

**PHARMACOKINETICS OF STILBENE
ANALOGUES AND THEIR APPLICATION AS
NOVEL ANTI-INFLAMMATORY AGENTS FOR
CHRONIC OBSTRUCTIVE PULMONARY DISEASE**

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Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Yeo Chao Ming Samuel
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5. Q.-Y. Choo, **S. C. Yeo***, P. C. Ho, Y. Tanaka, H.-S. Lin, Pterostilbene surpassed resveratrol for anti-inflammatory application: Potency consideration and pharmacokinetics perspective. *Journal of Functional Foods* 11, 352-362 (2014).
6. **S. C. Yeo**, W. Luo, J. Wu, P. C. Ho, H. S. Lin, Quantification of pinosylvin in rat plasma by liquid chromatography-tandem mass spectrometry: application to a pre-clinical pharmacokinetic study. *J Chromatogr B Analyt Technol Biomed Life Sci* 931, 68-74 (2013).

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Summary

Chronic obstructive pulmonary disease (COPD) is an escalating global health problem that is associated with massive healthcare costs. It is characterised by a progressive decline in lung function due to chronic inflammatory processes which lead to the destruction of peripheral airways and lung parenchyma. There are currently no effective therapies which can significantly ameliorate the underlying inflammation and alter the natural history of this disease. Corticosteroids, one of the most widely used anti-inflammatory agents, have proved to be largely ineffective. As such, the development of novel anti-inflammatory therapy is urgently needed.

Resveratrol, the stilbene analogue found naturally in berries, has antioxidant and anti-inflammatory effects in several disease models related to airway inflammation. However, its therapeutic application is limited by its relatively weak potency and poor pharmacokinetics and its anti-inflammatory mechanisms have not been fully elucidated. In recent years, other stilbene analogues have emerged as viable alternatives with superior pharmacokinetics and potency but their pharmacological properties are still not well understood. This project extensively investigated the pharmacology of stilbene analogues by first profiling the pharmacokinetics of some analogues to identify possible structural determinants of pharmacokinetics, followed by examining the anti-inflammatory effects and cellular mechanisms of these compounds to identify promising drug candidates.

Studies in this thesis using a pre-clinical rat pharmacokinetic model indicated that meta-hydroxy groups in the stilbene analogue structure are associated with poor pharmacokinetics and methylation appears to reduce its susceptibility to clearance. Furthermore, tissue distribution studies for pterostilbene demonstrated that stilbene analogues may be widely distributed into major drug target organs and the major biotransformation mechanisms of a novel biologically active stilbene analogue, *trans*-2,6-difluoro-4'-(N,N-dimethylamino)stilbene, were identified and its excellent pharmacokinetics were confirmed.

Using transformed and primary airway epithelial cell models, some stilbene analogues were shown to be more potent than resveratrol in inhibiting the release of inflammatory cytokines, IL-6 and CXCL8. Their anti-inflammatory actions involved the suppression of major inflammatory transcription factors, NF- κ B and AP-1, and more importantly, were mediated through abrogating the activation of the PI3K/Akt signalling pathway, which is distinct from the actions of dexamethasone. These studies established isorhapontigenin as the most potent stilbene analogue with better pharmacokinetics than resveratrol which substantiate its further development as a novel anti-inflammatory agent for COPD.

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List of Abbreviations

AP-1	Activator protein-1
ASK	Apoptosis signal-regulatory kinase 1
AUC _{0→last}	Area under curve from 0 min to last time measured time
BEBM	Bronchial epithelial basal medium
BPE	Bovine pituitary extract
bZIP	Basic region leucine zipper
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
CCL	CC-chemokine ligand
cDNA	complementary deoxyribonucleic acid
CE	Collision energy
Cl	Plasma clearance
C _{max}	Maximum plasma compound concentration
CMC	Carboxymethylcellulose
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclo-oxygenase 2
CREB	cAMP response element binding protein
CSE	Cigarette smoke extract
CV	Coefficient of variation
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
CXP	Collision exit cell potential
DAMPs	Damage-associated molecular patterns
DFS	<i>trans</i> -2,6-difluoro-4'-(N,N-dimethylamino)stilbene
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethyl sulphoxide
DP	Declustering potential
DPPH	2,2-diphenyl-1-picrylhydrazyl
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EMS	Enhanced Q3 single MS
EP	Entrance potential
ERK	Extracellular signal-regulated kinase
ESI	Electrospray ionisation
F	Oral bioavailability
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FEV ₁	Forced expiratory volume in one second
FGF	Fibroblast growth factor
FoxO3A	Forkhead box O3A

FVC	Forced vital capacity
GM-CSF	Granulocyte-macrophage colony stimulating factor
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GPCRs	G-protein coupled receptors
GRE	Glucocorticoid response element
GRs	Glucocorticoid receptors
H ₂ DCFDA	2',7'-dichlorofluorescein diacetate
HAT	Histone acetyltransferase
HBSS	Hanks' balanced salt solution
HDAC 2	Histone deacetylase 2
HDAC2	Histone deacetylase 2
HPLC	High performance liquid chromatography
HP- β -CD	Hydroxypropyl β -cyclodextrin
HRP	Horseradish peroxidase
IC ₅₀	50% inhibitory concentrations
IFN γ	Interferon gamma
IKK	I κ B kinase
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
IRAK	Interleukin-1 receptor-associated kinase
IS	Internal standard
IV	Intravenous
I κ B	Inhibitor of κ B
JNK	c-Jun NH ₂ -terminal kinase
K-SFM	Keratinocyte-serum free medium
LABA	Long-acting β agonist
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLOQ	Lower limit of quantification
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MEK	MAPK/ERK
MKK	Mitogen activated protein kinase kinase
MKP-1	MAPK phosphatase-1
MLCK	Myosin light chain kinase
MMPs	Matrix metalloproteinases
mMRC	Modified Medical Research Council
MOPS	3-(N-morpholino)propanesulfonic acid
MRM	Multiple reaction monitoring
MRM-IDA-EPI	Multiple reaction monitoring-information dependent acquisition-enhanced product ion
mTORC2	Mammalian target of rapamycin complex 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTT _{0→last}	Mean transition time 0 min to last time measured time
MYD88	Myeloid differentiation primary response gene 88

NAC	N-acetylcysteine
NEMO	NF- κ B essential modulator
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF- κ B inducing kinase
Nrf2	Nuclear factor E2-related factor-2
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDE-4	Phosphodiesterase-4
PDK1	3-phosphoinositide-dependent protein kinase 1
PH	Pleckstrin-homology
PHLPP	PH domain leucine-rich repeat protein phosphatase
PI / NL	Precursor ion and / or neutral loss
PI3K/Akt	Phosphatidylinositol 3-kinase/RAC-alpha serine/threonine-protein kinase
PKA	Protein kinase A
PKB	Protein kinase B
PP2A	Protein phosphatase 2A
PRRs	Pattern recognition receptors
PtdIns	Phosphatidylinositol
PTEN	Phosphatase and tensin homolog
QC	Quality control
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RAF	Rapidly accelerated fibrosarcoma kinase
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
SDS	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	Src homology 2
SHIP	Src homology 2-containing inositol 5-phosphate
SIRT	Sirtuin
SOD	Superoxide dismutase
$t_{1/2}$	Half-life
TAK1	Transforming growth factor- β -activated kinase 1
TBARS	Thiobarbituric acid reactive substances
TBS	Tris buffered saline
TBS	Tris buffered saline
TGF	Transforming growth factor
Th	T helper
TIMPs	Tissue inhibitors of MMPs
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptors
$t_{\max}$	Time to compound concentration

TMS	3,5,4'-trimethoxystilbene
TNF- α	Tumour necrosis factor- α
TPA	12-O-tetradecanoylphorbol-13-acetate
TRAF6	Tumor necrosis factor receptor associated factor 6
UV	Ultraviolet
V _c	Volume of distribution

CHAPTER 1

INTRODUCTION

1.1 Overview of chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) has high morbidity and mortality globally. Officially defined as a disease “characterised by persistent airflow limitation that is usually progressive and associated with enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases” (GOLD, 2016), COPD affects approximately 300 million people worldwide (Vos et al., 2015) and is expected to become the fourth leading cause of death and seventh most common cause of disability globally by 2030 (Mannino and Buist, 2007; Mathers and Loncar, 2006). The massive healthcare burden associated with COPD has driven more studies in recent decades which have significantly enhanced our understanding of the pathophysiological basis of this disease that will boost the development of effective therapies (Stockley et al., 2009).

1.2 Pathology and pathophysiology of COPD

COPD is associated with numerous pathological abnormalities which are found in the airways, lung parenchyma and the surrounding pulmonary vasculature (Hogg, 2004).

In the airways, there is presence of chronic inflammation characterised by increased number of macrophages, neutrophils and CD8+ T-lymphocytes (Finkelstein et al., 1995; Hogg et al., 2004; Pilette et al., 2007; Saetta et al., 1998). Goblet cell numbers are also elevated and mucous glands are enlarged leading to increased mucus secretion that is associated with chronic bronchitis. Increased fibrosis occurs in the

walls of the airways and there is narrowing and decline in the number of small airways leading to bronchiolitis or small airway disease (Figure 1.1). Collapse of the airways can eventually occur as a result of damage to the alveolar walls. This destruction of alveolar wall is a result of emphysema, whereby the terminal sections of the lungs, consisting of the respiratory bronchioles, alveolar ducts and alveoli and the surrounding capillaries and interstitium, which form the lung parenchyma, become damaged. In COPD, early aberrations in the pulmonary vasculature include intimal hyperplasia and are followed by smooth muscle hypertrophy and loss of the capillary bed.

The pathological manifestations described lead to various physiological abnormalities in the lungs including the characteristic airflow obstruction and air trapping, defects in gas exchange, mucus hypersecretion and, in some patients, pulmonary hypertension. In addition, systemic effects such as skeletal muscle wasting and cardiovascular disease are often present.

Chronic airflow obstruction in COPD occurs mainly in the small airways of less than 2 mm in diameter. Inflammation in the small airways (chronic bronchiolitis) and emphysema (Barnes et al., 2003) contributes to this obstruction. The degree by which each of these contributes to the disease in a COPD patient varies from one patient to another and this partly accounts for the heterogeneity of COPD (Miravittles et al., 2012). Airway obstruction leads to gradual trapping of air and hyperinflation of the lungs. Together with emphysema, it is also responsible for defects in gas exchange which cause hypoxemia and

hypercapnia. Hypercapnia results in respiratory acidosis which leads to compensatory increase in blood bicarbonate levels. If not fully compensated, the systemic effects of acidosis can range from arrhythmia and hypotension to respiratory muscle weakness and cachexia (Bruno and Valenti, 2012).

Mucus hypersecretion is a characteristic symptom of chronic bronchitis and results from the enlarged mucus glands and elevated goblet cells. In addition, pulmonary hypertension may occur in the later stages of COPD and it is due to the pathological abnormalities of the pulmonary vasculature described earlier (Cuttica et al., 2010). The airflow limitation and increased inflammatory mediators released into the circulation invariably leads to systemic effects such as skeletal muscle wasting and cardiovascular diseases.

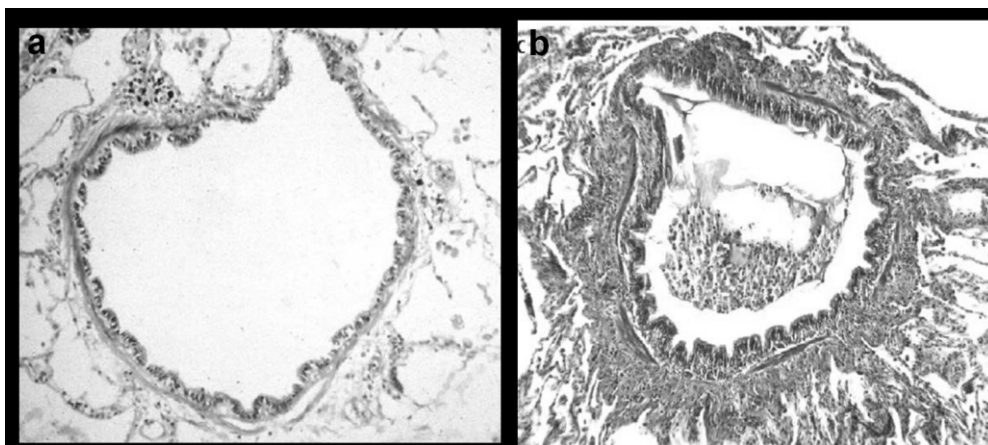


Figure 1.1 Histology of small airways in a) normal and b) COPD patients. In COPD patients, the airway wall is thickened; the airway lumen is narrowed and filled with mucus. Figure obtained from (Hogg, 2004).

1.3 Clinical presentation and diagnosis of COPD

Dyspnea, or shortness of breath, is a hallmark symptom for COPD and will worsen as the disease progresses. Patients may also present with

chronic cough and sputum production and in more severe disease, weight loss and depression can occur.

The diagnosis of COPD takes into account these symptoms but can only be confirmed with spirometry (GOLD, 2016). In spirometry, the volume of air forcibly exhaled from the point of maximal inspiration (forced vital capacity, FVC) and the volume of air exhaled during the first second of taking the FVC (forced expiratory volume in one second, FEV₁) are measured. A FEV₁/FVC ratio of < 0.7 after administration of short-acting bronchodilator denotes persistent airflow limitation and is strongly suggestive of COPD when symptoms and risk factors are also present (Figure 1.2).

Sudden worsening of symptoms and clinical parameters can occur during COPD exacerbation. This can occur as a result of bacterial or viral infections, environmental pollutants or other factors.

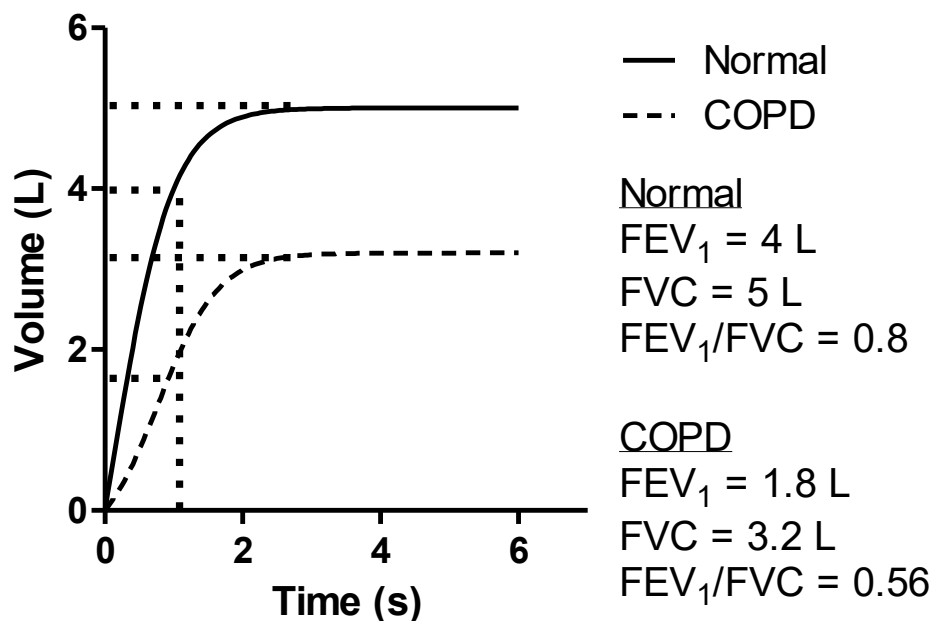


Figure 1.2 Spirometry tracing of typical normal and COPD patients. Figure adapted from (GOLD, 2016).

1.4 Risk factors of COPD

COPD is a complex disease which develops from the intricate interactions between environmental and host risk factors.

The major and most well-recognised risk factor for COPD is cigarette smoking (Løkke et al., 2006; Lundbäck et al., 2003; Terzikhan et al., 2016). Other environmental risk factors include exposure to particulate matter, dust and fumes. In particular, biomass smoke exposure is a significant risk factor in developing countries (Gordon et al., 2014; Miele et al., 2016). However, exposure to the same environmental risk factors does not lead to equal risk of developing COPD as host factors have an influence too (Mannino and Buist, 2007). Airway hyperresponsiveness to various external triggers is a notable host factor while atopy and impaired lung growth due to events such as preterm birth increase the susceptibility to COPD too. In addition, various genetic risk factors have been identified. The most well-established is the deficiency in α_1 -antitrypsin which results in widespread tissue damage by elastase and development of emphysema (Janciauskiene et al., 2011). Several other polymorphisms have been shown to be linked to COPD including genes related to antioxidant enzymes and metalloproteinases (He et al., 2002; Hirano et al., 2001).

1.5 Pathogenesis of COPD

The link between the risk factors and how they eventually contribute to the development of COPD have been mapped out in recent years from

extensive studies into the cellular and molecular mechanisms of COPD. When exposed to environmental insults, cells of the innate and adaptive immune system become activated to release a wide array of inflammatory mediators (Barcelo et al., 2006; Kent et al., 2008; Moermans et al., 2011). In COPD, this inflammatory response is augmented and remains unresolved while there is also increased oxidative burden and proteinases which can further orchestrate the inflammatory response.

1.5.1 Immune response and inflammation

1.5.1.1 Innate immune response

The innate immune response is the first-line, rapid and non-specific response to invading pathogen and cellular injury (Mogensen, 2009). Cells that are involved in this response include polymorphonuclear granulocytes (basophils, eosinophils and neutrophils), agranulocytes (monocytes and macrophages), dendritic cells, mast cells, natural killer cells, epithelial cells and endothelial cells (Mogensen, 2009; Robb et al., 2016). Innate immunity is based on the recognition of pathogen-associated molecular patterns (PAMPs) including bacterial nucleic acids and bacterial cell wall products such as lipopolysaccharide (LPS) from invading pathogens or damage-associated molecular patterns (DAMPs) such as heat-shock proteins and DNA released from injured tissues using pattern recognition receptors (PRRs) on the innate immune cells (Newton and Dixit, 2012; Pouwels et al., 2014). A wide variety of PRRs are present in cells and the toll-like receptors (TLR) constitute one of the major families of PRRs. A total of 10 different

TLRs have been identified in humans and they have been shown to be activated by different ligands (Newton and Dixit, 2012). Once activated, intracellular signalling pathways are triggered to result in the release of a repertoire of inflammatory mediators and activation of the adaptive immune system (Figure 1.3).

1.5.1.2 Adaptive immune response

The adaptive immune response is a slower and highly specific response which involves the T-lymphocytes and B-lymphocytes. Dendritic cells provide the link between innate and adaptive immune response whereby ligand binding of PRRs on dendritic cells promote their maturation and presentation of the antigens to CD4+ and CD8+ T cells. Activated CD4+ T cells release mediators which attract inflammatory cells to the lungs. Conversely, CD8+ T cells, which are the more abundant cells in the airways of COPD patients, produce proteolytic enzymes which can lead to emphysema (Freeman et al., 2010). The destruction of lung cells release antigens which promote the production of auto-antibodies by B-lymphocytes (Feghali-Bostwick et al., 2008). This activated adaptive immune system persists in COPD patients long after smoking cessation and has led to suggestions of autoimmunity in the pathogenesis of COPD (Cosio et al., 2009b).

1.5.1.3 Cellular and molecular mechanisms of inflammation in COPD

When cigarette smoke and other irritants enter the lungs, airway epithelial cells become damaged and release products that activate the PRRs including TLRs in lung macrophages and epithelial cells. The

activation of PRRs initiates a series of signalling mechanisms that result in the release of inflammatory mediators to recruit more inflammatory cells. Some examples of the mediators released are CC-chemokine ligand (CCL) 2, which binds to CC-chemokine receptor (CCR) 2 to attract monocytes, CXC-chemokine ligand (CXCL) 1 and CXCL8, which binds to CXC-chemokine (CXCR) 2 to recruit neutrophils and monocytes/macrophages, CXCL9, -10 and -11, which bind to CXCR3 to attract T helper (Th) 1 cells and type 1 cytotoxic T (Tc1) cells (Barnes, 2009; Costa et al., 2008). Other than these chemotactic factors, amplified release of cytokines including granulocyte-macrophage colony stimulating factor (GM-CSF) enhances the production and survival of granulocytes to prolong inflammation (Vlahos et al., 2006). Furthermore, pro-inflammatory cytokines such as tumour necrosis factor (TNF)- α , interleukin IL-1 β and IL-6 are released to further propagate inflammation through activation of several inflammatory signalling pathways which produce more inflammatory proteins (Hubeau et al., 2013; Reynolds et al., 2006).

In COPD, abnormalities in macrophages and airway epithelial cells have been shown to play vital roles in amplifying the inflammatory processes described above. Macrophage numbers are increased by approximately ten-fold in the airways and parenchyma of COPD patients as a result of increased attraction of monocytes to the lungs or prolonged survival of lung macrophages (Hogg et al., 2004; Tetley, 2002). These lung macrophages not only have enhanced basal and stimulated release of inflammatory proteins, but also impairment in

their phagocytic and efferocytic abilities which contribute to the amplified levels of inflammation in the lungs (Donnelly and Barnes, 2012; Hodge et al., 2003). Similarly, the airway epithelial cells which are a source of pro-inflammatory mediators as described earlier are more sensitive to stimulation by inflammatory cytokines in COPD (Patel et al., 2003).

1.5.2 Oxidative stress in COPD

Intracellular processes, occurring in cells such as the airway epithelial cells, neutrophils, eosinophils and macrophages (MacNee, 2001), and the external environment are sources of oxidative stress in the lungs. The mitochondrial respiratory chain and cellular processes including phagocytosis generate reactive oxygen species (ROS) in the cells while inhalation of cigarette smoke (contains more than 10^{14} free radicals per puff (Pryor et al., 1983)) and other environmental pollutants further increases the oxidative burden in the lungs. Other than their roles in modulating various signalling pathways discussed later (Thannickal and Fanburg, 2000), ROS including the hydroxyl and superoxide radicals are highly unstable and rapidly oxidise intracellular molecules such as proteins, lipids and DNA to form reactive carbonyls which can alter cellular processes or propagate further oxidative reactions through generation of secondary metabolic reactive species (Kirkham and Barnes, 2013). In normal cells, these damaging processes are controlled by various antioxidant mechanisms (Boots et al., 2003). Antioxidant molecules such as glutathione, ascorbic acid and α -tocopherol can effectively neutralise ROS as well as antioxidant

enzymes including superoxide dismutase (SOD) 2 and catalase. In addition, many important antioxidant enzymes are regulated by the transcription factors nuclear erythroid-2-related factor 2 (Nrf2) and forkhead box O3A (FoxO3A). In COPD, impairment of these defences escalates the oxidative burden in the lungs (Harju et al., 2002; Hwang et al., 2011; Tomaki et al., 2007), resulting in post-translational modifications which lead to alterations in activity of many redox-sensitive proteins (Di Stefano et al., 2002; Ito et al., 2004; Nakamaru et al., 2009). Some examples include activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), a key pro-inflammatory protein and deactivation of histone deacetylase 2 (HDAC 2), an important suppressor of inflammatory genes and also deactivation of Nrf2. Clearly, ROS can promote the inflammatory and oxidative processes in COPD.

