXY-3-GERANYLACETOPHENONE UPON TNF-α-INDUCED EPITHELIAL DYSFUNCTION

By

TEE YEE SIM

February 2016

Chairman: Professor Daud Ahmad Israf Ali, PhD Faculty: Medicine and Health Sciences

Asthma is characterized by activation of Th-2 (T-helper-2)-type T-cell, and patients clinically presented with breathlessness, wheezing, cough, and chest tightness. Current treatments for asthma is just symptomatic relieve during acute exacerbation and prevent from attacks using controllers. All this treatment is not actually treating the new pathogenesis of this disease which is targeting the airway epithelium barrier. Studies shown 5-10% of asthma patient does not have any responds towards usual corticosteroid treatment, therefore there is a need to develop a new drug which can work on the actual pathogenesis of asthma. Airway epithelium plays a critical role in inflammatory reactions by acting as a barrier to external environment, producing chemokines and expressing cell-surface adhesion molecules for recruitment of effector cells. Preliminary studies show that 2,4,6-trihydroxy-3- geranylacetophenone (tHGA) inhibits the synthesis of cysteinyl leukotrienes in activated macrophages via inhibition of 5-lipoxygenase (5-LO) enzymatic activity. This study aimed to determine inhibitory effect of tHGA on adhesion molecule, epithelium hyperpermeability, tight junction barrier and pro-inflammatory signaling pathways that are involved. A549 cell was induced with tumor necrosis factor alpha (TNF-a) 10ng/mL and co-treated with different concentration of tHGA (50µM, 12µM, 3µM) and Dexamethsone 10 µM act as drug control group. Epithelium hyperpermeability was measured by fluorescein isothiocyanate-dextran (FITC-Dextran) permeability assay and trans-epithelial electrical resistance (TEER). Adhesion assay and transepithelium migration assay was performed by using A549 cells and U937 cells. Enzyme-linked immunosorbent assay (ELISA) was used to determine soluble intercellular adhesion molecule-1 (sICAM-1) level and monocyte chemoattractant protein-1 (MCP-1) levels. Membrane expression of tight junction complexes proteins (Zonula occluden 1, occludin, and E-cadherin) was investigated under immunofluorescence, gene expression and protein expression studies. Phosphorylation of mitogen-activated protein kinases (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway was studied. The data obtained concluded that 50 μ M tHGA had 32.10 \pm 8.03% significantly suppressed leukocytes moving across epithelium. tHGA had reduced adhesion molecule expression by $43.45 \pm 0.16\%$, therefore inhibit adhesion of monocytes to tumor necrosis factor TNF- α induced epithelium (43 \pm 5.77%) and reduce transepithelium migration. tHGA is able to increase TEER, reduce epithelium hyperpermeability by 41 \pm 6.35%. tHGA enhance airway barrier integrity through redistribution of TJ proteins (ZO-1, occludin) and AJ protein (E-cadherin) and NF- κ B pathway and MAPK pathway be could involved in this process.



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

KESAN SINTETIK 2, 4, 6-TRIHYDROXY-3-GERANYLACETOPHENONE TERHADAP TNF-α-STIMULASI EPITHELIAL DISFUNGSI

Oleh

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Februari 2016

Pengerusi: Professor Daud Ahmad Israf Ali, PhD Fakulti: Perubatan dan Sains Kesihatan

Asma dicirikan oleh pengaktifan Th-2 (T-pembantu-2) -type T-sel, dan pesakit secara klinikal akan menghadapi sesak nafas, berdehit, batuk, dan sesak dada. Rawatan untuk pesakit asma hanya melegakan gejala semasa kepahitan akut dan mengelakkan daripada serangan dengan menggunakan ubat pengawal. Semua rawatan ini tidak benar-benar merawat patogenesis baru penyakit ini yang memfokuskan saluran udara epitelium. Kajian membuktikan 5-10% daripada pesakit asma tidak mempunyai sebarang kesan terhadap rawatan ubat kortikosteroid. Oleh itu, suatu ubat yang baru perlu dikaji dan targetkan patogenesis sebenar asma. Epitelium salur udara memainkan peranan kritikal dalam tindak balas keradangan, dengan bertindak sebagai satu halangan kepada persekitaran luaran, menghasilkan chemokines dan mengekspresikan molekul-mokelul pelekatan permukaan sel untuk pengambilan sel efektor. Kajian awal menunjukkan bahawa 2,4,6-trihydroxy-3- geranylacetophenone (tHGA) menghalang sintesis cysteinyl leukotrienes di makrofaj yang diaftikkan melalui perencatan aktiviti enzim 5-lipoxygenase (5-LO). Kajian ini bertujuan mengenalpasti kesan tHGA di molekul pelekatan, epitelium hyperpermeability, simpang dekat dan laluan proinflamasi, yang terlibat. Sel A549 telah distimulasi dengan tumor necrosis factor alpha $(TNF-\alpha)$ 10ng/mL dan dikulturkan bersama dengan kepekatan berbeza tHGA (50 μ M, 12μM, 3μM) dan Dexamethsone 10 μM berfungi sebagai kumpulan kawalan dadah. Epitelium hyperpermeability telah disukat oleh fluorescein isothiocyanate-dextran (FITC Dextran) kebolehtelapan assay dan transepitelium electrical resistance (TEER). Assay pelekat dan transepithelium penghijrahan assay dikaji dengan menggunakan selsel A549 dan sel-sel U937. Enzyme-linked immunosorbent assay (ELISA) telah digunakan untuk menentukan tahap larut lekatan intercellular molekul - 1 (sICAM-1) dan monosit chemoattractant protein-1 (MCP-1). Kompleks persimpangan ketat protein occludin, dan E-cadherin) (Zonula occluden 1. telah disiasat dengan immunofluorescence, ekspresi gen dan ekspresi protein. Pemfosforilan laluan mitogenactivated protein kinases (MAPK) dan nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB) dikaji. Data yang diperolehi, kesimpulannya 50 µM tHGA telah menghalangkan leukosit menyeberang epithelium sebanyak $32.10 \pm 8.03\%$. tHGA telah mengurangkan molekul pelekatan pada $43.45 \pm 0.16\%$, dengan itu

menghalang pelekatan monosit pada TNF-α stimulasi epithelium (43 ± 5.77%) dan mengurangkan penghijrahan transepithelium. tHGA mampu meningkatkan TEER, mengurangkan epitelium hyperpermeability sebanyak 41± 6.35%. tHGA meningkatkan integriti saluran udara melalui pengagihan semula protein TJ (ZO-1, occludin) dan AJ protein (E-cadherin) dan NF-κB laluan dan MAPK laluan terlibat dalam proses ini.



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I certify that a Thesis Examination Committee has met on 26 February 2016 to conduct the final examination of Tee Yee Sim on her thesis entitled "Effects of Synthetic 2, 4, 6-Trihydroxy-3-Geranylacetophenone Upon TNF- α -Induced Epithelial Dysfunction" 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 Master of Science.

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LIST OF ABBREVIATIONS AND ANNOTATION

Abbreviations

ANOVA	one way analysis of variance
BCA	bicinchoninic acid
BSA	bovine serum albumin
CAMs	cell adhesion molecules
ddH2O	double distilled water
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
E-CAD	E-cadherin
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
GADPH	glyceraldehydes-3-phosphate dehydrogenase
HRP	horseradish peroxidase
ICAM-1	intercellular adhesion molecules type 1
ІКК	I-κB kinase
IL	Interleukin
І-кВ	inhibitory protein κB
JAKs	Janus kinases
JNK	c-Jun N-terminal kinases
МАРК	mitogen-activated protein kinase

MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MCP-1	monocyte chemotactic protein -1
MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2 yl)-2,5-diphenyltetrazolium
NF-κB	nuclear factor-kappaB
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PD 98059	2'-amino- <mark>3'-methoxyflav</mark> one
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription-polymerase chain reaction
SB 203580	4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) Imidazole
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SP 600125	anthra[1,9-cd]pyrazol-6(2H)-one
TBE	tris/Borate/EDTA
TBST	tris buffered saline-tween 20
TEM	transepithelium migration
TEMED	tetramethylethylenediamine
ТҒПВ	transcription factor II B
	MAPKK MAPKKK MCP-1 MMP MRNA MTT MRNA MTT NF-KB PAGE PAGE PBS PD 98059 PVDF RNA PD 98059 PVDF RNA RT-PCR SB 203580 SEM SB 203580 SB 203580 SEM SB 203580 SB 203580

Annotations

α	alpha
β	beta
°C	degree celcius
γ	gamma
g	gram
bp	base pair
kg	kilogram
<	lesser than
≤	less or equal
μ	micro
μg	micro gram
mg	milligram
μL	microlitre
μΜ	micromolar
mL	millilitre
mM	milimolar
М	molar
%	percent
±	plus and/or minus

CHAPTER 1

INTRODUCTION

1.1 Background of study

Asthma is an inflammatory airway disease that affects 300 million people worldwide. In Malaysia, asthma has been ranked as third main chronic disease by the Third National Health and Morbidity Survey (NHMS III) 2006. Asthma prevalence for Malaysians aged 18 years and above over duration of 12 months was 4.5%; more than 50% of adult asthmatics seek medical attention due to acute exacerbation. The airway epithelium is a barrier between tissues of the airways and the external environment. Proteins and receptors in tight junctions (TJ) such as zonula occludens (ZO) 1-3, claudin 1-5, occludin, and trans-membrane adhesion proteins (E-cadherin, catenin and junctional adhesion molecule) regulate intercellular rephrases and interact with adjacent cells (Holgate 2008). Tight junctions, adherens junctions, desmosomes and hemidesmosomes form an epithelium junctional complex that functions as a unit. Studies shown that the airway epithelium was disrupted in asthma and involves detached columnar ciliated cells, interference of the junction molecule E-cadherin at sites of epithelial detachment, and the increase in permeability to allergens, and presence of epithelial aggregates in sputum (Holgate 2008).