1.5.3 Protease-antiprotease imbalance in COPD

The protease-antiprotease imbalance theory originated from the observation that patients with genetic deficiency of the antiprotease alpha-1 antitrypsin were more susceptible to COPD (Eriksson, 1965). This theory suggests that emphysema in COPD occurs due to the degradation of lung connective tissues, in particular elastin, from increased activity of proteases and/or reduced activity of antiproteases (Shapiro, 2003). Antiproteases are important in protecting against the effects of proteases. Serine proteases, specifically neutrophil elastase, and matrix metalloproteinases (MMPs) are the major proteases implicated in COPD and clinical studies have confirmed elevated levels

of these proteases in emphysema (Mercer et al., 2005; Russell et al., 2002). The amplified protease activities can subsequently elevate the inflammatory status as proteases such as neutrophil elastase are capable of inducing the release of inflammatory cytokines.

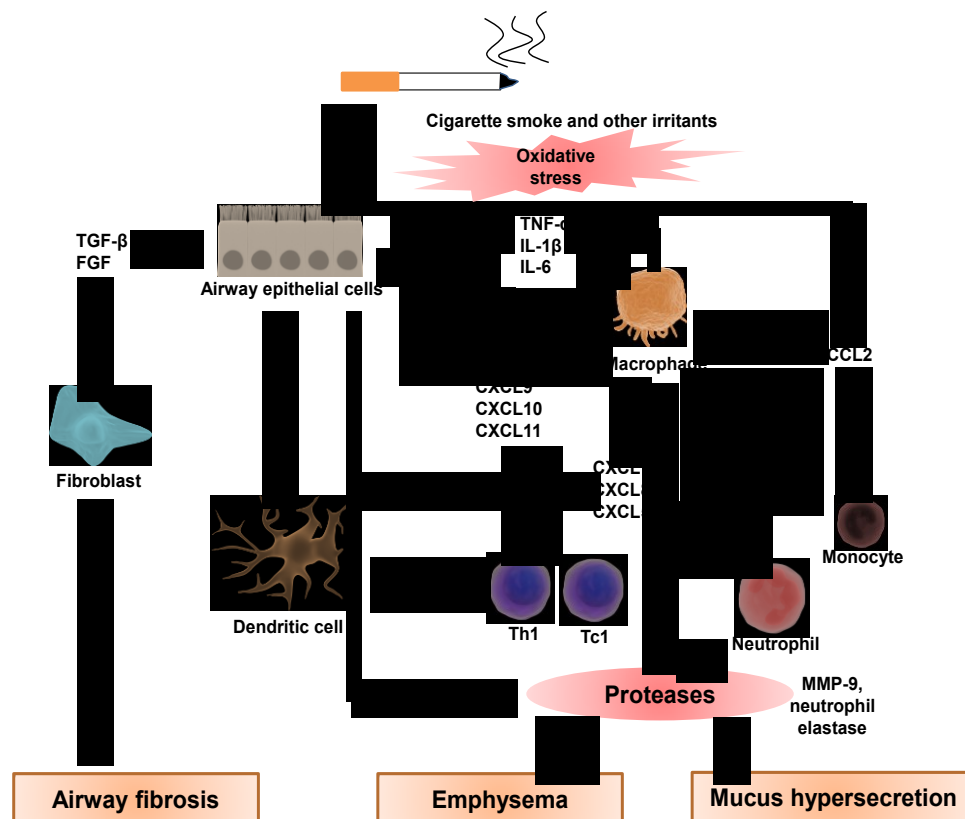


Figure 1.3 The key inflammatory cells and mediators involved in the pathogenesis of COPD. Inhaled cigarette smoke and other irritants stimulate airway epithelial cells and lung macrophages to release a repertoire of mediators including chemokines which attract more inflammatory cells to the lungs. These cells release further mediators such as transforming growth factor (TGF)- β and fibroblast growth factor (FGF) which result in airway fibrosis, emphysema and mucus hypersecretion. Figure is adapted from (Barnes, 2009).

1.6 Intracellular inflammatory signalling pathways in airway epithelial cells in COPD

Airway epithelial cells also play a pivotal role in COPD as they are amongst the first cells to encounter environmental irritants and can initiate and sustain inflammation through the release of inflammatory mediators. Many types of receptors can be engaged by the irritants or oxidative stress to activate a network of inflammatory proteins which culminate in the release of these mediators. Major signalling pathways involved are the mitogen activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase/RAC- α serine/threonine-protein kinase (PI3K/Akt) pathways (Barnes et al., 2003). In addition, the NF- κ B and activator protein (AP)-1 pathways are key transcription factors regulating expression of inflammatory genes (Rahman and MacNee, 1998). A summary of the key inflammatory pathways involved is presented in Figure 1.4.

1.6.1 MAPKs pathway

The MAPKs pathway can be triggered by activation of a variety of receptors including TLRs, IL-1 and TNF receptors on exposure to extracellular stresses such as inflammatory cytokines, growth factors and osmotic stress (Chang and Karin, 2001). MAPKs are a family of serine/threonine kinases of which there are three major groups, the extracellular signal-regulated kinase (ERK)1/2, c-Jun NH₂-terminal kinase (JNK) and p38, which are regulated in different ways through a three-tiered phosphorylation cascade. Activation of MAPKs occurs through dual phosphorylation by specific MAPK kinases (MAPKK or

MKK) (MAPK/ERK (MEK)1/2 for ERK 1/2; MKK4/7 for JNK; MKK3/6 for p38) which are in turn regulated by a wide variety of MAPKK kinases (MAPKKKs, also known as MEKKs) including apoptosis signal-regulatory kinase 1 (ASK), transforming growth factor- β -activated kinase 1 (TAK1) and rapidly accelerated fibrosarcoma kinase (RAF).

In airway epithelial cells, the role of MAPKs in inflammation can be observed from studies which have shown that CXCL8 release following cigarette smoke stimulation was dependent on the activation of p38 and ERK1/2 and that there was increased activation of p38 in cells from COPD patients (Gaffey et al., 2013; Moretto et al., 2012).

1.6.2 PI3K/AKT pathway

PI3Ks are a family of lipid kinases capable of phosphorylating the 3-OH group of the inositol ring of phosphatidylinositol (PtdIns) and can be classified into three main classes according to structural properties and substrate specificity, namely Class I, Class II and Class III (Fruman et al., 1998).

Class I PI3Ks are the most extensively studied and can be further categorised into Class IA and Class IB enzymes (Fruman et al., 1998). Class IA PI3Ks consist of heterodimers made up of the p110 catalytic subunit involved in enzymatic activity and p85 regulatory subunit responsible for activation and localization of the kinase. There are three variants of Class IA p110 subunit designated p110 α , β and δ and five variants of p85 subunit designated p85 α , p55 α , p50 α , p85 β and p55 γ . While p110 α , p110 β , p85 α and p85 β are ubiquitously expressed,

the other isoforms show tissue-specific distribution (Gout et al., 1992; Hirsch et al., 2006). For instance, p110 δ is found mainly in leukocytes. Class IA PI3Ks can be activated by receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs). Upon activation of the appropriate receptor, PI3K is recruited to the plasma membrane through interaction of the Src homology 2 (SH2) domain on its p85 subunit directly with tyrosine phosphate motifs on the activated receptors or with adaptor proteins bound to the receptors. Class IB PI3Ks are also heterodimeric, comprising of the catalytic p110 γ subunit and regulatory p101 or p84 subunit. They are found mainly in leukocytes and can only be activated by GPCRs. Once activated, the catalytic subunit of Class I PI3Ks are able to produce PtdIns (3)-phosphate (PIP), PtdIns (3,4)-bisphosphate (PIP₂) or PtdIns (3,4,5)-trisphosphate (PIP₃) from their precursors.

Class II and Class II PI3Ks are relatively less well studied and their roles in inflammation are less well defined. Class II PI3Ks only produce PIP and PIP₂ while Class III PI3Ks only produce PIP.

The formation of PIP₂ and PIP₃ at the inner side of cell membrane serves as docking sites for intracellular proteins with pleckstrin-homology (PH) domains such as protein kinase B (PKB also known as Akt) and 3-phosphoinositide-dependent protein kinase (PDK) 1 (Liu et al., 2009). Akt is a major effector of PI3K activation with numerous cellular functions through its serine/threonine kinase activity. The three isoforms of Akt, Akt1, Akt2 and Akt3 are widely expressed but different forms are predominant in each tissue (Lee et al., 2011). The activity of

Akt is regulated by phosphorylation whereby its translocation to the plasma membrane brings about phosphorylation at serine 473 (Ser473) by PDK2 (recently shown to be mammalian target of rapamycin complex (mTORC) 2 (Sarbasov et al., 2005)) followed by phosphorylation at threonine 308 (Thr308) by PDK1 which fully activates it. Full activation of Akt leads to phosphorylation of its various downstream targets including FoxO proteins (Manning and Cantley, 2007).

The activity of PI3K/Akt pathway is also regulated by negative regulators that target PIP_3 or Akt. Phosphatase and tensin homolog (PTEN) converts PIP_3 to $PI(4,5)P_2$ by removing the 3-phosphate group while Src homology 2-containing inositol 5-phosphate (SHIP) converts PIP_3 to $PI(3,4)P_2$ by removing the 5-phosphate group. Regulation of Akt involves protein phosphatase 2A (PP2A) which dephosphorylates Akt mainly at Thr308 and PH domain leucine-rich repeat protein phosphatase (PHLPP) which dephosphorylates Akt at Ser473 (Andjelkovic et al., 1996; Brognard et al., 2007; Stambolic et al., 1998).

In human lung epithelial cells, the PI3K/Akt pathway plays a role in inflammatory cell signalling. For instance, CXCL8 release induced by thrombin involved the PI3K/Akt pathway and MMP-9 expression induced by $TNF-\alpha$ was also shown to be mediated through the PI3K/Akt pathway (Lee and Yang, 2013; Lin et al., 2011).

1.6.3 NF- κ B pathway

The NF- κ B pathway is a key regulator of inflammatory genes including cytokines and inflammatory enzymes (Tak and Firestein, 2001). NF- κ B exists as homodimers or heterodimers of the p65 (RelA), RelB, c-Rel, p50 and p52 subunits, with different combinations having varying activities and functions. The p65/p50 heterodimer is the predominant transcriptional activating form in cells.

In the unstimulated state, NF- κ B dimers are sequestered in the cytoplasm by the inhibitory molecules of the inhibitor of κ B (I κ B) family which mask the nuclear localisation sequence on the NF- κ B proteins. Activation of the majority of NF- κ B dimers occurs through the classical pathway involving the activation of inflammatory receptors such as TLRs, IL-1 receptor and TNF receptor followed by phosphorylation, ubiquitination and degradation of I κ B proteins (Verstrepen et al., 2008). Phosphorylation is mediated by the I κ B kinase (IKK) complex which comprises three subunits: IKK- α and IKK- β which are the catalytic subunits and IKK- γ (also known as NF-kappa-B essential modulator (NEMO)) which is the regulatory subunit. The IKK complex can be activated by a variety of mechanisms including phosphorylation by TGF- β -activating kinase 1 (TAK1) and MEKKs. Ubiquitination of I κ B proteins occurs following phosphorylation and is carried out by the E3 ubiquitin ligase complex. The poly-ubiquitinated I κ B proteins will be rapidly degraded by the 26S proteasome which allows the NF- κ B proteins to translocate into the nucleus to exert their transcriptional activation effects. There is also the alternative NF- κ B signalling

pathway that involves activation of receptors such as CD40, followed by activation of NF- κ B inducing kinase (NIK) which phosphorylates the IKK- α homodimers (Sun, 2011). IKK- α can phosphorylate p100 which will then be processed to p52 and the p50/p52 dimers will translocate into the nucleus to activate genes involved in functions such as production of antibodies.

Other than the classical and alternative pathways described, the activity of the NF- κ B pathway is also further modulated by interactions with other proteins or other signalling pathways which lead to post-translational modifications. For instance, p65 can be phosphorylated at various sites by different kinases such as IKK- α and protein kinase C which influence its transcriptional activities. Other pathways including MAPKs, PI3K/Akt and ROS have also been linked to the activity of NF- κ B (Hoesel and Schmid, 2013).

Various studies have affirmed the crucial role of NF- κ B in inflammatory processes in human lung epithelial cells. Organic dust exposure to epithelial cells enhanced the release of pro-inflammatory cytokines through the NF- κ B pathway (Lidén et al., 2003). Similarly, the NF- κ B pathway also mediated the release of various inflammatory mediators such as IL-6, CXCL8, GM-CSF stimulated by IL-1 β and TNF- α (Newton et al., 1998).

1.6.4 AP-1 pathway

The AP-1 pathway is another major redox-sensitive transcription factor signalling pathway commonly implicated in inflammation. This pathway

involves basic region leucine zipper (bZIP) domain protein families, including Jun (comprising c-Jun, JunB and JunD), Fos (comprising c-Fos, FosB, Fra-1 and Fra-2) and some members of the ATF (ATF α , ATF-2 and ATF-3) and JDP (JDP-1 and JDP-2) protein family, which can form different combinations of dimers with different activities (Karin et al., 1997). The heterodimer of c-Jun and c-Fos is the major one which binds mainly to the 12-O-tetradecanoylphorbol-13-acetate (TPA) response element. Other than TPA, AP-1 activity can also be stimulated by many other factors including cytokines and growth factors (Karin, 1995). These factors regulate the activities of AP-1 by controlling the amount and activities of the AP-1 proteins.

The amount of AP-1 proteins is frequently regulated by the transcription of their genes. Many genes of AP-1 proteins such as c-Jun and c-Fos are immediate early genes whose transcription can be rapidly induced following stimulation. AP-1 protein abundance can also be modulated by regulation of their stability (Devary et al., 1991). For instance, serine phosphorylation of c-Jun by JNK can increase its stability by reducing the ubiquitination and degradation rate (Fuchs et al., 1997). The activities of AP-1 can also be controlled by post-translational modifications which affect the transcriptional activity. For instance, serine phosphorylation of c-Jun by JNK may upregulate its transcriptional activity due to increased affinity for the transcriptional coactivator CREB (cAMP response element binding protein) binding protein (CBP) (Reddy et al., 2013).

Many inflammatory genes relevant in airway inflammation are regulated by AP-1 in addition to NF- κ B (Adcock and Caramori, 2001). In human airway epithelial cells, it was observed that H₂O₂ or TNF- α -induced release of CXCL8 was mediated through the AP-1 pathway (Lakshminarayanan et al., 1998). Likewise, the release of MMP-9 and TGF- β from IL-1 β stimulation was dependent on the activation of AP-1 (Lee et al., 2006; Lin et al., 2011).

1.6.5 Other pathways

The activity of the various inflammatory pathways and expression of inflammatory genes are tightly regulated by a group of proteins known as the histone deacetylases (HDACs). There are four classes of HDACs, of which HDAC2, belonging to the Class I HDACs, and sirtuin 1 (SIRT1) and sirtuin 6 (SIRT6), belonging to the NAD⁺-dependent Class III HDACs, have been shown to be implicated in COPD (Ito et al., 2005; Rajendrasozhan et al., 2008; Takasaka et al., 2014).

Reduced levels and activities of HDAC2 have been reported in alveolar macrophages and lung epithelial cells from COPD patients (Ito et al., 2001; Qu et al., 2013). These have been attributed to post-translational modifications such as tyrosine nitration, S-nitrosylation of cysteine residues and serine phosphorylation due to increased oxidative stress and activation of inflammatory signaling (Adenuga et al., 2009; Osoata et al., 2009; Yang et al., 2006). The reduced HDAC2 level and activity impairs the action of corticosteroid as glucocorticoid receptors require HDAC2-mediated deacetylation to inhibit NF- κ B (Ito et al., 2006).

Furthermore, HDAC2 is also required for inflammatory gene silencing by glucocorticoids (Ito et al., 2000).

More recently, reduced levels of SIRT1 and SIRT6 have been reported in the lungs of COPD patients (Nakamaru et al., 2009; Rajendrasozhan et al., 2008; Takasaka et al., 2014). Similar to HDAC2, post-translational modifications by oxidants have been implicated in the reduced activity and levels of SIRT1 (Caito et al., 2010). The decrease in SIRT1 levels has been linked to increased activation of NF- κ B which elevates levels of CXCL8 and MMP-9 (Nakamaru et al., 2009; Rajendrasozhan et al., 2008). Likewise, SIRT6 has also been shown to be involved in the suppression of Akt activation in human airway epithelial cells (Takasaka et al., 2014).

In summary, a series of cell signalling events are initiated in the presence of inflammatory stimuli. These include the MAPKs and PI3K/Akt which can phosphorylate and activate a variety of proteins such as the transcription factors AP-1 and NF- κ B. In addition, the HDACs constitute additional epigenetic regulatory mechanism for inflammatory gene transcription. The final inflammatory response is a culmination of the complex interactions between these pathways.

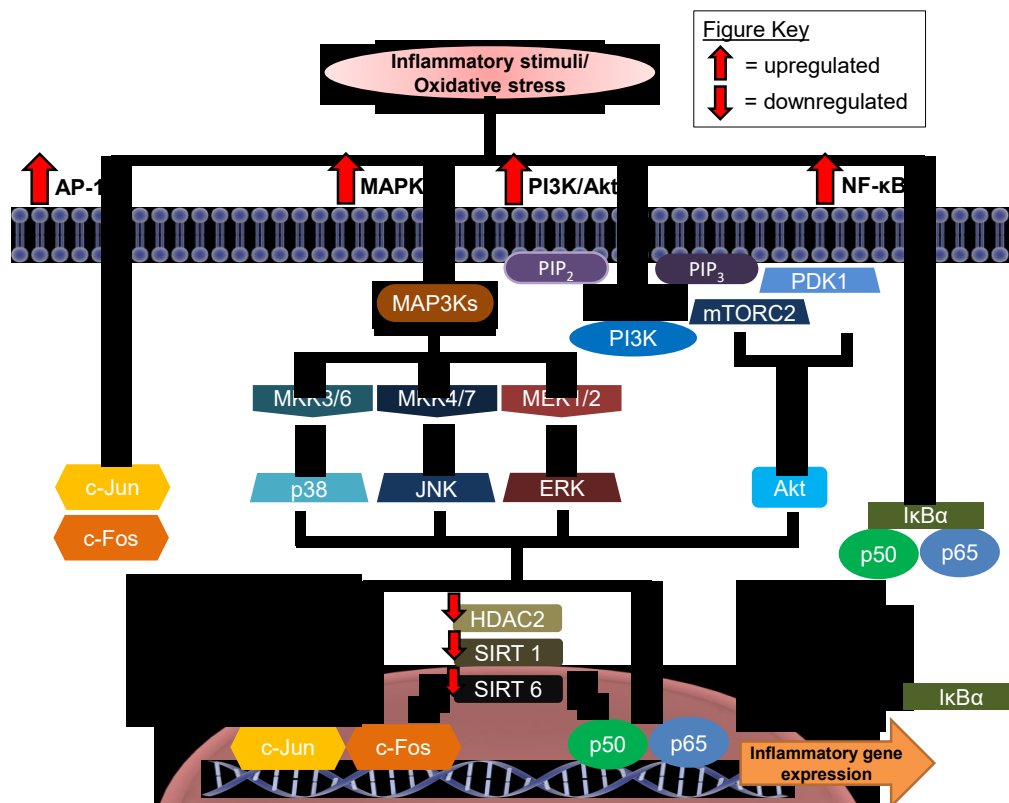


Figure 1.4 Intracellular inflammatory signalling pathways in airway epithelial cells in COPD. Major pathways including MAPK, PI3K/Akt and transcription factors such as AP-1 and NF-κB are activated when the cells are exposed to inflammatory stimuli or oxidative stress in COPD. Regulatory mechanisms such as HDAC2, SIRT1 and SIRT6 are effective in suppressing inflammation but are dysregulated in COPD. Red arrows show the upregulation or downregulation of these pathways.

1.7 Therapeutic options for COPD

Aside from limiting exposure to risk factors such as smoking cessation and pulmonary rehabilitation programmes, the management of stable, non-exacerbating COPD mainly involves the use of inhaled bronchodilators (β_2 agonist and anticholinergics) and inhaled corticosteroids (GOLD, 2016).

1.7.1 Bronchodilators

The most commonly used bronchodilators for COPD are the β_2 -agonists and anticholinergics. Bronchodilators reduce the tone of smooth muscles in the airways to effect widening of the airways, improvements in the symptoms and FEV₁ of patients and can reduce exacerbation risk (Sin et al., 2003). While short-acting bronchodilators (duration of effect within 6 h) can achieve rapid onset of action, long-acting bronchodilators (LABA) (duration of effect more than 12 h) are used in more severe cases to achieve better control of symptoms and improved quality of life (Tashkin and Fabbri, 2010)

β_2 -agonists stimulate the β_2 adrenergic receptors to increase the production of cyclic adenosine monophosphate (cAMP) (McGraw and Liggett, 2005). This increase in cAMP activates protein kinase A (PKA) which phosphorylates myosin light chain kinase (MLCK) to inhibit it. Myosin phosphorylation is reduced as a result and contraction of smooth muscle becomes diminished (Pelaia et al., 2008). Examples of short-acting β_2 -agonists are salbutamol and terbutaline and long-acting β_2 -agonists are formoterol and salmeterol.

Anticholinergics antagonise the effect of the endogenous neurotransmitter, acetylcholine, on the muscarinic cholinergic receptors (Belmonte, 2005). The three main muscarinic receptors found in the lungs are M1, M2 and M3. M1 receptors mediate the cholinergic nerve transmission, leading to the release of acetylcholine which stimulates the M3 receptors on smooth muscle to cause bronchoconstriction. Stimulation of M3 receptors activates phospholipase C which increases

intracellular calcium that binds to calmodulin. This activates MLCK, causing phosphorylation of myosin and resulting in bronchoconstriction. M2 receptors, on the other hand, inhibit the release of acetylcholine to counteract the effects of M1 and M3. Ipratropium is an example of short-acting acetylcholine inhibitor and tiotropium is an example of long-acting acetylcholine inhibitor (GOLD, 2016).

Combination of bronchodilators from two different classes has been shown to improve symptoms in cases whereby monotherapy is inadequate (Tashkin and Ferguson, 2013).