2,4,6 – trihydroxy – 3 – geranyl acetophenone (tHGA) was previously isolated from *Melicope ptelefolia* and subsequently a synthetic form was demonstrated to be effective in aberration of pulmonary inflammation (Ismail et al., 2012). Furthermore, Shaari et al., 2011, proved that tHGA inhibits the synthesis of cysLTs in activated macrophages via inhibition of 5-lipoxygenase (5-LO) enzymatic activity.

1.2 Problem statement

Asthma is a multi-factorial disease which is associated with genetic, allergic, infections, environment and emotional aspects. Asthma causes many deaths yearly and expensive hospitalization and medical bills that had been a burden to underdeveloped countries. Currently, treatments for asthma are just symptomatic relieve during acute exacerbation and no treatment is actually available to cure the actual pathogenesis of this disease. Furthermore, studies have shown that 5-10% of asthma patients do not respond to usual corticosteroid treatment; therefore there is a need to develop new drugs which aim to cure asthma.

Epithelial tight junctions act as physical barrier between the external environment and the internal tissues. In asthmatic patients, they presented with TJ disruptions even without exogenous stimuli. Subepithelial collagen deposition and thickening of the lamina reticularis were ways of healing responses within the airways in barrier dysfunction and act as the protective barrier in the absence of a functional epithelium in asthma. Airways play a fundamental role in the initiation of innate immunity. Activated epithelial cells will release cytokines and chemokines on stimulation due to expression of pattern-recognition receptors like Toll-like receptors (TLRs). When there is a disruption of the TJ integrity, external antigens will penetrate the TJ causing activations of DCs and innate immunity. These processes caused amplified recruitment of effector cells, and further diminish of barrier function, thus breakdown of barrier homeostasis (Swindle et al., 2009).

Shaari 2006 had proven anti-inflammatory properties of Melicope ptelefolia resulted in the identification of 2,4,6-trihydroxy-3-geranylacetophenone (tHGA), a drug-like compound containing the phloroglucinol as bioactive structural-core. Originally, this compound was found to have a dose-dependent effect towards inhibition of soybean 15-LOX. Subsequently, this compound exerts a dose-dependent inhibition of cysteinyl leukotriene secretion from activated macrophage cells. Further studies were carried out on both the chemistry and pharmacology of tHGA revealed that tHGA inhibited 5lipoxygenase (5-LOX) and both cyclooxygenase (COX) isoforms. tHGA was as effective as Zileuton, a commercial LOX inhibitor in an acute model of murine asthma. To date, tHGA is able to reduce pulmonary cellular infiltration, control airway hyperresponsiveness towards methacholine challenge, reduce goblet cell metaplasia, decrease cytokine (IL-4, IL-5, IL-13) and cysteinyl leukotriene secretion and systemic IgE concentrations. However, the study of tHGA properties on restoration of epithelium barrier integrity has not yet been done. Therefore, it is important to highlight the potential of tHGA on the epithelial tight junction restoration. Furthermore, in future many asthma patients can have the maximal beneficial of a non steroidal drug which can reverse and prevent the asthma disease from deteriorating, and further save up millions of expensive medical expenses.

1.3 Objectives

1.3.1 General Objective

The objective of the study was to determine the effect of tHGA upon TNF- α induced in-vitro model of airway epithelium dysfunction which mainly focus on the restoration of disrupted tight junctions properties.

1.3.2 Specific Objectives

- i. To determine the effects of tHGA on extracellular secretion and intracellular genes and protein expression of cell adhesion molecules, epithelium hyper permeability and chemokine synthesis in TNF- α induced A549 cells.
- ii. To determine the effects of tHGA on epithelium cells leukocytes interaction on adhesion and migration.
- iii. To determine the effects of tHGA upon dysregulated junctional protein expression.
- iv. To determine the effects of tHGA upon major epithelial tight junctions pathways mainly NF-κB and MAPK pathways which regulate tight junctions proteins expression.

1.4 Hypothesis

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tHGA will be able to reduce the recruitment and migration of leukocytes, reduce adhesion molecule expression and enhances TJ barrier integrity. tHGA exerts an effect on NF- κ B and MAPK pathways.



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