1.7.2 Corticosteroids

Corticosteroids are potent anti-inflammatory agents that are highly effective in asthma but show limited efficacy in COPD (Highland et al., 2003; To et al., 2010). Inhaled corticosteroids can slightly reduce exacerbations and progression rate of symptoms in COPD but cannot arrest the inexorable decline in lung function and mortality. This has been suggested to be a result of corticosteroid resistance - the failure of corticosteroids in treating the underlying inflammation in COPD.

Corticosteroids bind to the glucocorticoid receptors (GRs) in the cytoplasm to activate and facilitate nuclear translocation of receptors (Barnes, 2006a). The ligand-bound receptors dimerise and bind to glucocorticoid response elements (GRE) in the promoter regions of glucocorticoid-responsive genes. Gene activation occurs through interaction between the receptors and transcriptional co-activator

proteins such as CBP (Adcock et al., 2004). Anti-inflammatory genes activated in this trans-activation mode of action include interleukin-10, I κ B- α and MAPK phosphatase-1 (MKP-1). In addition, numerous inflammatory genes such as IL-6, CXCL8 and cyclo-oxygenase 2 (COX-2) are switched off in the trans-repression mode of action of corticosteroids. In trans-repression, activated glucocorticoid receptors can recruit histone deacetylase 2 (HDAC2) to the activated inflammatory gene complex to reduce histone acetylation and bring about gene silencing (Ito et al., 2000). Inflammatory gene silencing is also achieved through inhibition of histone acetyltransferase (HAT) activities of transcriptional co-activators by the activated glucocorticoid receptor.

The mechanisms behind corticosteroid resistance have been gradually unravelled in recent years (Adcock and Barnes, 2008; Eltom et al., 2011; Sousa et al., 1999). Some of the proposed mechanisms include increased activation of p38 and JNK which lead to higher GR phosphorylation that reduces binding to GRE; and over-activation of AP-1 which binds GR to hinder its interaction with GRE and other transcription factors. In addition, defective epigenetic mechanisms of GR such as failure to acetylate lysine residues on histone-4 which causes reduced transactivation of anti-inflammatory genes and oxidative stress-mediated reduction in HDAC2 levels which leads to diminished silencing of inflammatory genes have been shown in studies (Ito et al., 2004; Matthews et al., 2004; Osoata et al., 2009). More recently, the activation of the P2X7/NLRP3 inflammasome

pathway which cannot be modulated by the action of corticosteroids has been linked to the corticosteroid-resistant inflammation in COPD (Eltom et al., 2011).

Clinically, corticosteroids are only used in more severe COPD patients and always in combination with bronchodilators as this have been shown to be effective in reducing exacerbations and improving lung function compared to either drug alone (GOLD, 2016). Furthermore, triple therapy involving β_2 -agonist, anticholinergic and corticosteroid have proved to be more effective in severe cases of COPD (Gaebel et al., 2011).

1.7.3 Other therapies

In more severe cases of COPD, other pharmacological agents that are used include theophylline, phosphodiesterase-4 (PDE-4) inhibitors, mucolytics/antioxidants and macrolide antibiotics (GOLD, 2016).

Theophylline is a methylxanthine with mild bronchodilator effect and is able to reduce symptoms and exacerbations. The exact mechanism of methylxanthines is still unknown although they have been suggested to be non-selective inhibitors of phosphodiesterase or PI3K- δ isoform selective inhibitor (Barnes, 2006b; To et al., 2010).

PDE-4 inhibitors reduce the degradation of cAMP to exert anti-inflammatory and bronchodilatory effects. Roflumilast is a potent PDE-4 inhibitor approved for reduction of exacerbations in COPD (Price et al., 2010).

Mucolytics such as *N*-acetylcysteine (NAC) and carbocysteine are used to reduce mucus viscosity in COPD although no improvements in clinical outcomes have been observed. In addition, NAC is a glutathione precursor with anti-oxidant effects but studies to date have not found conclusive evidence of its beneficial effects in COPD (Sadowska et al., 2006).

Chronic therapy with macrolide antibiotics is also used in severe COPD patients to reduce exacerbations. Other than reducing bacteria-induced exacerbations, the anti-inflammatory effects of macrolides such as azithromycin have been suggested to contribute to their efficacy (Yamaya et al., 2012).

1.7.4 Classification of COPD patients and recommended pharmacotherapy

In order to guide treatment plan, the GOLD guidelines considered the spirometric classification (Table 1.1), exacerbation history and symptoms (measured using Modified Medical Research Council (mMRC) or COPD Assessment Test (CAT) questionnaires) to classify COPD patients into one of four categories according to risk of exacerbations and symptoms (Table 1.2) (GOLD, 2016). The recommended pharmacotherapy for stable COPD patients is shown in Table 1.3.

Table 1.1 Spirometric classification of severity of airflow limitation (Based on post bronchodilator FEV₁) (In patients with FEV₁/FVC < 0.7)

Classification	Description	FEV ₁ *
GOLD 1	Mild	≥ 80% predicted
GOLD 2	Moderate	≥ 50% and < 80% predicted
GOLD 3	Severe	≥ 30% and < 50% predicted
GOLD 4	Very Severe	< 30% predicted

*FEV₁ % predicted is calculated by comparison with reference values based on age, height, sex and race ((GOLD, 2016; Pellegrino et al., 2005))

Table 1.2 Categorisation of COPD patients according to risk and symptoms evaluation (GOLD, 2016)

Category	Characteristics	Spirometric Classification	Exacerbations per year	CAT	mMRC
A	Low Risk Less Symptoms	GOLD 1-2	≤ 1	<10	0-1
B	Low Risk More Symptoms	GOLD 1-2	≤ 1	≥ 10	≥ 2
C	High Risk Less Symptoms	GOLD 3-4	≥ 2 or ≥ 1 leading to hospitalisation	<10	0-1
D	High Risk More Symptoms	GOLD 3-4	≥ 2 or ≥ 1 leading to hospitalisation	≥ 10	≥ 2

Table 1.3 Pharmacological therapy for stable COPD patients (GOLD, 2016)

Patient Category	First Choice	Alternative Choice	Other Possible Treatment
A	S.A anticholinergic prn or S.A β_2 -agonist prn	L.A anticholinergic or L.A β_2 -agonist or S.A anticholinergic + S.A β_2 -agonist	Theophylline
B	L.A anticholinergic or L.A β_2 -agonist	L.A anticholinergic + L.A β_2 -agonist	S.A anticholinergic and/or S.A β_2 -agonist Theophylline
C	Inhaled corticosteroid+ L.A β_2 -agonist and/or L.A anticholinergic	L.A anticholinergic + L.A β_2 -agonist or L.A anticholinergic +PDE-4 inhibitor or L.A β_2 -agonist + PDE-4 inhibitor	S.A β_2 -agonist and/or S.A anticholinergic Theophylline

Patient Category	First Choice	Alternative Choice	Other Possible Treatment
D	Same as C	Inhaled corticosteroid + L.A β_2 -agonist + L.A anticholinergic or Inhaled corticosteroid + L.A β_2 -agonist + PDE-4 inhibitor or L.A anticholinergic + L.A β_2 -agonist or L.A anticholinergic + PDE-4 inhibitor	Carbocysteine N-acetylcysteine S.A anticholinergic and/or S.A β_2 -agonist Theophylline

S.A = short acting; L.A = long acting; PDE = phosphodiesterase;
 prn=when necessary

1.7.5 Evaluation of COPD pharmacological agents and drug development

1.7.5.1 Lack of safe and effective anti-inflammatory agents

Current COPD therapies are mainly focused on alleviating symptoms without having substantial effects on the underlying inflammation which drives the progressive decline in lung function (Barnes, 2013b). In fact, considerable advances have been made in the development of longer-acting LABAs and LAMAs and their combinations, clearly evident by their dominance of the list of newly approved drugs by Food and Drug Administration (FDA) for COPD in the past few years (FDA, 2016). On the other hand, little progress has been made in the development of effective and safe anti-inflammatory agents for COPD which have disease-modifying effects. Corticosteroids are poorly effective as described in Chapter 1.7.2 and are associated with increased risk of pneumonia (Suissa et al., 2013). Roflumilast, a PDE-4 inhibitor which is a novel class of anti-inflammatory agent, has shown efficacy but its use is hindered by side effects (Rabe, 2011). Similarly, theophylline and macrolides with anti-inflammatory effects are of limited value due to their toxicity and concerns with antibiotic resistance (Barnes, 2006b; Li et al., 2014).

As such, it is well-recognised that the lack of safe and effective anti-inflammatory agent in COPD is a pressing issue which needs to be addressed.

1.7.5.2 COPD drug development

The inflammation in COPD involves the activation of multiple kinases as described in Chapter 1.6, making them appealing drug targets. However, no kinase inhibitors are currently in clinical use for COPD and none has reached the large-scale Phase III clinical trials (Barnes, 2016). The discovery of novel molecules with effects on these pathways will be imperative in the quest to combat the steroid-resistant inflammation in COPD.

1.8 Stilbene analogues

1.8.1 Overview of stilbene analogues

The natural environment, with its rich diversity of molecules, may hold promise in tackling the challenges in COPD therapy. In fact, in the past two decades, natural products and natural product-derived drugs have continued to dominate the new small molecule drug approvals (Newman and Cragg, 2012). This is in spite of a shift in focus to high throughput screening of synthetic combinatorial chemical libraries in drug discovery. In particular, plants have been a major source of medication with records dating back to as early as 2600 BC (Cragg and Newman, 2013). At the turn of the new century, plant-derived small molecules have continued to generate much excitement in the medical science field. One outstanding class of compounds is the stilbene family of compounds.

Naturally occurring stilbene analogues are produced in plants via the phenylpropanoid pathway which is an important pathway in secondary

plant metabolism (Roupe et al., 2006a). These secondary metabolites, also known as phytoalexins, are integral in the defence of plants against stressful conditions such as ultraviolet radiation and fungal infections. Resveratrol, the well-known stilbene analogue, can be found in various plants such as blueberries, bilberries and grapes (Lyons et al., 2003). Since it was first isolated from the roots of white hellebore in 1940, resveratrol has enjoyed escalating interest especially after its meteoric rise to fame for its postulated role in the “French Paradox” (Constant, 1997). Encouraging results from the early *in vitro* experiments acted as the catalyst for the comprehensive probe into the pharmacological effects of resveratrol that has culminated into a substantial library of information today (Baur and Sinclair, 2006; Fulda, 2010; Saiko et al., 2008)

1.8.2 Pharmacological effects of stilbene analogues

The myriad pharmacological effects of resveratrol were elegantly demonstrated in a seminal study on its chemopreventive activity where all three stages of carcinogenesis were inhibited (Jang et al., 1997). Specifically, its anti-oxidant and anti-inflammatory effects were demonstrated to be important in these activities. Various other studies have also documented its anti-inflammatory, anti-oxidative, anti-cancer, neuroprotective and anti-ageing effects (Baur et al., 2006; Baur and Sinclair, 2006; Fulda, 2010) in pre-clinical as well as clinical settings (Bo et al., 2013; Brasnyo et al., 2011; Witte et al., 2014) (Figure 1.5). One of its most noteworthy effects is the activation of sirtuin 1 (SIRT1) (Hubbard et al., 2013), a protein deacetylase involved in regulation of

many proteins including those related to inflammation (Kotas et al., 2013; Yang et al., 2012). These multiple effects of resveratrol form the basis for its potential application against a wide range of common diseases such as cardiovascular diseases, inflammatory diseases (including asthma, chronic obstructive pulmonary disease and arthritis) and cancer.

The promising properties of resveratrol have sparked exploration of the other stilbene analogues (Figure 1.6). Many of the naturally-derived analogues can be found in fruits and plants and they show close structural resemblance to resveratrol, displaying differences only in number of hydroxyl or methoxy moieties. Other than these, a diversity of functional groups has also been introduced into the stilbene structure through synthetic methods. One example is *trans*-2,6-difluoro-4'-(N,N-dimethylamino)stilbene (DFS), a biologically active analogue which displayed 1000-fold higher potency compared to resveratrol (Zhang et al., 2013; Zhang et al., 2011a). The other analogues have also demonstrated biological activities in a wide range of disease models (Fulda, 2010; Kang et al., 2009; Reinisalo et al., 2015; Richard et al., 2005; She et al., 2003) (Table 1.4). Notably, several analogues were more potent than resveratrol against inflammation and oxidative stress (Table 1.4). Furthermore, mechanistic studies have revealed that these analogues altered key inflammatory pathways including NF- κ B, AP-1, PI3K/Akt and MAPKs (Choi et al., 2010; Cichocki et al., 2008; Costa et al., 2012; Deck et al., 2008; Paul et al., 2009; Qureshi et al., 2012). Likewise, stilbene analogues have also demonstrated

direct radical scavenging effects and regulation of anti-oxidant enzymes such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Amorati et al., 2004; Chiou et al., 2011; Lee et al., 2010a; Stivala et al., 2001).

The pleiotropic anti-inflammatory and anti-oxidative mechanisms of resveratrol and its analogues make them attractive therapeutic agents against the complex inflammatory processes in COPD. Several investigations have already confirmed the potential of resveratrol in COPD. When alveolar macrophages from cigarette smokers and COPD patients were treated with resveratrol, it was found that there was a marked reduction in the release of inflammatory markers (GM-CSF and CXCL8) from both groups of patients which was poorly inhibited by dexamethasone (Culpitt et al., 2003a; Culpitt et al., 2003b). In addition, a mechanistic study in A549 lung carcinoma cells revealed that resveratrol appeared more efficacious than dexamethasone in the inhibition of NF- κ B and AP-1-dependent transcription and the anti-inflammatory action was independent of the glucocorticoid pathway, which further ascertains the promise of stilbene analogues in COPD treatment (Donnelly et al., 2004). Given that several stilbene analogues have shown more potent anti-inflammatory and anti-oxidative effects, it is conceivable that there are more potent alternatives to resveratrol that remain to be uncovered.

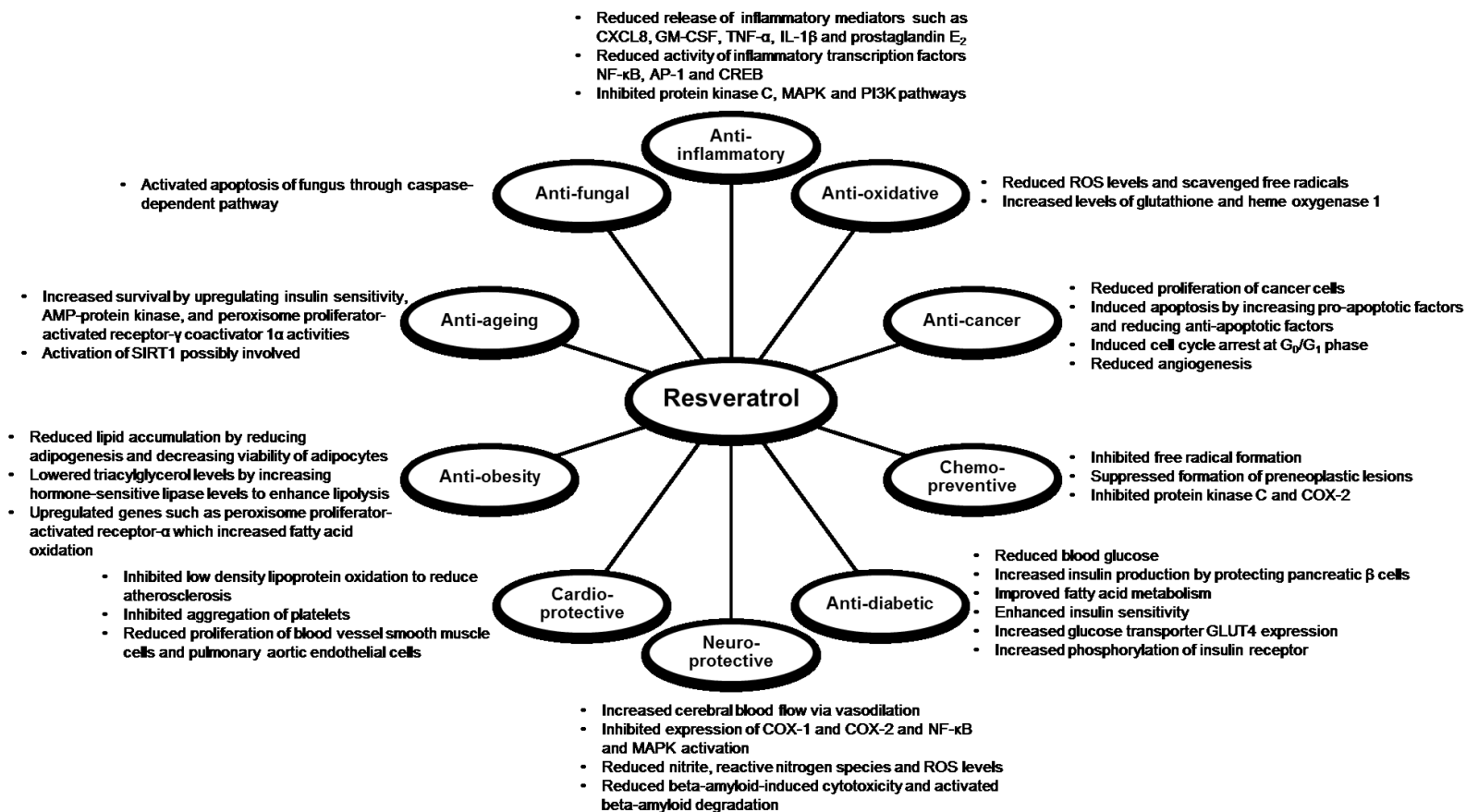


Figure 1.5 Selected examples of the pharmacological effects of resveratrol in pre-clinical studies (Aguirre et al., 2014; Aluyen et al., 2012; Bastianetto et al., 2015; Baur et al., 2006; Bhat and Pezzuto, 2002; Birrell et al., 2005; Donnelly et al., 2004; Kasiotis et al., 2013; Lee and Lee, 2015; Szkudelski and Szkudelska, 2015; Wu and Hsieh, 2011).

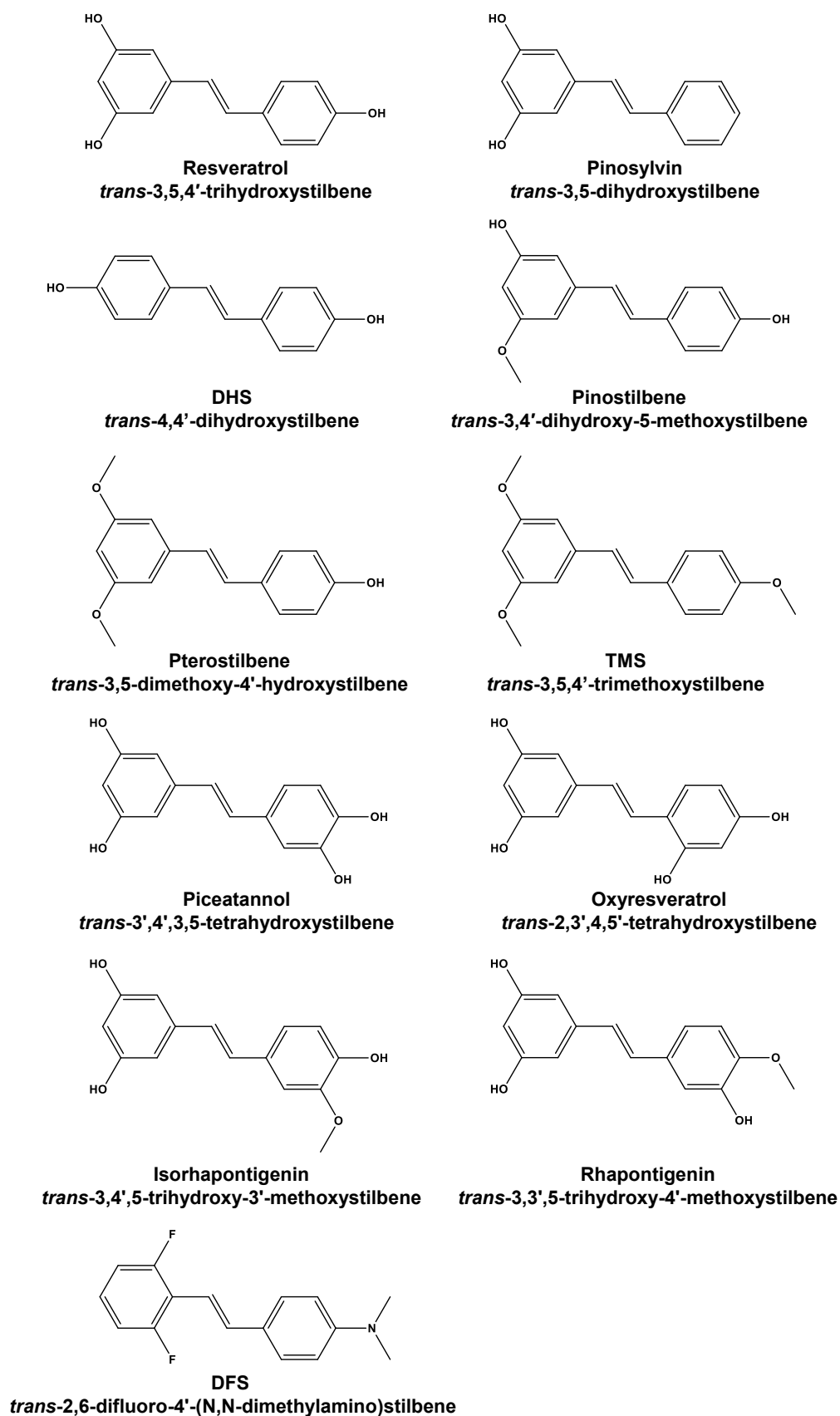


Figure 1.6 Structures of stilbene analogues.

Table 1.4 Anti-inflammatory or anti-oxidative effects of stilbene analogues compared to resveratrol

Compound	Assay	Effect compared to resveratrol	Reference
Pinosylvin	Leukotriene inhibition in human neutrophils	More potent ($IC_{50} = 22.7 \mu M$ vs $> 50 \mu M$)	(Adams et al., 2005)
Found in <i>Pinus sylvestris</i> and <i>Gnetum cleistostachyum</i>	NF- κ B inhibition in SW1353 cells	Similar potency ($IC_{50} = 14.5 \mu g/mL$ vs $18.5 \mu g/mL$)	(Sobolev et al., 2011)
	ROS inhibition in HL-60 cells	Less potent ($IC_{50} < 20 \mu g/mL$ vs $0.28 \mu g/mL$)	
	NO production inhibition in RAW284.7 cells	More potent ($IC_{50} = 6.9 \mu g/mL$ vs $10 \mu g/mL$)	
	Cu ²⁺ -induced low density lipoprotein peroxidation inhibition	Lower activity (Inhibition time = 43 min vs 58 min)	(Cheng et al., 2006)
	Extracellular chemiluminescence inhibition in granulocytes (antioxidant assay)	Lower activity (Inhibition = 87% vs 99%)	(Perecko et al., 2008)
	Thiobarbituric acid reactive substances (TBARS) production inhibition in Fe ²⁺ -induced rat liver microsomal peroxidation	Lower activity (Inhibition at $1 \mu M = 50\%$ vs 100%)	(Cai et al., 2003)

DHS Found in <i>Yucca periculosa</i>	TBARS production inhibition in Fe ²⁺ /ascorbate-induced human erythrocyte lipid peroxidation	Higher activity (TBARS formation = 0.45 nmol/mg protein vs 0.61 nmol/mg protein)	(Fan et al., 2009)
	TBARS production inhibition in Fe ²⁺ -induced rat liver microsomal peroxidation	Higher activity (Inhibition at 0.25 µM = 70% vs 25%)	(Cai et al., 2003)
	Cu ²⁺ -induced low density lipoprotein peroxidation inhibition	Higher activity (Inhibition time = 77 min vs 58 min)	(Cheng et al., 2006)
	β-carotene bleaching inhibition (antioxidant assay)	Higher activity (Inhibition = 81.5% vs 66.9%)	(Privat et al., 2002)
Pinostilbene	Free radical scavenging activity in cell-free system	Less potent (IC ₅₀ = 47.1 µM vs 36.9 µM)	(Chao et al., 2010)
Found in <i>Gnetum venosum</i> and <i>Pinus sibirica</i>	Cyclooxygenase-2 enzyme inhibition	More potent (IC ₅₀ = 0.35 µM vs 3.40 µM)	(Kutil et al., 2015)

Pterostilbene	Mouse model of accelerated ageing	Increased manganese superoxide dismutase levels and reduced NF- κ B p65 levels	(Chang et al., 2012)
Found in blueberries, grapes and almonds	Cyclooxygenase-2 enzyme inhibition	Less potent (IC_{50} = 27.04 μ M vs 3.40 μ M)	(Kutil et al., 2015)
	5-lipoxygenase enzyme inhibition	More potent (IC_{50} = 9.32 μ M vs >50 μ M)	
	Nitric oxide and TNF- α release inhibition in RAW264.7 cells stimulated with LPS	Similar inhibition	(Qureshi et al., 2012)
	Lipid peroxidation inhibition in H_2O_2 -stimulated human erythrocytes	More potent (IC_{50} = 27.04 μ M vs >200 μ M)	(Mikstacka et al., 2010)
	TBARS production inhibition in normal human fibroblast stimulated with t-butyl hydroperoxide	Similar activity (Inhibition = 61% vs 67%)	(Stivala et al., 2001)
	NO production inhibition in microglial cells stimulated with LPS	More potent (IC_{50} = 1.9 μ M vs 15.5 μ M)	(Meng et al., 2008)

TMS	TBARS production inhibition in normal human fibroblast stimulated with t-butyl hydroperoxide	Poorer activity (no inhibition vs 67%)	(Stivala et al., 2001)
Found in <i>Dacrydium balansae</i> , <i>Scirpus holoschoenus</i> L. and <i>Virola elongata</i>	IL-1 β and TNF- α release inhibition in RAW264.7 cells stimulated with LPS	Slightly lower potency	(Son et al., 2014)
	HO-1 expression in HT22 cells	Less induction	(Kim et al., 2012)
	NO production inhibition in microglial cells stimulated with LPS	More potent (IC ₅₀ = 10.8 μ M vs 15.5 μ M)	(Meng et al., 2008)
Piceatannol	LPS-induced NO production inhibition in RAW264.7 cells	More potent (IC ₅₀ = 8.96 μ M vs 29.9 μ M)	(Djoko et al., 2007)
Found in <i>Picea abies</i> , <i>Aiphanes horrida</i> , <i>Gnetum cleistostachyum</i> , peanuts and grapes	NF- κ B activation in LPS-induced RAW264.7 cells	More potent (40% inhibition with 15 μ M vs 30 μ M)	
	NF- κ B activation in TNF- α -induced KBM-5 cells	Slightly higher inhibition	(Ashikawa et al., 2002)
	NO production in LPS-stimulated mouse peritoneal macrophages	More potent (IC ₅₀ = 23 μ M vs 68 μ M)	(Kageura et al., 2001)
	I κ B α activation in 12-O-tetradecanoylphorbol-13-aceate (TPA)-stimulated MCF-10A cells	Higher inhibition	(Son et al., 2010)

Found in <i>Artocarpus lakoocha</i> , <i>Artocarpus xanthocarpus</i> and white mulberry,	Piceatannol	IL-1 β and TNF- α release inhibition in RAW264.7 cells stimulated with LPS	Slightly higher potency	(Son et al., 2014)
		DPPH free radical scavenging activity	Higher potency (IC ₅₀ = 7.42 μ g/mL vs 12.9 μ g/mL)	(He and Yan, 2013)
		Superoxide anion scavenging activity	Higher potency (IC ₅₀ = 0.45 μ g/mL vs 37.9 μ g/mL)	
	Oxyresveratrol	DPPH free radical scavenging activity	Higher potency (IC ₅₀ = 28.9 μ M vs 38.5 μ M)	(Lorenz et al., 2003)
		Dichlorofluorescein activity in H ₂ O ₂ -stimulated primary glial cells	Higher inhibition (250% increase vs 370%)	
		Nitrite production inhibition in LPS/IFN- γ -stimulated microglial cells	Lower potency (IC ₅₀ = 45.3 μ M vs 22.4 μ M)	
		Cyclooxygenase-2 enzyme inhibition	Less potent (IC ₅₀ = 14.71 μ M vs 3.40 μ M)	(Kutil et al., 2015)
		5-lipoxygenase enzyme inhibition	More potent (IC ₅₀ = 18.5 μ M vs >50 μ M)	

Rhapontigenin	NO production in LPS-stimulated mouse peritoneal macrophages	More potent (IC ₅₀ = 48 µM vs 68 µM)	(Kageura et al., 2001)
Found in <i>Gnetum cleistostachyum</i> , <i>Rheum undulatum</i> , <i>Rheum rhaponticum</i> L, crimson glory vine, rhubarb	Cyclooxygenase-2 enzyme inhibition	Less potent (IC ₅₀ = 36.12 µM vs 3.40 µM)	(Kutil et al., 2015)
	ABTS ^{•+} radical scavenging	Similar activity	(Ngoc et al., 2008)
	Lipoxygenase inhibition	Similar potency (IC ₅₀ = 10.7 µM vs 12.3 µM)	
Isorhapontigenin	NO production in LPS-stimulated mouse peritoneal macrophages	Similar potency (IC ₅₀ = 63 µM vs 68 µM)	(Kageura et al., 2001)
Found in <i>Gnetum cleistostachyum</i> , <i>Gnetum parvifolium</i> , and <i>Aiphanes aculeata</i>	H ₂ O ₂ release and p44/42 MAPK activation inhibition in oxidised low density lipoprotein-stimulated bovine aortic smooth muscle cells	Similar inhibitory effect	(Liu and Liu, 2004)
	Cyclooxygenase-2 enzyme inhibition	Less potent (IC ₅₀ = 24.00 µM vs 3.40 µM)	(Kutil et al., 2015)
	Superoxide anion scavenging activity	Lower potency (IC ₅₀ = 29 µM vs 15 µM)	(He and Yan, 2013)

1.8.3 Pharmacokinetics of stilbene analogues

Pharmacokinetics, the time course of compound concentration in the body, is another critical aspect that determines the biological effect of a compound *in vivo*. This compound concentration is a factor of its absorption, distribution, metabolism and excretion characteristics and understanding these will be essential in optimising variables in drug development such as the dosing route, formulation and dose. Some key pharmacokinetics parameters and their definitions are presented in Table 1.5.

The oral pharmacokinetics of a drug is of particular interest since oral dosing is the preferred administration route. To achieve good oral bioavailability, a drug should ideally possess good aqueous solubility so that it can dissolve easily in gastric or intestinal fluids; high membrane permeability such that the dissolved drug can pass through membranes into the blood and high metabolic stability so that it will not be substantially metabolised before entering the systemic circulation (Hurst et al., 2007). Local administration such as inhalation should be considered if oral bioavailability is poor but the systemic exposure of the drug will not necessarily be high as it will be dependent on its systemic clearance.

The pharmacokinetics of resveratrol has been extensively investigated in various pre-clinical and clinical studies (Boocock et al., 2007; Das et al., 2008; Kapetanovic et al., 2011; Meng et al., 2004; Walle et al., 2004). Generally, these studies have concluded that resveratrol is rapidly absorbed but highly metabolised by Phase II conjugation

reactions, resulting in low plasma exposure and oral bioavailability although there is evidence of enterohepatic recirculation (Marier et al., 2002). This partly explains the modest effects of resveratrol in clinical studies despite the substantial positive pre-clinical data (Poulsen et al., 2015). In addition, these studies also highlight the utility of the commonly-employed rat pharmacokinetic model as a tool to study the pharmacokinetics of stilbene analogues as the data correlated well with clinical profiling. Clearly, pharmacokinetic investigations of the other stilbene analogues should be an essential component of the pre-clinical evaluation of these compounds.

Compared to resveratrol, the pharmacokinetic parameters of the other stilbene analogues are not well-studied. Using the same rat pharmacokinetic model, the pharmacokinetics of several fully methoxylated stilbene analogues and resveratrol have been profiled which allow direct comparisons. These fully methoxylated analogues were found to have slower clearance and longer elimination half-life after intravenous injection compared to resveratrol. However, the oral bioavailability of the analogues varied from being poorly bioavailable (Lin et al., 2010a; Lin et al., 2012; Ng et al., 2014) to having much better bioavailability (Lin et al., 2010b; Lin and Ho, 2011; Lin et al., 2010c) compared to resveratrol. Of all the stilbene analogues known so far, the partially methoxylated stilbene analogue, pterostilbene, has emerged as an analogue with one of the most favourable pharmacokinetic parameters (Lin et al., 2009). Its clearance was lower compared to resveratrol and its oral bioavailability could be increased

through dose escalation. Similarly, the synthetic analogue, *trans*-2,6-difluoro-4'-(N,N-dimethylamino)stilbene (DFS), displayed favourable pharmacokinetics and could achieve a higher bioavailability when its dose was escalated (Lin et al., 2013).

Preliminary studies performed on other stilbene analogues have provided some guidance on their pharmacokinetics. For example, the plasma exposure of pinosylvin was the lowest compared to piceatannol and rhapontigenin after intravenous injection (Roupe et al., 2006b). However, methodological variations between studies such as dosing vehicles used and design of studies impede meaningful comparisons. Further studies of these analogues would be required for a better understanding of their pharmacokinetics compared to resveratrol.

Table 1.5 Pharmacokinetic parameters and their definitions

Parameter	Definition
$AUC_{0 \rightarrow last}$	• Area under the curve from 0 min to last measured time • Measure of the exposure of compound in plasma or tissue
C_{max}	• Maximum plasma compound concentration • Measure of the maximum concentration of compound in the plasma achieved
Cl	• Plasma clearance • Measure of the apparent volume of plasma cleared of compound per unit time
F	• Oral bioavailability • Measure of the percentage of unchanged compound that enters systemic circulation after oral administration
$MTT_{0 \rightarrow last}$	• Mean transit time measured from 0 min to last measured time • Measure of the average time a molecule stays in the body from the start of absorption to end of

elimination	
$t_{1/2}$	• Terminal elimination half-life • Measure of time taken for compound in plasma to be reduced to half after reaching pseudo-equilibrium
t_{max}	• Time to maximum plasma compound concentration • Measure of the time required to achieve maximum concentration of compound in the plasma
V_c	• Apparent volume of distribution of the central compartment • Measure of how widely the compound is distributed in the plasma and highly perfused tissues after administration

1.9 Hypothesis and aims of thesis

Previous studies have demonstrated the promising effects of resveratrol in counteracting the inflammation in COPD but the poor pharmacokinetics and weak potency of resveratrol are major barriers to its clinical development and better alternatives are required. It is hypothesised that some analogues of resveratrol have superior *in vivo* pharmacokinetics and *in vitro* anti-inflammatory effects compared to resveratrol and they exert their effects through a pathway distinct to that of corticosteroid.

To test this hypothesis, this project aims to achieve a comprehensive understanding of the pharmacology of stilbene analogues by characterising their pharmacokinetics and also examining their pharmacological effects in airway inflammation cell models.

The plasma pharmacokinetics of some stilbene analogues will be profiled using the same pharmacokinetic model to shed some light on the structural-pharmacokinetic relationship of stilbene analogues. More advanced pharmacokinetic studies involving profiling of tissue distribution will be carried out for pterostilbene, the stilbene analogue with one of the best pharmacokinetics, to give an insight into its movement across different body compartments and possible target organs as well as offer a general idea on the tissue distribution of stilbene analogues. Detailed pharmacokinetic studies involving metabolite identification will be performed for DFS, the synthetic analogue which has shown 1000-fold higher biological activity compared to resveratrol and good oral bioavailability (approximately 40%) in preliminary studies, to identify major biotransformations on its functional groups that are distinct from the naturally-occurring stilbene analogues.

The pharmacological effects of stilbene analogues will be studied in *in vitro* airway inflammation cell models to identify candidates with superior anti-inflammatory profiles. The key anti-inflammatory mechanisms of stilbene analogues will be clarified and compared to dexamethasone to evaluate their suitability as alternative anti-inflammatory agents for COPD.

With the insights of their pharmacological properties gained from this project, suitable stilbene analogues with decent pharmacokinetic profiles (such as slow clearance and high oral bioavailability) and anti-

inflammatory effects will be identified for further development as novel anti-inflammatory therapeutics against COPD.

CHAPTER 2

MATERIALS & METHODS

2.1 Materials

Unless otherwise mentioned, the following materials were obtained from the stated suppliers:

Material	Supplier
2-hydroxypropyl- β -cyclodextrin (degree of substitution around 0.6)	Wacker (Burghausen, Germany)
Acetonitrile	Tedia, OH, USA
Albumin, bovine serum (BSA)	Sigma-Aldrich, Dorset, UK
Anti-Akt (pan) antibody, rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti-c-Fos antibody, rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti-c-Jun antibody, rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti-ERK1/2 antibody, rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti-FoxO3A antibody rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti-I κ B α antibody, rabbit polyclonal	Cell Signaling Technology, MA, USA
Anti-JNK antibody, rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti-mouse IgG, HRP-linked antibody	Cell Signaling Technology, MA, USA
Anti-NF- κ B p50 antibody, rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti-p38 antibody, rabbit polyclonal	Cell Signaling Technology, MA, USA
Anti-phospho-Akt (Ser473) antibody, rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti-phospho-Akt (Thr308) antibody, rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti-phospho-ERK1/2 (Thr202/Tyr204) antibody, rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti-phospho-I κ B α (Ser32) antibody, rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti-phospho-JNK (Thr183/Tyr185) antibody, rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti-phospho-p38 antibody, rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti-rabbit IgG, HRP-linked antibody	Cell Signaling Technology, MA, USA

Anti-SIRT1 antibody, mouse monoclonal	Cell Signaling Technology, MA, USA
Anti-SIRT1 antibody, rabbit polyclonal	Abcam, Cambridge, UK
Anti-TBP antibody, rabbit monoclonal	Cell Signaling Technology, MA, USA
Anti- α -tubulin antibody, mouse monoclonal	Sigma-Aldrich, Dorset, UK
Anti- β -Actin antibody, mouse monoclonal	Cell Signaling Technology, MA, USA
Bio-Rad Protein Assay	Biorad, Hertfordshire, UK
Bis-Tris polyacrylamide gel, 4-12%	Invitrogen, Paisley, UK
Bronchial epithelial growth media	Lonza, Slough, UK
Carboxymethylcellulose, sodium salt	Sigma-Aldrich, MO, USA
Cell Lysis Buffer	Cell Signaling Technology, Danvers, USA
Collagenase from <i>Clostridium histolyticu</i> , Type IA-S	Sigma-Aldrich, Dorset, UK
CXCL8 ELISA capture antibody	Invitrogen, Paisley, UK
CXCL8 ELISA detection antibody	Invitrogen, Paisley, UK
CXCL8 ELISA standard protein	R&D Systems, Abingdon, UK
Deoxyribonuclease I from bovine pancreas	Sigma-Aldrich, Dorset, UK
Dexamethasone	Sigma-Aldrich, Dorset, UK
Dimethyl sulphoxide	Sigma-Aldrich, Dorset, UK
dNTP mix	Applied Biosystems, MA, USA
Dulbecco's modified eagle medium (DMEM)	Sigma-Aldrich, Dorset, UK
Dulbecco's phosphate buffered saline	Sigma-Aldrich, Dorset, UK
Dynabeads Protein A for Immunoprecipitation	Fisher Scientific, Loughborough, UK
ECL Western Blotting Detection Reagents	GE Healthcare, Buckinghamshire, UK
ELISA plate, Nunc MaxiSorp®	Fisher Scientific, Loughborough, UK
Ethylenediaminetetraacetic acid (EDTA)	Biorad, Hertfordshire, UK
Fetal bovine serum	Gibco, Paisley, UK
Full range rainbow molecular weight marker	GE Healthcare, Buckinghamshire, UK
Glycerol	Sigma-Aldrich, Dorset, UK
H ₂ DCFDA	Sigma-Aldrich, Dorset, UK
Hank's balanced salt solution	Sigma-Aldrich, Dorset, UK
Hyperfilm	GE Healthcare, Buckinghamshire, UK

IL-6 ELISA capture antibody	Invitrogen, Paisley, UK
IL-6 ELISA detection antibody	Invitrogen, Paisley, UK
IL-6 ELISA standard protein	R&D Systems, Abingdon, UK
Keratinocyte serum free medium with bovine pituitary extract and recombinant human epidermal growth factor	Invitrogen, Paisley, UK
L-ascorbic acid	Sigma-Aldrich, Dorset, UK
L-glutamine	Sigma-Aldrich, Dorset, UK
LHC-9 medium	Invitrogen, Paisley, UK
Luciferase Assay System	Promega
Methanol	Tedia, OH, USA
MicroAmp® 96 Well Reaction Plate	Invitrogen, Paisley, UK
Minisart® syringe filter, 0.2 µm	Sartorius, Gottingen, Germany
Multiscribe™ reverse transcriptase	Applied Biosystems, MA, USA
NADPH Regenerating Solutions A and B	BD Biosciences, MA, USA
NE-PER™ Nuclear and Cytoplasmic Extraction Reagents	Fisher Scientific, Loughborough, UK
Nitrocellulose Membrane	Amersham, GE Healthcare Life Sciences, Buckinghamshire, UK
NuPAGE MOPS SDS Running Buffer (20 X)	Invitrogen, Paisley, UK
Nylon Cell Strainer, 70 µm	Fisher Scientific, Loughborough, UK
Oxyresveratrol	Sigma-Aldrich, Dorset, UK
Phenol red free DMEM	Fisher Scientific, Loughborough, UK
Phosphatase Inhibitor Cocktail	Sigma-Aldrich, Dorset, UK
Piceatannol	Tokyo Chemical Industry, Tokyo, Japan
Pinostilbene	Tokyo Chemical Industry, Tokyo, Japan
Pinosylvin	Tokyo Chemical Industry, Tokyo, Japan
Pooled human liver microsomes	BD Biosciences, MA, USA
Pooled male B6C3F1 mouse liver microsomes	BD Biosciences, MA, USA
Pooled male Sprague-Dawley rat liver microsomes	BD Biosciences, MA, USA
Potassium phosphate	Sigma-Aldrich, MO, USA
Protease Inhibitor Cocktail Tablet	Sigma-Aldrich, Dorset, UK

Pterostilbene	Tokyo Chemical Industry, Tokyo, Japan
Purified bovine collagen solution	Advanced BioMatrix, California, USA
qPCR Human Reference cDNA, random primed	Clontech Laboratories, Inc., California, USA
Random primers	Applied Biosystems, MA, USA
Resveratrol	Tokyo Chemical Industry, Tokyo, Japan
Resveratrol, ¹³ C	Sigma-Aldrich, MO, USA
Rhapontigenin	Tokyo Chemical Industry, Tokyo, Japan
RNase free water	Qiagen, Manchester, UK
RNeasy Plus Mini Kit	Qiagen, Manchester, UK
Sample Buffer, Laemmli	Sigma-Aldrich, Dorset, UK
Sample Buffer, Laemmli 2× Concentrate	Sigma-Aldrich, Dorset, UK
Sodium dodecyl sulphate	Sigma-Aldrich, Dorset, UK
Sulphuric acid	Sigma-Aldrich, Dorset, UK
TaqMan® gene expression assay, HPRT1, human	Applied Biosystems, MA, USA
TaqMan® gene expression assay, JUN, human	Applied Biosystems, MA, USA
TaqMan® universal PCR master mix	Applied Biosystems, MA, USA
<i>trans</i> -2,4'-dihydroxystilbene	Apin Chemicals Limited, Oxfordshire, UK
<i>trans</i> -2,6-difluoro-4'-(N,N-dimethylamino)stilbene (DFS) and its synthetic analogues	Professor David Watt Lab
<i>trans</i> -3,5,4'-trimethoxystilbene	Tokyo Chemical Industry, Tokyo, Japan
Tris base	Sigma-Aldrich, Dorset, UK
Tris buffered saline	Fisher Scientific, Loughborough, UK
Tween® 20	Sigma-Aldrich, Dorset, UK
β-mercaptoethanol	Sigma-Aldrich, Dorset, UK

2.2 Methods

2.2.1 Stock solutions preparation for HPLC-UV and LC-MS/MS assays

Stock solutions of all compounds used for high performance liquid chromatography (HPLC) analysis were prepared by dissolving in dimethylsulphoxide (DMSO) and stored at room temperature. Calibration standards and quality control (QC) samples were prepared by further dilution using blank rat plasma. The stock solution of isotopically labelled ^{13}C resveratrol was dissolved in acetonitrile to 10 $\mu\text{g/mL}$ and stored in $-80\text{ }^{\circ}\text{C}$ as aliquots. This solution was further diluted to the required working concentrations when used for sample preparation and stored at $4\text{ }^{\circ}\text{C}$.

2.2.2 Instrumentation for HPLC-UV and LC-MS/MS assays

A HPLC-UV assay was established for analysis of pterostilbene in tissue samples while three different liquid chromatography tandem mass spectrometry (LC-MS/MS) assays were set up for analysis of DHS, isorhapontigenin and pinosylvin individually.

All HPLC-UV assays were carried out on a Shimadzu (Kyoto, Japan) 2010A liquid chromatography with a reversed-phase HPLC column (Agilent ZORBAX Eclipse Plus C18: $250 \times 4.6\text{ mm}$, $5\text{ }\mu\text{m}$), which was protected by a guard column (Agilent ZORBAX Eclipse Plus C18: $12.5 \times 4.6\text{ mm}$, $5\text{ }\mu\text{m}$). Data acquisition and integration were executed using Shimadzu Class-VP Version 6.12 software.

All LC-MS/MS assays were carried out on Agilent 1290 Infinity Liquid Chromatography system (Agilent Technologies, CA, USA) coupled to ABSciex QTRAP® 5500 (AB Sciex, MA, USA) mass spectrometer equipped with Turbolon SprayTM (electrospray ionisation (ESI)) probe which was controlled by the Analyst 1.5.2 software (AB Sciex, Framingham, MA, USA). Chromatography was carried out using reversed-phase HPLC columns described in Chapter 3. Chromatographic data analysis was performed using the same software used to control the system.

In the mass spectrometer, nitrogen was used as nebulising, curtain and collision gases. Optimisation of the operating parameters of the ESI ion source for the different compounds were carried out using 150 ng/mL solutions ((dissolved in ACN–H₂O (50:50, v/v)) of compounds separately. These tuning solutions were delivered directly into the mass spectrometer at flow rate of 10 µL/min using a syringe pump. Both the negative and positive ion mode were used to detect the parent ion and the ion mode with highest detection sensitivity for the parent ion was used to further optimize the operating parameters of the MS. Sequential ramping of the operation potentials identified the optimal compound parameters, comprising of declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit cell potential (CXP), for the most intense precursor-to-product ion transitions for the different compounds. Using the optimised parameters, the MS detector was operated in multiple reaction monitoring (MRM) mode at unit mass resolution. In the MRM mode,

parent ion of specific mass is sent into the collision cell where fragmentation occurs and only fragment ion of specific mass is allowed to pass into the detector. This process selects only ions of specific characteristics for detection and allows highly specific detection of compounds.

2.2.3 Method validation for HPLC-UV and LC-MS/MS assays

Validation of all HPLC-UV and LC-MS/MS assays were performed by assessing their specificity, sensitivity, linearity, accuracy, precision, recovery, dilution integrity and stability. In addition, matrix effect was ascertained for all LC-MS/MS assays.

Specificity was determined by comparing the chromatograms of six individual blank rat plasma samples with plasma samples spiked with compounds and IS. The specificity was further confirmed in actual pharmacokinetic study by examining the pre-dosing plasma samples.

The sensitivity of this assay was represented by the lower limit of quantification (LLOQ) which was defined as signal-to-noise ratio of at least 10, precision (CV < 15%) and accuracy (analytical recovery within $100 \pm 15\%$) in five samples

The analytical response was defined as the ratio of the peak area of compound and IS. Linear regression analysis was performed where x was the concentration of compound, y was the analytical response and $1/x^2$ was chosen as the weighting factor (Lin et al., 2013; Yeo et al., 2013). The goodness-of-fit of linear regression is represented by the R^2 . Calibration standards were used to assess linearity on at least 3

consecutive days. Five replicates were prepared for intra-day assay and duplicate samples were prepared for the inter-day assay. Similarly, the quality control samples at low, mid and high concentrations were prepared to assess the intra- and inter-day accuracy and precision. The accuracy was determined by expressing the measured concentrations as percentage of the corresponding nominal concentrations (analytical recovery) and precision was measured by determining the coefficient of variation (CV). The assay was considered accurate and precise if the analytical recovery was between 85 – 115% and CV < 15%.

The absolute recoveries of compounds were determined by expressing the peak area of the compounds in precipitated plasma samples in five replicates as a percentage of the peak area of the corresponding pure standards. Recovery for compounds was determined at the three QC concentrations while that for IS was measured at the working concentration. Dilution integrity was assessed by expressing the peak area ratio of the samples diluted from an appropriate higher concentration as a percentage of the mean peak area ratio of the target concentration (mid QC). The dilution was considered acceptable if the calculated percentage is $100 \pm 15\%$.

The stability of compound DMSO solutions and IS stock solution were evaluated after storing at room temperature and 4 °C respectively for the number of days stated in Chapter 3. Stability of the plasma samples were evaluated at the three QC concentrations using five replicates under conditions that were likely to be encountered during

sample storage, preparation and analysis. These include short term stability, long term stability, freeze thaw stability after three cycles (-80°C to 25°C) and post preparative stability. Different conditions were used for different compounds depending on the actual conditions the pharmacokinetic samples are exposed to and these are detailed in Chapter 3. Samples were considered stable if the analytical response were more than 85% of the corresponding freshly prepared samples.

Matrix effect was determined by comparing the peak areas of neat standards spiked into post-precipitated blank samples from six individual rats with those of neat solutions. Matrix effect was considered to be insignificant if the CV of the six samples was less than 15%.

2.2.4 Pharmacokinetic studies

All pharmacokinetic studies were executed in accordance with the animal handling protocols that were reviewed and approved by the Institutional Animal Care and Use Committee of the National University of Singapore (NUS) (IACUC Protocol No. 114/12). Adult male Sprague Dawley rats (9–10 weeks old) were purchased from Centre for Animal Resources (NUS) or InVivos (Singapore) and housed in a specific pathogen-free animal facility with 12 h light/dark cycle at the Comparative Medicine, NUS (temperature = $22 \pm 1^{\circ}\text{C}$; humidity = 60 – 70%). They were allowed free accesses to food and water throughout the study period.

For plasma pharmacokinetic studies, on the day before the pharmacokinetic study, a polyethylene tube (i.d.= 0.58 mm, o.d.= 0.965 mm, Becton Dickinson, Sparks, MD, USA) was inserted into the right jugular vein under isoflurane anesthesia. This cannula was used for intravenous dosing and blood sampling, and its patency was maintained by flushing 0.3 mL heparin-saline (10 IU/mL) following each use. The blood samples (150 μ L from each rat at each time point) were centrifuged at 5000 *g* for 5 min and the plasma layer was retrieved and stored at -80 °C until analysed. This preclinical pharmacokinetic model has been extensively utilized in our laboratory (Lin et al., 2013; Yeo et al., 2013).

For tissue distribution study, at about 15 min before the sampling time, the rats were put under isoflurane anaesthesia and a polyethylene tube (i.d.= 0.58 mm, o.d.= 0.965 mm, Becton Dickinson, Sparks, MD, USA) was implanted into the right carotid artery for exsanguination to ensure the accurate measurement of compound concentration in tissues. The liver, kidney, lung, heart and brain of each rat were harvested immediately after bloodletting. These organs were cut into small pieces and rinsed with phosphate buffered saline (PBS) to remove residual blood before blotting dry. The collected plasma and tissue samples were stored at -80 °C until HPLC analysis.

The specific dosing formulations, dosing routes and blood sampling schedule for each compound are detailed in Chapter 4 and Chapter 5.

2.2.5 Subject selection for *in vitro* studies

Lung tissue samples were obtained from non-smokers, smokers and COPD patients. COPD patients fulfilled the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (GOLD, 2016). All subjects provided informed written consent and the study was approved by London-Chelsea National Research Ethics Committee (09/H0801/85).

2.2.6 Cell culture

2.2.6.1 Cell lines

A549 human alveolar epithelial cells and BEAS-2B human bronchial epithelial cells were obtained from American Type Culture Collection (ATCC). A549 cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 2 mM L-glutamine. BEAS-2B was grown in keratinocyte-serum free medium (K-SFM) containing 50 µg/mL bovine pituitary extract (BPE) and 5 ng/mL epidermal growth factor (EGF). Cells were kept in a humidified incubator supplied with 5% (v/v) CO₂ and maintained at 37 °C.

2.2.6.2 Isolation of primary human airway epithelial cells

Human airway epithelial cells isolated from lung tissues were cultured in LHC-9 medium on flasks coated with 3% (w/v) bovine collagen in Hanks' balanced salt solution (HBSS). Primary human airway epithelial cells were also isolated from lung tissue using an established protocol (Fenwick et al., 2015). Briefly, a small section of lung tissue approximately 5 cm³ was repeatedly flushed with HBSS to remove residues. The tissue was cut into small pieces before deoxyribonuclease I and collagenase dissolved in HBSS were added at

a final concentration of 0.16 mg/mL and 0.83 mg/mL respectively. This mixture was incubated at 37 °C for 30 min with shaking. The partially digested tissue was further washed with DMEM and broken down using Pasteur pipette. The resulting digest was then filtered through a 70 µm cell strainer and centrifuged at 500 g for 10 min to obtain the cell pellet. Cells were then resuspended in LHC-9 medium and transferred into a 75 cm² cell culture flask coated with 3% (w/v) bovine collagen. Medium was changed every 2-3 days until cells reached confluency.

2.2.7 Preparation of cigarette smoke extract (CSE)

Cigarette smoke extract was prepared freshly before each experiment by bubbling mainstream smoke from one Marlboro Red cigarette (with filter removed) into 5 mL of phenol red free DMEM (for A549 cells), K-SFM (for BEAS-2B cells) or bronchial epithelial basal medium (BEBM) (for primary airway epithelial cells) using a peristaltic pump set at 50 rpm. CSE was filter sterilised using a 0.2 µm filter and the concentration normalised by measuring absorbance at 320 nm using a spectrophotometer and adjusted such that 100% CSE was designated as having optical density of 0.15 measured at 320 nm (Wirtz and Schmidt, 1996).

2.2.8 Enzyme linked immunosorbent assay (ELISA)

The concentration of cytokines, IL-6 and CXCL8, in cell culture supernatants were quantified using sandwich ELISA kits according to the manufacturer's instructions. Briefly, the appropriate capture antibody was first coated on 96-well ELISA plate (Fisher Scientific, Loughborough, UK) at a concentration of 1.0 µg/mL in phosphate

buffered saline (PBS) overnight at room temperature. The plate was then washed three times using ELISA wash buffer (1% (v/v) Tween 20 in PBS). The plates were then blocked with ELISA blocking buffer (1% (w/v) BSA, 5% (w/v) sucrose, 0.05% (w/v) NaN₃ in PBS) for 2 h at room temperature. Plates were next washed three times using ELISA wash buffer. Subsequently, 100 µL of appropriately diluted cell culture supernatant samples were loaded onto the plate and incubated for 2 h at room temperature. Calibration standards consisting of the appropriate cytokine standard solution serially diluted to the range of 31.25 pg/mL and 2000 pg/mL were also loaded alongside the samples. ELISA dilution buffer (0.5% (w/v) BSA in PBS) was used to dilute the calibration standards and samples as appropriate. After incubation, plates were washed three times and the corresponding biotin-conjugated detection antibody was added to each well. This was followed by another 2 h of incubation at room temperature. Streptavidin-horseradish peroxidase solution diluted to 1 µg/mL using ELISA dilution buffer was added to the wells after three washing cycles. After a 0.5 h incubation at room temperature, plates were thoroughly washed five times before the mixture of TMB reagents was added. When sufficient blue colour has developed in the solution of all the wells, the reaction was terminated by addition of the ELISA stop solution (0.35 M H₂SO₄) which turned the solution yellow. The absorbance of this final solution was measured using a Spectramax microplate reader (Molecular Devices, California, USA) at 450 nm and the corresponding values at 590 nm were deducted to account for

background variation between wells. The limit of detection for the ELISA assay was 31.25 pg/mL.

2.2.9 Western blotting

The intracellular levels of proteins were studied using western blotting. Cells were lysed to release the proteins which were separated on the basis of size using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Electric current was applied to transfer the proteins on the gel onto nitrocellulose membrane. Subsequently, the membrane was incubated in a solution of primary antibody against the target protein. This was followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody which will bind to the primary antibody. Finally, a chemiluminescent reaction was initiated and the signal was captured by exposure to a photographic film which can be quantified using densitometry to allow comparison of the relative amount of proteins in a blot.

2.2.9.1 Whole cell lysis

At the end of treatment, cells were washed with ice-cold PBS and lysed using Cell Lysis Buffer (Cell Signaling Technology, Danvers, USA) containing 20 mM Tris-HCl (pH 7.5) with 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄ and 1 μ g/mL leupeptin, supplemented with additional protease inhibitor and phosphatase inhibitor cocktail. The lysates were incubated on ice for 15 min with periodic mixing using the vortex to ensure complete cell lysis. Following this, the lysates were centrifuged at 10 000 g for 10 min

at 4 °C and the supernatants containing solubilised proteins were used for further analysis.

2.2.9.2 Nuclear extraction

Nuclear extracts of cells after various treatments were prepared using the NE-PER™ Nuclear and Cytoplasmic Extraction Kit according to the manufacturer's instructions. Briefly, cells were washed with ice-cold PBS and scrapped in PBS. After centrifugation at 500 *g* for 5 min at 4 °C, the cell pellet was re-suspended in 100 µL of cytoplasmic extraction reagent (CER) I, mixed well by vigorous vortexing and incubated on ice for 10 min. Following this, 5.5 µL of CER II was added to further disrupt the cell membrane and release cytoplasmic contents. After brief vortexing and incubation on ice for 1 min, the nucleus was isolated following centrifugation where the supernatant containing cytoplasmic extract was removed. Nuclear extraction reagent at 30 µL was then added to lyse the nuclear membrane and solubilise the nuclear proteins. The samples were incubated on ice for 40 min with vigorous vortexing every 10 min. Finally, the samples were centrifuged at 10 000 *g* for 10 min at 4 °C and the supernatant containing nuclear extract was harvested.

2.2.9.3 Protein quantification

To ensure equal loading of samples in each gel, the amount of proteins in the samples were first quantified using Bio-Rad Protein Assay. Each assay consisted of calibration curve serially diluted from 1.0 mg/mL BSA to cover the range of 0.2 mg/mL and 1.0 mg/mL and appropriately diluted unknown samples in duplicates. The protein assay solution

(diluted 5-fold to the working concentration using distilled water) was added to each sample at the volume of 200 μ L. The absorbance of each sample at 595 nm was recorded using Spectramax microplate reader (Molecular Devices, California, USA) and the concentrations of unknown samples were interpolated from the calibration curve constructed for each assay (Figure 2.1).

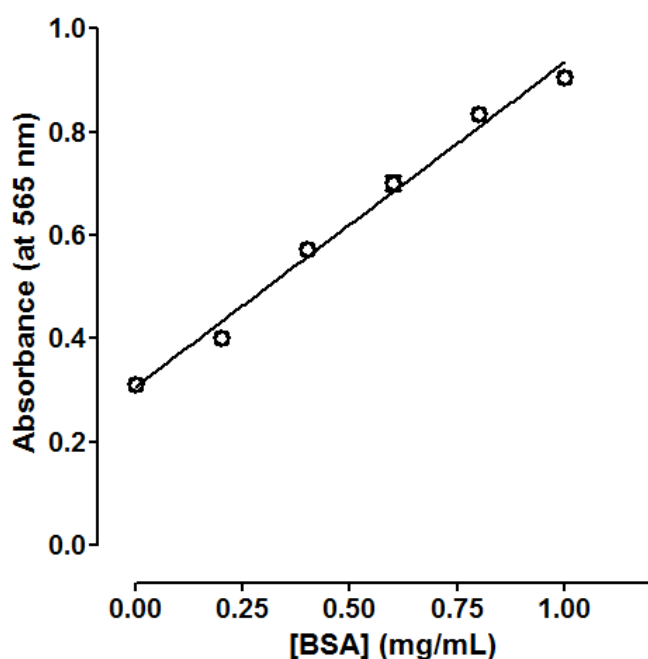


Figure 2.1 Typical calibration standard curve for protein quantification. BSA was serially diluted in PBS and protein assay solution was added. Absorbance was recorded at 595 nm.

2.2.9.4 Gel electrophoresis and protein transfer

Laemmli sample buffer (2 X concentrate) (containing 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β -mercaptoethanol, 0.004% (w/v) bromophenol blue and 0.125 M Tris HCl) was added to each sample which has been normalised to contain the same amount of total proteins. These samples were heated on a heating block for 10 min at

90 °C to denature the proteins. Subsequently, the samples were loaded into the wells of 4-12% gradient Bis-Tris polyacrylamide gels to separate the proteins based on size. The gels were secured in a gel tank filled with 3-(N-morpholino)propanesulfonic acid (MOPS) running buffer (2.5 mM MOPS, 2.5 mM Tris base, 0.05 mM EDTA, 0.005% (w/v) SDS) and resolved using a power source set at 115 V and 115 mA for 2 h. Transfer of the separated proteins from the gel to nitrocellulose membrane was carried out in a transfer tank filled with transfer buffer (25 mM Tris, 190 mM glycine, 20% (v/v) methanol) using voltage and current set at 200 V and 200 mA respectively for 1 h. This procedure was maintained at low temperature using ice pack.

2.2.9.5 Immunoblotting

The membrane with transferred proteins was incubated with 5% (w/v) skimmed milk dissolved in Tris buffered saline (TBS)-0.05% (v/v) Tween 20 (milk/TBS-T) for 1 h at room temperature to block unspecific binding sites. Subsequently, this was changed to a solution of the appropriate primary antibody in milk/TBS-T and incubated overnight at 4 °C. Unbound antibody was removed by washing the membrane 3 times using TBS-T before the appropriate HRP-conjugated secondary antibody in milk/TBS-T was added and incubated for 1 h at room temperature. After another round of 3 wash cycles, enhanced chemiluminescence (ECL) detection reagents were added for 1 min to initiate a chemiluminescent reaction which was captured by exposing the membrane to Hyperfilm. This film was developed using Imaging Developer (AFP Imaging, New York, United States) and the protein

bands were analysed using UVP GelDoc-It imaging system (UVP, California, USA) and VisionWorks LS software (UVP, California, USA).

When necessary, antibodies on the membrane were stripped using 25 mL of stripping buffer (50 mM Tris base, 2% (w/v) SDS, 0.1 M β -mercaptoethanol) at 50 °C for 20 min and washed 3 times in TBS-T. Additional proteins were detected by repeating the procedures described earlier, starting from the blocking step.

2.2.10 Cell viability assay

Cell viability was monitored using methyl thiazol tetrazolium (MTT) assay. This assay relies on the ability of viable cells to reduce the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye to insoluble formazan crystals. Cell culture medium was removed and 1 mg/mL of MTT solution in HBSS was added to the cells and incubated for 30 min at 37°C. The solution was replaced with DMSO to solubilise the crystals and the absorbance at 550 nm was recorded using a spectrophotometer (Molecular Devices, California, USA). Non-treated cells were used as controls and the viability was expressed as percentage absorbance compared to control cells (100%).

2.2.11 Sirtuin 1 (SIRT 1) enzyme activity assay

The ability of the compounds to activate SIRT 1 enzyme directly was measured using SIRT1 fluorometric drug discovery kit (Enzo Life Sciences, Exeter, UK) according to the manufacturer's instructions. This assay is based on the deacetylation of the Fluor de Lys®-SIRT 1 substrate by SIRT 1 which exposes the lysine moiety that reacts with

the Developer solution to form a fluorophore. Briefly, stilbene compounds were dissolved in DMSO and further diluted with Assay Buffer before pre-incubation with 1 U SIRT 1 enzyme at 37 °C for 5 min. Fluor de Lys®-SIRT 1 substrate and NAD⁺, both at final concentration of 25 µM, were added to the mixture and incubated for a further 45 min at 37 °C. Finally, the reaction was terminated by addition of Developer solution containing 2 mM nicotinamide. After incubation at room temperature for 45 min, fluorescence readings at excitation wavelength of 370 nm and emission wavelength of 450 nm were recorded using FLUOstar OPTIMA microplate reader (BMG Labtech, Bucks, UK). Time zero samples, consisting of samples that were terminated using the method as described previously before addition of substrate and NAD⁺, provided the background value which was deducted from all sample readings. These background corrected values were expressed as a ratio of the mean vehicle control value.

2.2.12 Sirtuin 1 (SIRT 1) immunoprecipitation

To evaluate the effects of any structural modifications on SIRT 1 by the compounds which can affect its activity, immunoprecipitation of SIRT 1 was carried out. After the treatment period, cells were washed with ice cold PBS and scrapped in PBS. Thereafter, the cell pellet was collected after centrifugation at 500 g for 5 min and the cells were lysed using 100 µL of cell lysis buffer (550 mM Tris HCl (pH 7.4), 0.5% (v/v) IGEPAL CA-630, 150 mM NaCl in water) containing protease and phosphatase inhibitors. The lysates were incubated on ice for 15 min with periodic mixing using the vortex to ensure complete cell lysis.

Following this, the lysates were centrifuged at 10 000 *g* for 10 min at 4 °C and the concentration of proteins in the supernatants were measured using the protein quantification assay described earlier. Lysates containing 500 µg of total proteins were incubated with SIRT 1 antibody (rabbit polyclonal) at dilution of 1:40 overnight at 4 °C. This antigen-antibody complex was isolated by incubating with Dynabeads Protein A which has been washed three times using cell lysis buffer prior to use. Incubation was carried out for 1 h at room temperature. Subsequently, the antigen-antibody complex bound to Dynabeads was collected using magnet and washed three times using cell lysis buffer. Finally, the beads were suspended in 65 µL of Assay Buffer and SIRT 1 activity assay was carried out as described earlier.

2.2.13 Luciferase assay

The modulation of NF-κB activity was measured using A549 cells stably transfected with the NF-κB reporter 6kBtkluc.neo that have also been used in a previous study in our laboratory (Donnelly et al., 2004). At the end of treatment, luciferase assay system (Promega) was used to assess luciferase activity. The cells were first lysed using 20 µL of cell lysis buffer. Next, 40 µL of luciferase assay reagent was added and the emitted light was recorded immediately using FLUOstar OPTIMA microplate reader (BMG Labtech, Bucks, UK). The NF-κB activity was represented by the percentage luminescence calculated by the formula:

$$\frac{\text{Luminescence of sample} - \text{mean luminescence of non stimulated cells}}{\text{Difference in mean luminescence of non-stimulated and stimulated cells}} \times 100\%$$

2.2.14 Measurement of ROS

A cell permeable probe, H₂DCF-DA, was used to assess the levels of ROS in the cells after each treatment. Upon entering the cells, this probe becomes deacetylated by intracellular esterases to H₂DCF which is rapidly oxidised to the highly fluorescent 2',7'-dichlorodihydrofluorescein by ROS. Cells were washed with warm PBS and 10 µM H₂DCF-DA was loaded into the cells by incubation for 30 min at 37 °C. Following this, fluorescence of each sample was measured at excitation wavelength of 485 nm and emission wavelength of 520 nm. The background corrected value of each sample was expressed as a percentage of the stimulated cells which were used as controls.

2.2.15 Measurement of gene expression using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Gene expression was studied using qRT-PCR where the isolated RNA was first converted to cDNA by reverse transcription. This was followed by quantitative PCR which allows monitoring of the amplification of specific DNA molecules.

2.2.15.1 RNA isolation

Total RNA from cells was isolated using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. At the end of treatment, cells were lysed using RLT plus buffer containing 1% (v/v) β-mercaptoethanol. This lysate was mixed well by vortexing and then homogenised by centrifugation through QIAshredder spin column at 10 000 g for 2 min. Following this, the homogenised lysate was

transferred to a gDNA Eliminator spin column and centrifuged for 30 s at 10 000 *g*. An equal volume of 70% (v/v) ethanol was next added to the flow through and this sample was transferred to an RNeasy spin column. After a brief centrifugation for 15 s at 10 000 *g*, buffer RW1 was added and the same centrifugation step was repeated to wash the column membrane. The membrane was subsequently washed by addition of buffer RPE and centrifugation for 15 s at 10 000 *g*. An additional wash using buffer RPE was carried out before centrifugation for 2 min at 10 000 *g*. The spin column was transferred to a new collection tube and centrifuged for 1 min at 10 000 *g* to thoroughly remove any residual buffer. Finally, 30 μ L of RNase-free water was added and centrifuged for 1 min at 10 000 *g* to elute the RNA. This step was repeated by using the eluate to maximize the yield of RNA.

2.2.15.2 RNA analysis

The purified RNA samples were analysed by taking spectrophotometric measurements using NanoDrop ND-1000 (ThermoFisher Scientific, Waltham, USA). The quantities of RNA were recorded and the purity of the samples were assessed using the ratio of absorbance at 260 nm and 280 nm. A ratio between 1.9 and 2.1 was considered acceptable.

2.2.15.3 Reverse transcription

A master mix consisting of dNTP mix, random primers and MultiScribe™ recombinant moloney murine leukemia virus reverse transcriptase was added to 500 ng of RNA. cDNA was synthesised from this mixture using a thermal cycler (G-Storm, Somerset, UK) with the following conditions: 25 °C for 10 min → 37 °C for 120 min → 85 °C

for 5 min. cDNA was diluted to 5 ng/μL using RNase-free water to be used for subsequent assays.

2.2.15.4 Quantitative PCR

Synthesized cDNA (10 ng) was added to a master mix consisting of 5 μL TaqMan® Universal PCR Master Mix, 2.5 μL RNase-free water and 0.5 μL appropriate TaqMan® gene expression assay (Jun TaqMan gene expression assay: Hs01103582_s1) and HPRT-1 TaqMan gene expression assay: Hs99999909_m1) in a MicroAmp® 96 well reaction plate. Calibration standards, comprising of random-primed human reference cDNA diluted to the range of 16 pg/μL and 10 000 pg/μL using RNase-free water, were present for each primer in each plate. The plate was sealed and centrifuged briefly before amplification and detection on a 7500 Real Time PCR system (Applied Biosystems, Paisley, UK). The PCR cycle consisted of: 50 °C for 2 min → 95 °C for 1 min and 40 cycles of 95 °C for 15 s → 60 °C for 1 min. mRNA expression levels of the samples were calculated using their C_t values by interpolation from the standard curve constructed. The mRNA expression levels for all samples were normalised to the level of the housekeeping gene HPRT-1.

2.2.16 Statistics

All statistical analyses were carried out using GraphPad Prism Version 5.03 (GraphPad Software Inc., CA, USA). The distribution of experimental data was first assessed using Kolmogorov-Smirnov test. If data was found to follow Gaussian distribution, parametric tests (two-tailed unpaired t test for comparison between two groups or one way

ANOVA for comparison among three or more groups) were carried out. For ANOVA, Tukey post-test was used to discern differences amongst all groups while Bonferroni post-test was used to compare against a control group. If data was not normally distributed, non-parametric tests (Mann-Whitney test for comparison between two groups or Kruskal-Wallis test with Dunns post-test for comparison among three or more groups) were used. 50% inhibitory concentrations (IC_{50}) were calculated by log transforming the concentration values followed by curve fitting using non-linear regression. Statistical significance was achieved if p value was < 0.05 .

CHAPTER 3
BIOANALYTICAL METHOD DEVELOPMENT
AND VALIDATION

3.1 Introduction

In order to study the pharmacokinetic characteristics of stilbene analogues, sensitive and reliable analytical methods will be essential to measure the concentrations of the compounds in biological samples. High performance liquid chromatography (HPLC) is a commonly employed technique to separate the components in complex biological matrices to allow quantitation of compounds (Pandey et al., 2010). For stilbene analogues, detection using the ultraviolet (UV) detector has been commonly used (Lin and Ho, 2011; Lin et al., 2013; Roupe et al., 2006b) and mass spectrometry is gaining popularity for its higher sensitivity and selectivity (Kapetanovic et al., 2011; Muzzio et al., 2012; Tian et al., 2014).

Prior to instrumental analysis, the stilbene analogues have to be extracted from the biological matrices. Amongst the wide variety of extraction techniques (Bylda et al., 2014), protein precipitation is the first choice due to its simplicity and excellent recovery of compounds. This sample preparation step removes proteins such that it becomes compatible with the subsequent HPLC analysis.

The aims of this chapter are to develop and validate high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for the quantification of DHS, isorhapontigenin and pinosylvin in rat plasma. A HPLC-UV method will also be developed for the quantification of pterostilbene in the major organs (heart, lungs, liver,

kidneys and brain) in rats. The methods will be applied to the study of the pharmacokinetic characteristics of stilbene analogues in Chapter 4.

3.2 Methods

3.2.1 Sample preparation

A simple protein precipitation procedure was used for plasma sample cleanup prior to LC-MS/MS analysis. For isorhapontigenin, L-ascorbic acid (final concentration = 0.8 mg/mL) was spiked into plasma to enhance stability. Three volumes of internal standard (IS) (isotopically labelled ^{13}C resveratrol) solution in ACN (10 ng/mL for DHS and 20 ng/mL for pinosylvin samples) or in ACN-0.18 μM sodium EDTA solution (10 ng/mL for isorhapontigenin samples) were added to one volume of plasma sample. After vigorous vortex, the samples were centrifuged at 10 000 g for 10 min at 4 °C. Subsequently, the clear supernatants were transferred into HPLC vials.

For analysis of pterostilbene in plasma samples, three volumes of internal standard (IS) (200 ng/mL *trans*-stilbene) solution in ACN were added to one volume of plasma sample and processed as described in the earlier paragraph from the vortex step. This method has been validated in an earlier study in the laboratory (Yeo et al., 2013). For tissue sample cleanup, approximately 350 mg of each tissue was weighed out and finely minced with scissors. The samples were homogenised (1:3, w/v) in 0.2% L-ascorbic acid solution for up to 5 cycles using a sonicator (Sonic Dismembrator Model 500, Fisher Scientific) operating at an amplitude of 20% and 20 s for each cycle.

Subsequently, five volumes of IS (200 ng/mL *trans*-stilbene solution in ACN), were added to one volume of tissue homogenate and following vigorous vortex, the sample was processed as described in the earlier paragraph from the vortex step.

3.2.2 Instrumental analysis

The analysis of pinosylvin, DHS and isorhapontigenin were carried out on the LC-MS/MS system described in Chapter 2.2.2 while that for pterostilbene was performed on the HPLC-UV system described in the same chapter.

Reversed-phase HPLC columns were used for all analysis as listed in Table 3.1. Chromatographic separation of the respective compounds, IS and any endogenous interference were achieved using gradient elution with mobile phase comprising of a mixture of ACN and water as detailed in Table 3.2.

After chromatographic separation, the eluent was directly introduced to the mass spectrometer via an ESI source operating in the negative mode (for DHS, isorhapontigenin and pinosylvin) or UV detector (for pterostilbene). The operation parameters of the mass spectrometer, following optimisation described in Chapter 2.2.2, are described in Table 3.3. The UV detector for pterostilbene analysis was set at 320 nm.

For the IS (^{13}C Resveratrol), the source parameters were the same as the respective analyte it was used with while the optimised compound parameters were -74.7 V, -3.9 V, -26.9 V and -12.2 V for DP, EP, CE

and CXP respectively for the most intense precursor-to-product ion transition of 233→191.

Table 3.1 HPLC columns and column conditions used

Compound	Column
DHS	• Agilent Poroshell 120 EC-C18: 50×3.0 mm, 2.7 µm • Guard column: Agilent Poroshell 120 EC-C18 5 × 3.0 mm, 2.7 µm • Maintained at 40 °C
Isorhapontigenin	• Agilent Poroshell 120 EC-C18: 75×3.0 mm, 2.7 µm • Guard column: Agilent Poroshell 120 EC-C18 5 × 3.0 mm, 2.7 µm • Maintained at 40 °C
Pinosylvin	• Agilent Poroshell 120 EC-C18: 75×3.0 mm, 2.7 µm • Guard column: Phenomenex Security Guard 4 × 2.0 mm • Maintained at 40 °C
Pterostilbene	• Agilent ZORBAX Eclipse Plus C18: 250×4.6 mm, 5 µm) • Guard column: Agilent ZORBAX Eclipse Plus C18 12.5 × 4.6 mm, 5 µm • Maintained at 50 °C

Table 3.2 HPLC programme used for analysis of different compounds

Compound	Gradient schedule	Injection volume (µL)
DHS	<u>0.0–0.5 min</u> : ACN 35% <u>0.5–0.7 min</u> : ACN 35→40% <u>0.7–1.5 min</u> : ACN 40% <u>1.5–2.0 min</u> : ACN 40→90% <u>2.0–3.0 min</u> : ACN 90% <u>3.0–3.5 min</u> : ACN 90→35% <u>3.5–5.0 min</u> : ACN 35%	2
Isorhapontigenin	<u>0.0–1.0 min</u> : ACN 20% <u>1.0–1.5 min</u> : ACN 20→45% <u>1.5–2.5 min</u> : ACN 45% <u>2.5–3.0 min</u> : ACN 45→90% <u>3.0–4.0 min</u> : ACN 90% <u>4.0–4.5 min</u> : ACN 90→20% <u>4.5–5.5 min</u> : ACN 20%	1
Pinosylvin	<u>0.0–1.0 min</u> : ACN 45% <u>1.0–1.5 min</u> : ACN 45→80% <u>1.5–2.5 min</u> : ACN 80% <u>2.5–3.0 min</u> : ACN 80%→45% <u>3.0–6.0 min</u> : ACN 45%	2
Pterostilbene	<u>0.0–4.0 min</u> : ACN 35% <u>4.0–5.0 min</u> : ACN 35→90% <u>5.0–9.0 min</u> : ACN 90% <u>9.0–12.5 min</u> : ACN 35%	75

Table 3.3 Optimised MS source and compound parameters for DHS, isorhapontigenin and pinosylvlin

Compound	MS source and compound parameters
DHS (Ion transition = 211→117)	<u>Source parameters</u> Curtain gas: 20 psi; Gas 1: 40 psi; Gas 2: 30 psi; Temperature: 600 °C; IonSpray Voltage: -4500 V <u>Compound parameters</u> DP: -35.0 V; EP: -9.1 V; CE: -50.0 V; CXP: -5.81 V Dwell time = 100 ms
Isorhapontigenin (Ion transition = 257→241)	<u>Source parameters</u> Curtain gas: 20 psi; Gas 1: 40 psi; Gas 2: 40 psi; Temperature: 600 °C; IonSpray Voltage: -4500 V <u>Compound parameters</u> DP: -105.0 V; EP: -9.0 V; CE: -26.0 V; CXP: -11.0 V Dwell time = 100 ms
Pinosylvlin (Ion transition = 257→241)	<u>Source parameters</u> Curtain gas: 20 psi; Gas 1: 40 psi; Gas 2: 40 psi; Temperature: 600 °C; IonSpray Voltage: -4500 V <u>Compound parameters</u> DP: -68.5V; EP: -7.0V; CE: -24.1 V; CXP: -10.1 V Dwell time = 150 ms

DP = Declustering potential; EP = Entrance potential;
CE = Collision energy; CXP = Collision exit potential

3.2.3 Assay validation

Validation of the analytical assays were performed by assessing their specificity, sensitivity, linearity, accuracy, precision, recovery, dilution integrity and stability, in addition to matrix effect for LC-MS/MS assays, as described in Chapter 2.2.3. For pterostilbene, validation of the tissue analytical method was performed but not plasma as it has already been reported in a previous study in our laboratory (Yeo et al., 2013). The concentrations of the calibrations standards and QC samples (low, mid and high) used in the different assays are shown in Table 3.4.

Table 3.4 Calibration standards and QC samples concentrations

Analyte	Calibration standards concentrations (ng/mL)	QC samples concentrations (ng/mL)
DHS	2, 10, 50, 100, 500, 1000, 1500	5, 600, 1400
Isorhapontigenin	1, 5, 10, 50, 100, 500, 1000	2, 400, 800
Pinosylvin	1, 5, 10, 50, 100, 500, 1000	2, 450, 800
Pterostilbene (Tissue)	20, 50, 100, 500, 1000, 2000, 3000	40, 1500, 2800

3.3 Results

3.3.1 Analytical method development

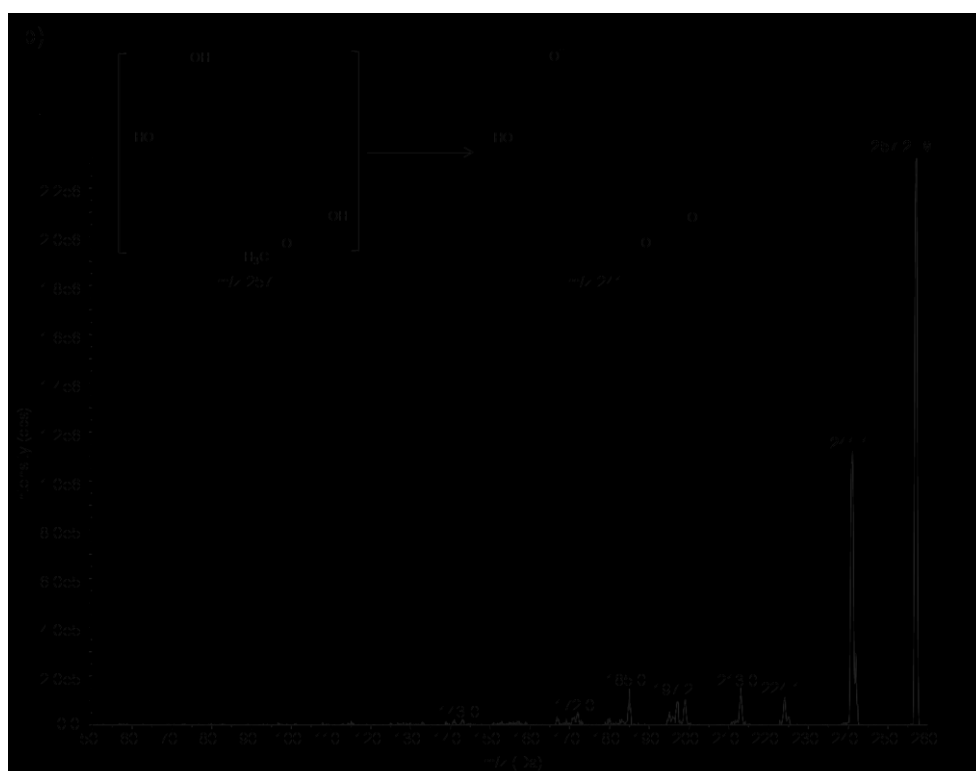
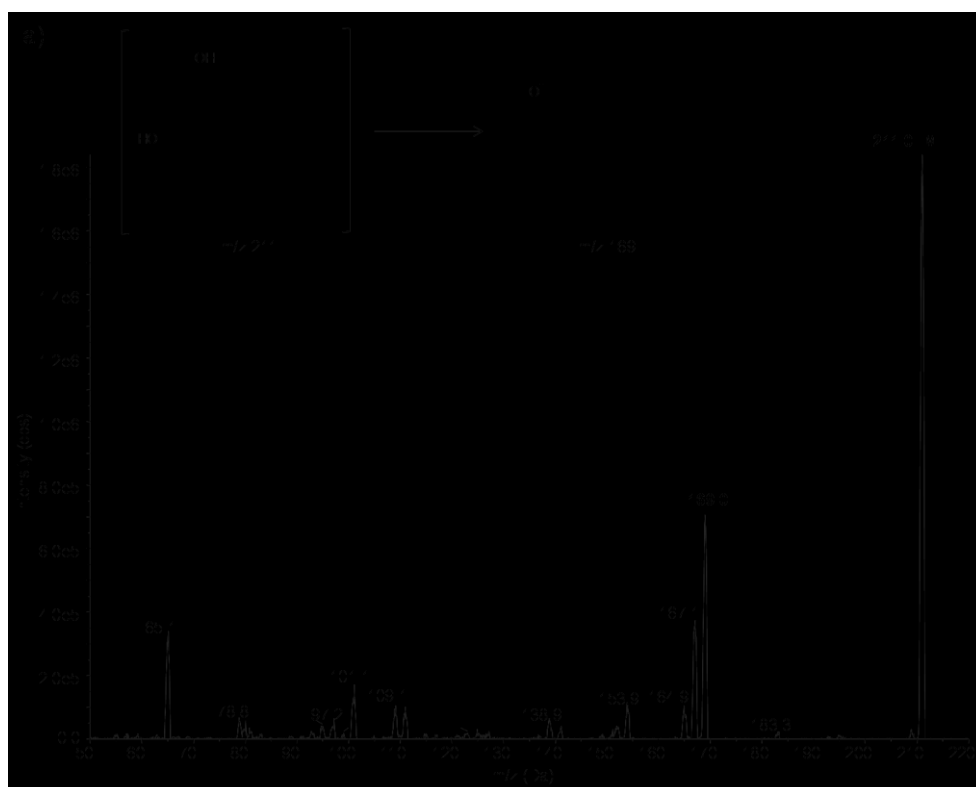
For detection using MS, the MS spectra of the compounds were first compared to the blank vehicle sample to confirm the presence of the major ion $[M-H]^-$ when the ESI source is operated in the negative mode. Following optimisation of the collision energy, the most sensitive transition observed in the MS/MS spectra was selected for quantification of the compounds and they are shown in Table 3.3. The

MS/MS spectra for the different compounds and the possible structure of the fragment ions are presented in Figure 3.1.

Various HPLC conditions including the gradient schedule and mobile phases were varied in order to obtain symmetrical and sharp peak shapes with adequate separation of analyte, IS and endogenous interference peaks, The optimised conditions are presented in Table 3.2. Under the conditions used, the retention times of the various analytes in the different assays are presented in Table 3.5. Representative chromatograms are presented in Figures 3.2-3.5.

Table 3.5 Chromatographic retention times of analytes and IS in the different optimised assays

Analyte	Chromatographic retention time (min)	
	Analyte	IS
DHS	1.3	0.7
Isorhapontigenin	2.2	2.1
Pinosylvlin	1.7	0.9
Pterostilbene	7.4	8.5



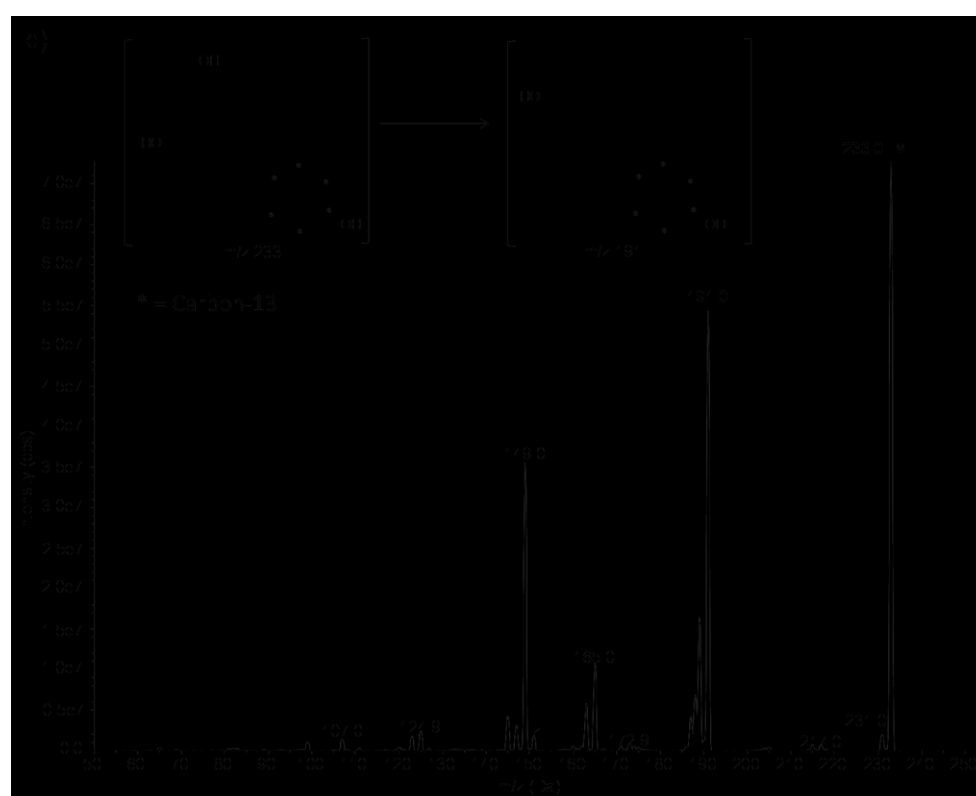
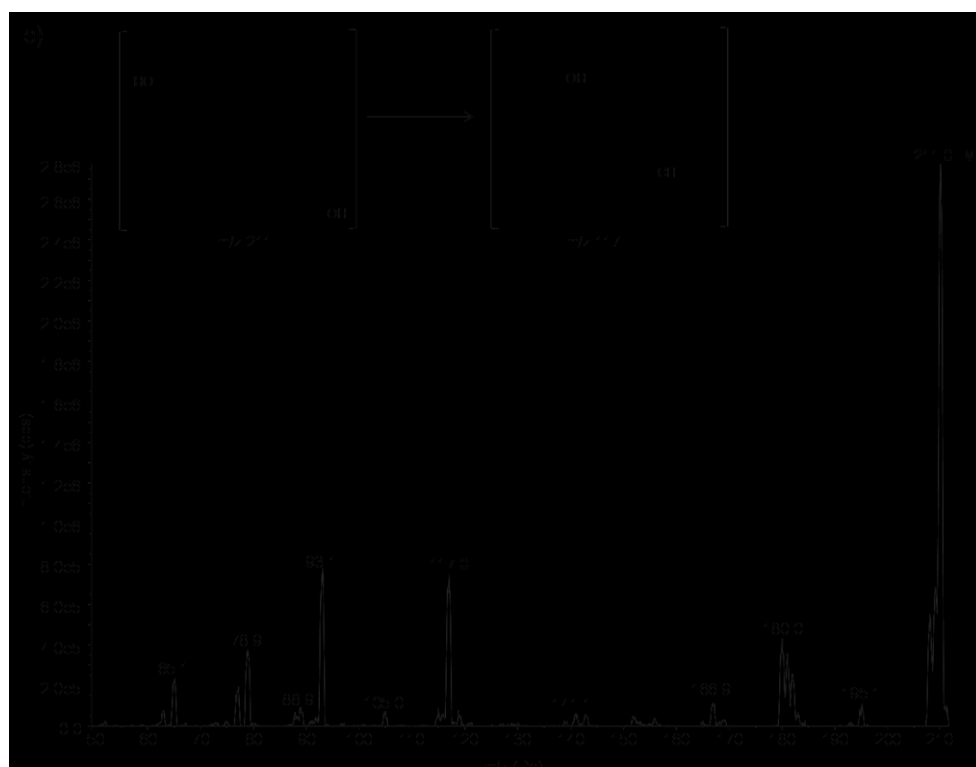


Figure 3.1 MS/MS spectrum of stilbene analogues a) pinosylvin, b) isorhapontigenin, c) DHS and d) IS (isotopically labelled ^{13}C resveratrol). The proposed structures of the major fragments ions are shown.

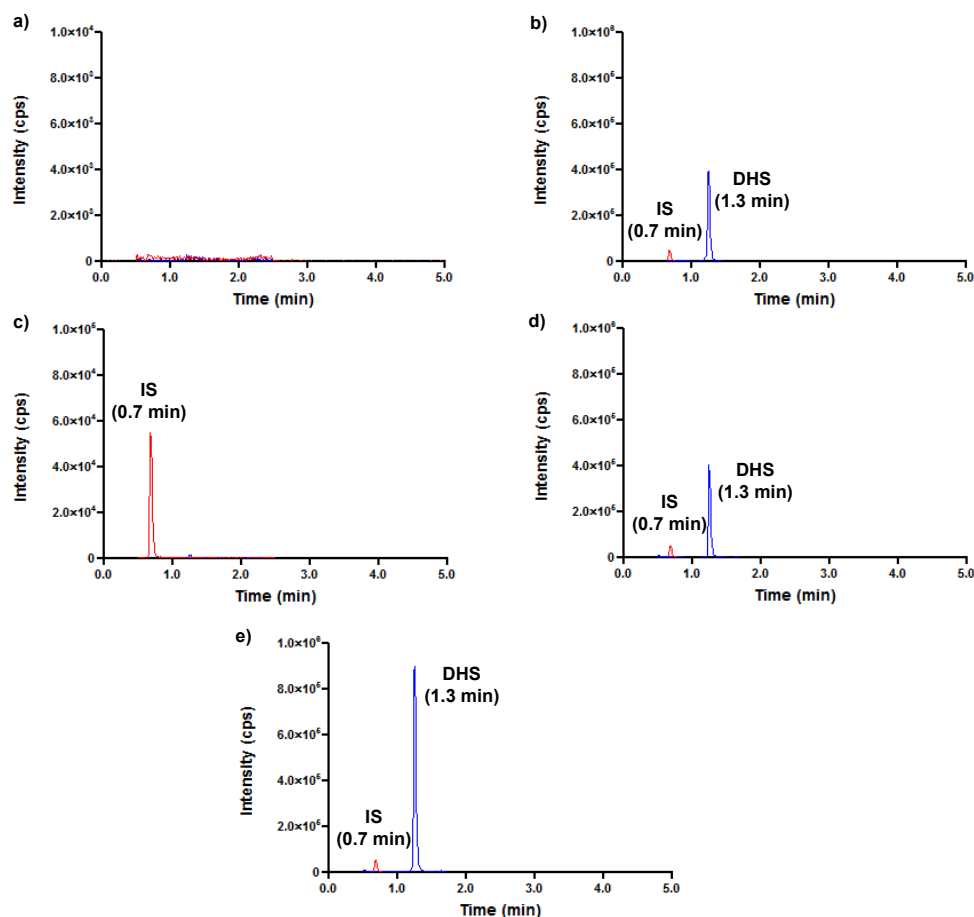


Figure 3.2 LC-MS/MS chromatograms of DHS rat plasma samples a) pre-dosing plasma; b) blank plasma spiked with 100 ng/mL DHS and 30 ng/mL IS; c) blank plasma spiked with 30 ng/mL IS; d) plasma sample collected 1 h after 4 mg/kg DHS solution intravenous injection spiked with 30 ng/mL IS; e) plasma sample collected 1 h after 10 mg/kg DHS solution oral administration spiked with 30 ng/mL IS. DHS and IS eluted at 1.3 min and 0.7 min respectively. Blue trace = DHS (m/z 211 $\rightarrow$ 117); Red trace = IS (m/z 233 $\rightarrow$ 191).

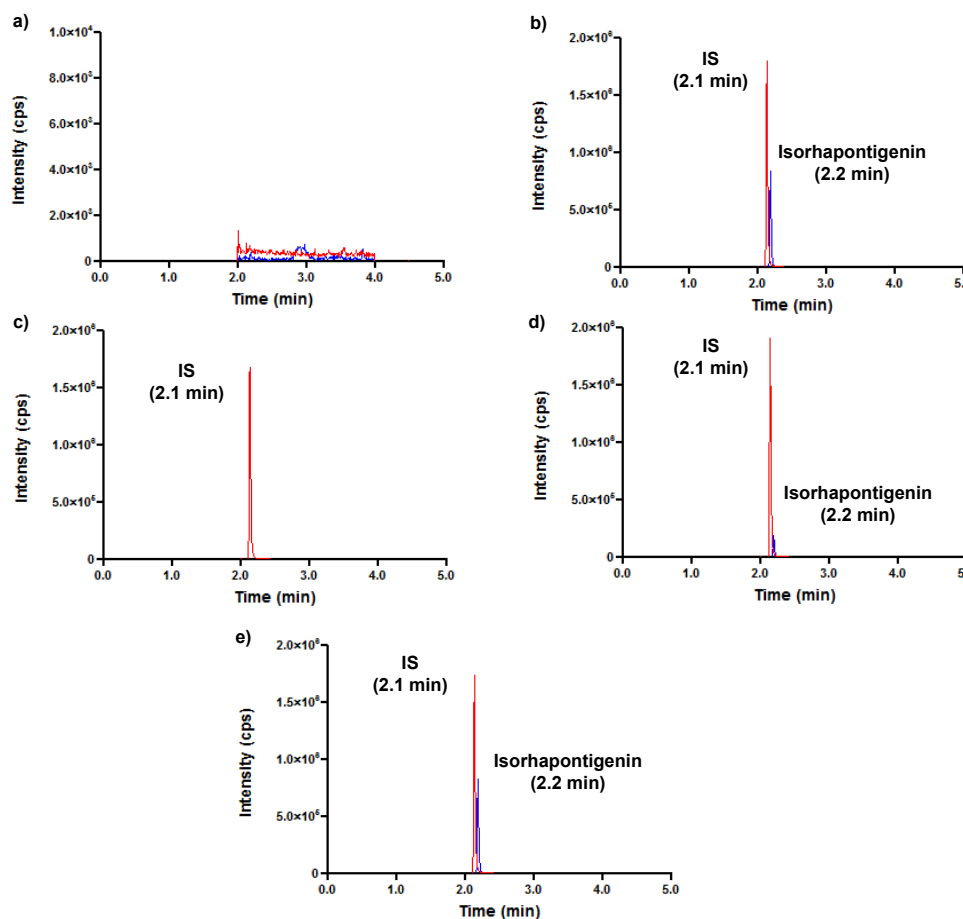


Figure 3.3 LC-MS/MS chromatograms of isorhapontigenin rat plasma samples a) pre-dosing plasma; b) blank plasma spiked with 100 ng/mL isorhapontigenin and 30 ng/mL IS; c) blank plasma spiked with 30 ng/mL IS; d) plasma sample (diluted 20 fold) collected 30 min after 7.74 mg/kg isorhapontigenin solution intravenous injection spiked with 30 ng/mL IS; e) plasma sample (diluted 20 fold) collected 1.5 h after 154.8 mg/kg isorhapontigenin solution oral administration spiked with 30 ng/mL IS. Isorhapontigenin and IS eluted at 2.2 min and 2.1 min respectively. Blue trace = Isorhapontigenin (m/z 257→241); Red trace = IS (m/z 233→191).

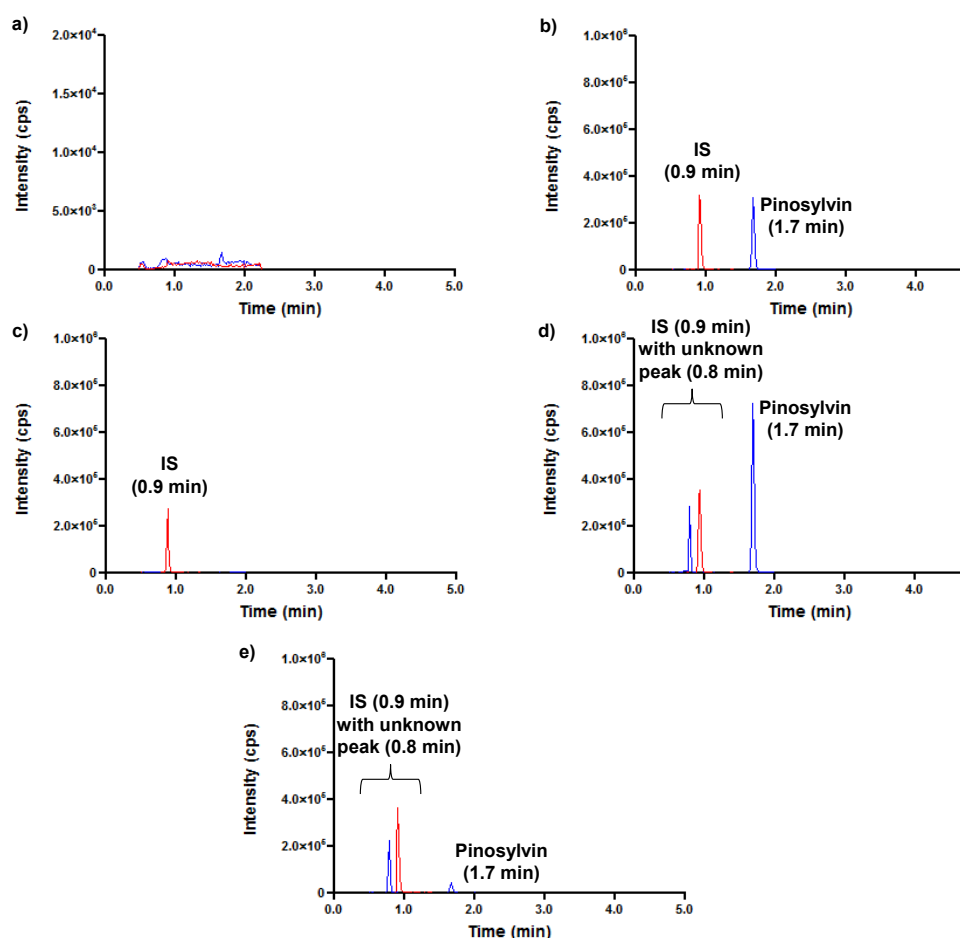


Figure 3.4 LC-MS/MS chromatograms of pinosylvin rat plasma samples a) pre-dosing plasma; b) blank plasma spiked with 100 ng/mL pinosylvin and 60 ng/mL IS; c) blank plasma spiked with 60 ng/mL IS; d) plasma sample collected 15 min after 5 mg/kg pinosylvin solution intravenous injection spiked with 30 ng/mL IS; e) plasma collected 15 min after 50 mg/kg pinosylvin solution oral administration spiked with 30 ng/mL IS. Pinosylvin and IS eluted at 1.7 min and 0.9 min respectively. Blue trace = Pinosylvin (m/z 211→169); Red trace = IS (m/z 233→191).

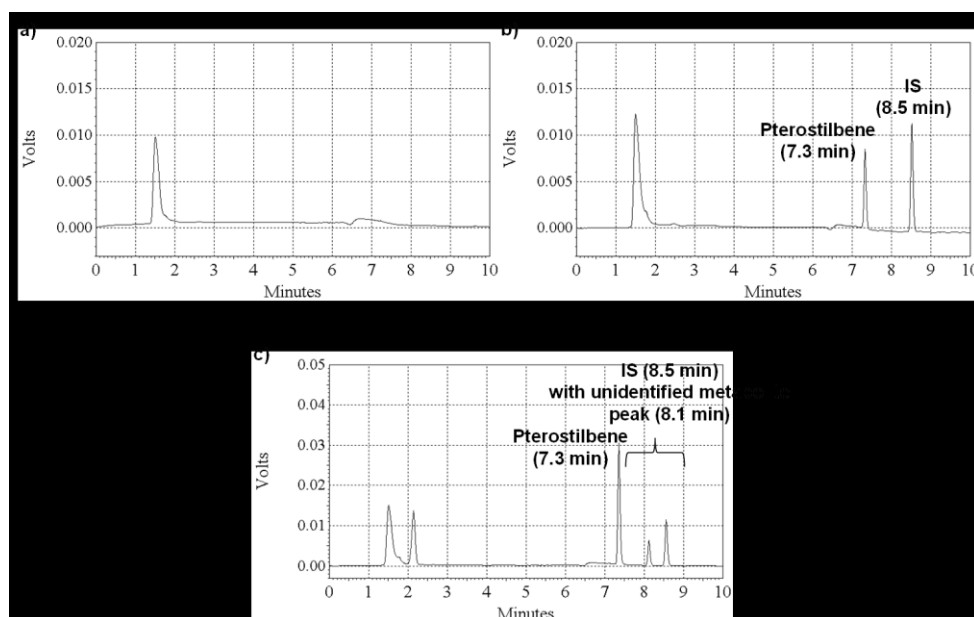


Figure 3.5 HPLC-UV chromatograms of pterostilbene rat tissue samples a) pre-dosing lung homogenate; b) blank lung homogenate spiked with 500 ng/mL pterostilbene and 1 µg/mL IS; c) lung homogenate of sample collected 3 h min after 40 mg/kg pterostilbene solution oral administration spiked with 1 µg/mL IS. Pterostilbene and IS eluted at 7.3 min and 8.5 min respectively.

3.3.2 Assay validation

Six different sources of plasma or tissue samples and pre-dosing samples were analysed in the different assays. These showed no notable interference from endogenous compounds at the retention times of the compound and IS, which confirms the specificity of the assays developed. Furthermore, co-elution of metabolite peaks were not seen in the pharmacokinetic samples. Although a metabolite peak was observed in the chromatograms of pinosylvin PK samples at 0.8 min (Figure 3.4 (d)), it did not interfere with the quantification of the IS. This peak with the same MRM transition as pinosylvin but with higher polarity is likely from the in-source fragmentation of conjugated pinosylvin metabolites (Yan et al., 2003).

The LLOQ of the different assays were 1 ng/mL for isorhapontigenin and pinosylvin; 2 ng/mL for DHS; and 20 ng/mL for pterostilbene tissue samples. Calibration curves of all the assays had good linearity with $R^2 > 0.99$. Similarly, the assays achieved high accuracy (analytical recovery = $100 \pm 15\%$) and precision ($CV \leq 15\%$) as reflected in the analysis of the QC samples shown in Table 3.6 and Table 3.7. The simple protein precipitation used to clean up the samples also yielded excellent absolute recovery in the different assays (Table 3.8). Dilution integrity of the five-fold (for DHS), 20-fold (for isorhapontigenin), five-fold (for pinosylvin) dilutions were $110.6 \pm 6.9\%$, $98.2 \pm 4.2\%$ and $94.9 \pm 2.4\%$ respectively. For pterostilbene tissue assay, only liver samples required dilution and the five-fold dilution used was $104.2 \pm 8.4\%$ of the target concentration. These accurate and precise dilutions indicate that they were suitable for use in the different assays. Investigations into the stability of the samples confirmed that conditions encountered during sample and processing did not cause significant degradation to the compounds (Table 3.9 and Table 3.10).

Table 3.6 Intra-day and inter-day analytical recovery (accuracy) and CV (precision) of DHS, isorhapontigenin and pinosylvin

Parameters	Concentrations of QC samples		
	Low	Mid	High
DHS			
<u>Intra-day</u> (n = 5)			
Analytical recovery (%)	94.8 ± 8.5	107.4 ± 5.0	109.1 ± 3.8
CV (%)	9.0	4.7	3.5
<u>Inter-day</u> (n = 3)			
Analytical recovery (%)	95.0 ± 6.0	106.5 ± 6.0	109.7 ± 3.6
CV (%)	6.3	4.7	3.3
Isorhapontigenin			
<u>Intra-day</u> (n = 5)			
Analytical recovery (%)	102.9 ± 7.3	105.9 ± 7.2	101.9 ± 4.9
CV (%)	7.2	6.8	4.8
<u>Inter-day</u> (n = 3)			
Analytical recovery (%)	93.1 ± 9.4	92.9 ± 7.7	91.2 ± 3.7
CV (%)	10.1	8.2	4.1
Pinosylvin			
<u>Intra-day</u> (n = 5)			
Analytical recovery (%)	92.8 ± 5.4	102.0 ± 3.4	92.7 ± 5.4
CV (%)	5.8	3.3	5.9
<u>Inter-day</u> (n = 5)			
Analytical recovery (%)	100.2 ± 12.0	95.2 ± 2.8	92.3 ± 1.9
CV (%)	12.0	3.0	2.1

Table 3.7 Intra-day and inter-day analytical recovery (accuracy) and CV (precision) of pterostilbene in different tissues

Tissue	Nominal Concentration (ng/mL)	Intra-day		Inter-day	
		Analytical recovery (%)	CV (%)	Analytical recovery (%)	CV (%)
Brain	40	106.1 ± 5.8	5.5	101.2 ± 3.9	3.9
	1500	96.8 ± 3.9	4.0	93.8 ± 8.6	9.2
	2800	96.9 ± 4.3	4.4	96.5 ± 4.8	5.0
Heart	40	100.1 ± 3.3	3.3	96.5 ± 3.2	3.3
	1500	99.8 ± 4.1	4.1	97.5 ± 2.0	2.0
	2800	102.0 ± 4.1	4.1	98.9 ± 2.2	2.2
Kidney	40	106.2 ± 4.5	4.2	100.0 ± 6.0	6.7
	1500	108.0 ± 6.2	5.8	103.2 ± 4.6	4.5
	2800	103.4 ± 5.8	5.6	95.3 ± 3.4	3.5
Liver	40	105.1 ± 3.2	3.0	99.8 ± 2.9	2.9
	1500	100.2 ± 3.7	3.7	95.1 ± 1.5	1.5
	2800	97.8 ± 5.1	5.2	90.5 ± 4.1	4.5
Lung	40	101.2 ± 4.0	3.9	96.3 ± 3.3	3.4
	1500	103.6 ± 2.3	2.2	99.4 ± 3.3	3.3
	2800	100.8 ± 2.1	2.1	99.9 ± 2.3	2.3

Data presented as mean ± SD (n = 5)

Table 3.8 Absolute recovery of DHS, isorhapontigenin and pinosylvin from plasma and pterostilbene from the various tissues

Analyte	QC samples (n = 5)			IS (n = 15)
	Low	Mid	High	
DHS	94.8 ± 8.5	107.4 ± 5.0	109.1 ± 3.8	
Isorhapontigenin	102.9 ± 7.3	105.9 ± 7.2	101.9 ± 4.9	97.0 ± 2.9
Pinosylvin	92.8 ± 5.4	102.0 ± 3.4	92.7 ± 5.4	
Pterostilbene				
Brain	92.3 ± 4.9	103.7 ± 4.0	92.0 ± 3.1	103.4 ± 1.1
Heart	98.4 ± 4.2	100.2 ± 4.6	93.0 ± 4.2	99.5 ± 1.6
Kidney	100.9 ± 5.4	104.8 ± 7.1	102.5 ± 8.1	102.6 ± 2.9
Liver	96.8 ± 4.7	89.5 ± 3.4	90.2 ± 3.2	104.7 ± 2.3
Lung	98.8 ± 6.1	114.4 ± 2.9	99.4 ± 3.2	104.1 ± 2.2

Data presented as mean ± SD (n = 5 except for IS where n = 15)

Table 3.9 Stability of plasma samples and stock solution of DHS, isorhapontigenin and pinosylvin in the different assays

Storage conditions	QC samples (% remaining)		
	Low	Mid	High
DHS			
Short term (24 °C, 3 h)	99.7 ± 7.0	99.8 ± 2.2	103.1 ± 1.9
Long term (-80 °C, 1 month)	103.8 ± 4.4	94.7 ± 2.9	86.0 ± 2.2
Post preparative (4 °C, 12 h)	101.9 ± 2.8	98.8 ± 3.4	102.5 ± 2.0
Freeze thaw (3 cycles)	99.8 ± 4.8	111.0 ± 6.8	116.3 ± 4.6
Stock solution stability (24 °C, 10 days)		89.2 ± 4.5	
Isorhapontigenin			
Short term (on ice, 3 h)	104.0 ± 6.0	102.0 ± 5.4	98.7 ± 6.4
Long term (-80 °C, 14 days)	87.9 ± 2.5	101.0 ± 3.0	97.0 ± 6.5
Post preparative (4 °C, 6 h)	108.0 ± 6.1	108.0 ± 1.0	108.0 ± 4.1
Freeze thaw (3 cycles)	98.4 ± 7.6	94.0 ± 2.4	92.3 ± 1.2
Stock solution stability (24 °C, 10 days)		96.7 ± 3.0	
Pinosylvin			
Short term (24 °C, 4 h)	93.7 ± 3.0	99.6 ± 4.9	105.7 ± 2.3
Long term (-80 °C, 20 days)	100.2 ± 6.8	105.7 ± 4.0	104.3 ± 1.8
Post preparative (4 °C, 12 h)	107.7 ± 6.2	101.1 ± 4.6	104.8 ± 6.2
Freeze thaw (3 cycles)	104.1 ± 3.7	104.3 ± 3.6	101.8 ± 2.7
Stock solution stability (24 °C, 5 days)		106.7 ± 2.6	

Data presented as mean ± SD (n = 5)

Table 3.10 Stability of pterostilbene in different tissue samples

Tissue	Storage conditions	QC samples (% remaining)		
		Low	Mid	High
Brain	Short term (24 °C, 6 h)	102.3 ± 3.5	106.3 ± 5.3	91.5 ± 3.9
	Post preparative (24 °C, 12 h)	97.1 ± 4.3	99.5 ± 1.3	99.5 ± 0.7
Heart	Short term (24 °C, 6 h)	100.2 ± 2.3	102.5 ± 4.1	93.0 ± 1.8
	Post preparative (24 °C, 12 h)	101.8 ± 2.5	100.2 ± 0.8	99.7 ± 0.6
Kidney	Short term (24 °C, 6 h)	103.1 ± 2.3	100.1 ± 6.7	96.2 ± 3.9
	Post preparative (24 °C, 12 h)	92.0 ± 4.9	101.2 ± 1.3	101.5 ± 1.6
Liver	Short term (24 °C, 6 h)	92.9 ± 3.0	101.1 ± 3.6	104.0 ± 5.1
	Post preparative (24 °C, 12 h)	99.1 ± 2.2	100.0 ± 1.3	99.5 ± 0.7
Lung	Short term (24 °C, 6 h)	102.6 ± 2.8	93.4 ± 4.4	95.4 ± 3.7
	Post preparative (24 °C, 12 h)	98.9 ± 8.7	97.4 ± 4.8	99.3 ± -.8

Data presented as mean ± SD (n = 5)

For LC-MS/MS analysis, it is also crucial to examine the effects of matrix in different sources of plasma as they can lead to varying ion suppression or enhancement resulting in accuracy. The mean matrix factor and CV of the matrix factor for DHS, isorhapontigenin and pinosylvin at the tested low QC concentration and that for their corresponding IS are listed in Table 3.11. As the matrix factors were all consistent (CV < 15%), matrix effect did not have a significant effect on the analysis of the compounds.

Table 3.11 Matrix factors for the analytes in different assays

Analyte	Compound	Matrix factor, CV
DHS	DHS	0.949 ± 0.098, 10.3%
	IS	0.826 ± 0.045, 5.4 %
Isorhapontigenin	Isorhapontigenin	1.203 ± 0.043, 3.6 %
	IS	1.187 ± 0.036, 3.0 %
Pinosylvin	Pinosylvin	0.985 ± 0.080, 8.1 %
	IS	0.935 ± 0.065, 7.0 %

Data presented as mean ± SD (n = 6)

3.4 Discussion

A HPLC-UV assay was successfully developed and validated for the analysis of pterostilbene in tissue samples while three different LC-MS/MS assays were developed and validated for the analysis of DHS, isorhapontigenin and pinosylvin. These assays are essential for reliable quantification of the different compounds in the pharmacokinetic studies described in Chapter 4.

At the point of study, no other reports have described the analysis of pterostilbene in tissues and the method developed in this study was the

first to facilitate such analysis. This method was developed with reference to the various pre-clinical studies on the tissue distribution of resveratrol (Azorin-Ortuno et al., 2011; Liang et al., 2013). Although autoradiography using radioisotopes of resveratrol is another possible method (Vitrac et al., 2003), this method was not considered due to safety and resource considerations. L-ascorbic acid was added to the homogenization solution as it was found to help reduce degradation of pterostilbene in preliminary studies.

The sample preparation methods for analysis of DHS, pinosylvin and isorhapontigenin were also optimised to reduce degradation of the compounds. The method for isorhapontigenin required addition of L-ascorbic acid in the plasma and EDTA in the IS solution for protein precipitation to ensure minimal degradation. This stability issue has also been noted in another study for a highly structurally similar analogue, piceatannol (Setoguchi et al., 2014).

No bioanalytical method has been reported for DHS at the point of study. For isorhapontigenin, although a study has reported using LC-MS/MS to quantify this compound in mouse plasma, details such as retention times and stability were missing which hinder the application of their method in other studies (Fang et al., 2013). For pinosylvin, only HPLC-UV has been used for its quantification in biological samples (Roupe et al., 2005; Roupe et al., 2006b). The LC-MS/MS methods developed in this study are novel methods which allow more sensitive and rapid quantification of the compounds in plasma samples

compared to any earlier methods. The methods developed have also been rigorously validated to ensure their reliability.

3.5 Conclusion

In summary, a HPLC-UV method for pterostilbene analysis in tissue samples and LC-MS/MS methods for analysis of DHS, isorhapontigenin and pinosylvin in plasma have been developed and validated. These methods will be suitable for analysis of the pharmacokinetic samples in Chapter 4.

CHAPTER 4

**PHARMACOKINETICS OF NATURALLY-
OCCURRING STILBENE ANALOGUES: DHS,
ISORHAPONTIGENIN, PINOSYLVIN AND
PTEROSTILBENE**

4.1 Introduction

Various naturally-occurring stilbene analogues have demonstrated promising therapeutic effects as described in Chapter 1.8. However, unlike resveratrol, the pharmacokinetic profiles of many of these stilbene analogues are not well-studied. As the poor pharmacokinetics of resveratrol is known to be a contributing factor impeding its development for clinical use, it is vital that identification of stilbene analogues which may have the same issues early in the drug discovery stage. This is especially applicable to the partially or fully hydroxylated stilbene analogues which have seen accumulating evidence of efficacy in recent years but knowledge of their pharmacokinetic properties is relatively scarce.

Pharmacokinetic studies usually measure the plasma level of compound as a surrogate for its concentrations in the body. This is sufficient as a first step to understand the compound's pharmacokinetic characteristics. However, as plasma levels may not accurately reflect the compound concentrations at the target organs, bio-distribution studies are important in more comprehensive studies.

The aim of this chapter is to further our understanding of the pharmacokinetic profiles of naturally-occurring stilbene analogues. Firstly, the plasma pharmacokinetic profiles of three analogues, namely DHS, pinosylvin and isorhapontigenin will be studied in rats following respective intravenous and oral administration. This will not only allow us to grasp the pharmacokinetics of these analogues but will also shed

light on the structural-pharmacokinetics relationship. Establishing such relationship can facilitate prediction of the pharmacokinetic characteristics of other analogues and also aid in the design of synthetic analogues with better pharmacokinetic properties. The tissue bio-distribution of pterostilbene, a naturally-occurring stilbene analogue with one of the best potency and pharmacokinetic profiles known to date, will be studied in rats following oral administration. This will reveal the plausible target organs of this compound as well as give an indication of the bio-distribution profile of the other stilbene analogues.

4.2 Methods

4.2.1 Preparation of dosing formulations

The preparation of dosing formulations was optimised based on previous studies in the same laboratory (Lin and Ho, 2011; Yeo et al., 2013). Solutions of DHS, isorhapontigenin and pinosylvin were prepared by dissolving the powder in 0.3 M hydroxypropyl β -cyclodextrin (HP- β -CD) 1 h before dosing while suspension formulations were prepared by suspending the powder in 0.3 % (w/v) carboxymethylcellulose (CMC) just before dosing. For isorhapontigenin, addition of L-ascorbic acid (final concentration = 0.01 % (w/v)) to all formulations was required to ensure stability of the compound.

Pterostilbene solution was prepared according to a previous study in the same laboratory (Yeo et al., 2013). 0.3 M HP- β -CD was added to pterostilbene powder and the suspension was shaken on a rotary

shaker at 80 °C for two hours followed by overnight at room temperature.

4.2.2 Pharmacokinetic studies

4.2.2.1 Single dose plasma pharmacokinetic studies

The single dose pharmacokinetic studies were conducted as described in Chapter 2.2.4. The design of the different studies is summarised in Table 4.1. Unless otherwise stated, the blood sampling schedule for bolus intravenous (IV) injection was before dosing and at 5, 15, 30, 60, 90, 120, 180, 240, 300, 420 and 600 min after administration while that for oral (PO) administration via oral gavage was before dosing and at 0, 15, 30, 45, 60, 90, 120, 180, 240, 300, 420 and 600 min after administration. For isorhapontigenin, blood samples were collected in tubes containing L-ascorbic acid (final concentration = 0.8 mg/mL) to ensure stability.

Table 4.1 Study design for the pharmacokinetic study of the different compounds

Study Compound	Group	Formulation	Dose and dosing route	Blood sampling schedule (min)
DHS	1 (n = 5)	Solution	4 mg/kg IV	0 – 600
	2 (n = 5)	Suspension	10 mg/kg PO	0 – 600
	3 (n = 5)	Solution	10 mg/kg PO	0 – 600
Isorhapontigenin	1 (n = 5)	Solution	7.74 mg/kg (30 μ mol/kg) IV	0 – 600
	2 (n = 4)	Suspension	154.8 mg/kg (600 μ mol/kg) PO	0 – 720*
Pinosylvin	1 (n = 5)	Solution	5 mg/kg IV	0 – 120
	2 (n = 5)	Solution	10 mg/kg IV	0 – 120
	3 (n = 3)	Solution	15 mg/kg PO	0 – 180
	4 (n = 6)	Solution	50 mg/kg PO	0 – 180

*Sampling schedule after dosing: 15, 30, 45, 60, 90, 120, 180, 240, 300, 420, 540, 720 min.

4.2.2.2 Single dose tissue distribution study following oral pterostilbene administration

The tissue distribution study for pterostilbene was conducted using 15 rats. Rats were administered pterostilbene solution by oral gavage at the dose of 40 mg/kg. Tissue samples were collected at 60, 180, 300, 420 and 540 min after dosing and three rats were used at each time point. The sampling method is described in Chapter 2.2.4.

4.2.3 HPLC-UV and LC-MS/MS analysis

All plasma and tissue samples were processed and analysed using the methods described in Chapter 3. When the measured plasma concentrations were below LLOQ, the data points were removed from analysis.

4.2.4 Pharmacokinetic calculations

All pharmacokinetic parameters were calculated using WinNonlin standard version 1.0 (Scientific Consulting, Apex, NC, USA).

After IV administration, the log plasma DHS levels declined with a rapid distribution phase followed by a prolonged terminal elimination phase typical of a bi-exponential decay. Therefore, the classical two compartmental first-order open model ($C = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t}$) was chosen to describe the IV pharmacokinetic profiles of DHS. For isorhapontigenin, its log plasma levels also showed a general biphasic decline although there was a slight increase at some time points possibly due to enterohepatic recirculation. To obtain an approximate apparent volume of distribution, the two compartmental first-order open model was used.

The logarithmic plasma pinosylvin concentration–time curves for all rats which received IV administration showed a linear decline and thus a one compartment first-order open model ($C = C_0 \cdot e^{-k \cdot t}$) was chosen to represent the IV pharmacokinetic profiles.

For rats receiving IV dosing, the plasma exposure (area under the plasma concentration–time curve from 0 min to last measurable point

(AUC_{0→t}) was calculated using the linear/log trapezoidal method while for the rats receiving oral administration, the AUC_{0→last} was calculated using linear trapezoidal method (Lin et al., 2013; Yeo et al., 2013). Similarly, the tissue exposure (area under the tissue concentration–time curve) was calculated with the mean value of the three rats at each time point. Clearance (Cl), mean transition time (MTT_{0→last}) and half-life (t_{1/2}) were also calculated using non-compartmental analysis without extrapolation to infinity. 1/y² was used as the weighting factor in all pharmacokinetic modelling (Lin et al., 2013; Yeo et al., 2013). The absolute oral bioavailability (F) of compounds was calculated using the following equation:

$$F(\%) = \frac{\text{AUC (individual values of oral dose group)}}{\text{Oral dose}} \div \frac{\text{AUC (mean value of IV dose group)}}{\text{IV dose}} \times 100\%$$

All statistical calculations were performed as described in Chapter 2.2.16.

4.3 Results

4.3.1 Pharmacokinetics of DHS

Upon bolus intravenous injection of DHS at 4 mg/kg (DHS Group 1), its plasma levels declined with a rapid distribution phase followed by a prolonged terminal elimination phase typical of a bi-exponential decay (Figure 4.1 (a)). Thus, the classical two compartmental first-order open model ($C = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t}$) was chosen to represent the intravenous pharmacokinetic profile of DHS with good fitting ($R^2 > 0.97$). The major pharmacokinetic parameters of DHS are listed in Table 4.2. DHS

displayed a moderate apparent volume of distribution of the central compartment ($V_c = 887 \pm 297$ mL/kg), clearance ($Cl = 44.7 \pm 5.1$ mL/min/kg) and a fairly short mean transit time ($MTT_{0 \rightarrow last} = 24.1 \pm 8.8$ min). As secondary peak was observed in the plasma concentration–time curve after 300 min post dosing in some rats, the terminal elimination half-life could not be determined accurately. The presence of secondary peak suggests the involvement of enterohepatic recirculation where the compound is excreted in the bile to be reabsorbed again in the small intestines. Such phenomenon has been reported for resveratrol too (Das et al., 2008; Marier et al., 2002).

When DHS suspension was given through oral gavage (10 mg/kg) (DHS Group 2), DHS was absorbed slowly (Figure 4.1 (b)). At the first two post-dosing time points (15 and 30 min), although DHS could be detected in 8 of 10 samples, only one sample was above the LLOQ (2 ng/mL). Subsequently, the plasma DHS level increased slowly and peaked between 180 to 300 min after oral administration. In general, the plasma exposure was very limited ($AUC_{0 \rightarrow last} = (0.471 \pm 0.153) \times 10^4$ min·ng/mL), with a low maximal plasma concentration ($C_{max} = 15.3 \pm 7.4$ ng/mL) and poor absolute oral bioavailability ($F = 2.22 \pm 0.72\%$). On the other hand, when DHS was dosed orally as solution (10 mg/kg) (DHS Group 3), it was absorbed rapidly ($t_{max} = 30$ or 45 min; Group 3 vs Group 2, Mann–Whitney test: $p < 0.01$). Moreover, the extent of oral absorption of DHS was substantially enhanced (Figure 4.1 (b)). In comparison to the suspension formulation, solution formulation showed more than 15-fold increase in C_{max} (356 ± 106 ng/mL vs 15.3 ± 7.4

ng/mL, two-tailed unpaired t test: $p < 0.01$), $AUC_{0 \rightarrow \text{last}} ((7.71 \pm 1.02) \times 10^4 \text{ min} \cdot \text{ng/mL}$ vs $(0.471 \pm 0.153) \times 10^4 \text{ min} \cdot \text{ng/mL}$, two-tailed unpaired t test: $p < 0.001$) and F ($36.3 \pm 4.8 \%$ vs $2.22 \pm 0.72 \%$, two-tailed t test: $p < 0.001$). Similar to IV dosing, secondary peak was also observed in the plasma DHS-time curve from 180 min post dosing.

Table 4.2 Pharmacokinetic parameters of DHS

Parameters	Grouping		
	1	2	3
Route	Intravenous	Oral	Oral
Formulation	Solution	Suspension	Solution
Dose (mg/kg)	4	10	10
V_c (mL/kg)	887 ± 297	-	-
$AUC_{0 \rightarrow \text{last}} (10^4 \times \text{min} \cdot \text{ng/mL})$	8.50 ± 1.17	0.471 ± 0.153	$7.71 \pm 1.02^{***}$
Cl (mL/min/kg)	44.7 ± 5.1	-	-
$MTT_{0 \rightarrow \text{last}}$ (min)	24.1 ± 8.8	285 ± 29	256 ± 25
C_{max} (ng/mL)	-	15.3 ± 7.4	$356 \pm 106^{**}$
t_{max} (min)	-	180 or 300	30 or 45 ^{**}
F (%)	-	2.22 ± 0.72	$36.3 \pm 4.8^{***}$

Results are presented as mean $\pm$ SD ($n = 5$). $^{**}p < 0.01$; $^{***}p < 0.001$ between 0.3 % CMC suspension and HP- β -CD solution.

Two tailed unpaired t-test was used to compare between groups except for t_{max} where Mann–Whitney test was used.

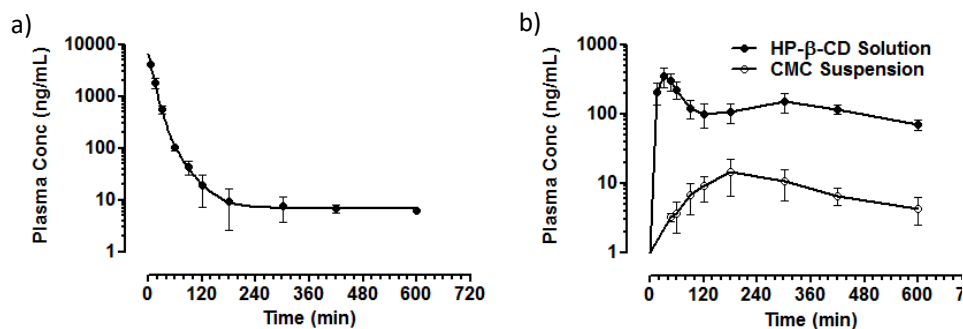


Figure 4.1 Pharmacokinetic profiles of DHS in Sprague Dawley rats after a) intravenous administration of DHS solubilised in 0.3 M HP- β -CD (4 mg/kg), $n = 5$ except at 300, 420 and 600 min where $n = 3$; b) oral administration of DHS solubilised in 0.3 M HP- β -CD or suspended in 0.3 % (w/v) CMC (10 mg/kg), $n = 5$ except 45 min in CMC suspension where $n = 3$. Symbols represent mean plasma concentrations and error bars represent SD.

4.3.2 Pharmacokinetics of isorhapontigenin

Upon bolus intravenous administration of isorhapontigenin at 7.74 mg/kg, the plasma isorhapontigenin levels first declined rapidly before a more prolonged terminal phase where secondary peaks were observed from 90 min (Figure 4.2 (a)). Appearance of secondary peaks suggests the involvement of enterohepatic recirculation described earlier for DHS. As such, the two compartmental first-order open model ($C = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t}$) was used only to obtain the V_c while non compartmental modelling was applied to calculate the other pharmacokinetic parameters (Table 4.3). The presence of secondary peaks also made the accurate calculation of elimination half-life difficult. Isorhapontigenin displayed a moderate V_c (696 ± 82 mL/kg), $MTT_{0 \rightarrow \text{last}}$ (14.5 ± 2.3 min) and rather fast CI (86.7 ± 13.3 mL/min/kg).

When isorhapontigenin was administered through oral gavage as suspension (154.8 mg/kg), it was absorbed rapidly ($t_{\text{max}} = 30 - 60$ min)

(Figure 4.2 (b)). The plasma exposure of isorhapontigenin was fairly abundant ($AUC_{0 \rightarrow \text{last}} = (57.9 \pm 10.7) \times 10^4 \text{ min} \cdot \text{ng/mL}$) and similar to IV dosing, secondary peak was also observed from 180 min post dosing.

Table 4.3 Pharmacokinetic parameters of isorhapontigenin

Parameters	Route	
	Intravenous	Oral
Formulation	Solution	Suspension
Dose (mg/kg)	7.74	154.8
V_c (mL/kg)	696 ± 82	-
$AUC_{0 \rightarrow last}$ ($10^4 \times$ min·ng/mL)	8.87 ± 1.31	57.9 ± 10.7
Cl (mL/min/kg)	86.7 ± 13.3	-
$MTT_{0 \rightarrow last}$ (min)	14.5 ± 2.3	246 ± 19
C_{max} (µg/mL)	-	2.31 ± 0.20
t_{max} (min)	-	30 – 60
F (%)	-	32.6 ± 6.0

Results are presented as mean ± SD (n = 5 for intravenous and n = 4 for oral group).

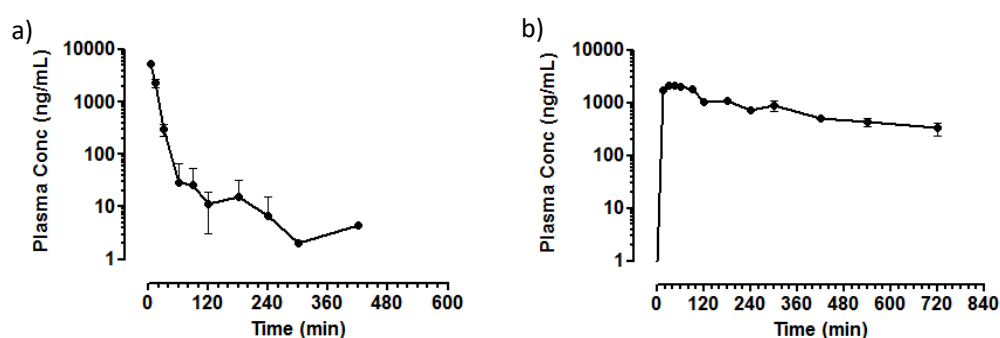


Figure 4.2 Pharmacokinetic profiles of isorhapontigenin in Sprague Dawley rats after a) intravenous administration of isorhapontigenin solubilised in 0.3 M HP-β-CD (7.74 mg/kg), n = 5 except at 300 and 420 min where n = 2; b) oral administration of isorhapontigenin suspended in 0.3 % (w/v) CMC (154.8 mg/kg), n = 4. Symbols represent mean plasma concentrations and error bars represent SD.

4.3.3 Pharmacokinetics of pinosylvlin

The intravenous pharmacokinetic profiles of pinosylvlin are shown in Figure 4.3. As the plasma levels declined exponentially, the one compartment first-order open model was applied in pharmacokinetic modelling with good fitting ($R^2 > 0.98$). The major pharmacokinetic parameters are listed in Table 4.4. Upon bolus intravenous administration at the dose of 5 mg/kg, pinosylvlin showed a moderate V_c (2.26 ± 0.81 L/kg), rapid Cl (0.29 ± 0.04 L/min/kg), short $t_{1/2}$ (5.16 ± 0.71 min) and $MTT_{0 \rightarrow \text{last}}$ (7.91 ± 0.91 min). From 60 min, plasma concentrations of pinosylvlin fell below the LLOQ of the assay. When the intravenous dose was increased to 10 mg/kg, although V_c and Cl remained unchanged (Mann–Whitney test: $p > 0.05$), $t_{1/2}$, $MTT_{0 \rightarrow \text{last}}$ and $AUC_{0 \rightarrow \text{last}}$ were significantly longer (two tailed unpaired t test: $p < 0.01$).

Oral administration of 15 mg/kg pinosylvlin solution resulted in negligible plasma exposure as all the plasma samples analysed were below the LLOQ (1 ng/mL). When the dose was increased to 50 mg/kg, pinosylvlin could be quantified in some plasma samples obtained from each rat. However, the profiles were highly erratic (profiles not shown) with low maximal plasma concentration ($C_{\text{max}} = 27.9 \pm 53.1$ ng/mL) and short exposure period. To obtain an approximation of the oral bioavailability of pinosylvlin, all plasma samples up to 2 h that were below the LLOQ were assigned as 1 ng/mL. Using this approach, the estimated mean absolute oral bioavailability (F) was determined to be < 1%.

Table 4.4 Pharmacokinetic parameters of pinosylvlin

Parameters	Grouping	
	1	2
Dose (mg/kg)	5	10
V_c (L/kg)	2.26 ± 0.81	2.67 ± 0.64
$t_{1/2}$ (min)**	5.16 ± 0.71	7.56 ± 1.29
$AUC_{0 \rightarrow \text{last}}$ ($10^4 \times$ min·ng/mL)**	1.73 ± 0.28	4.07 ± 0.65
Cl (L/min/kg)	0.29 ± 0.04	0.25 ± 0.05
$MTT_{0 \rightarrow \text{last}}$ (min)**	7.91 ± 0.91	11.36 ± 1.30

Results are presented as mean $\pm$ SD (n = 5). **p<0.01 between 5 mg/kg and 10 mg/kg groups.

Two tailed unpaired t-test was used to compare between groups.

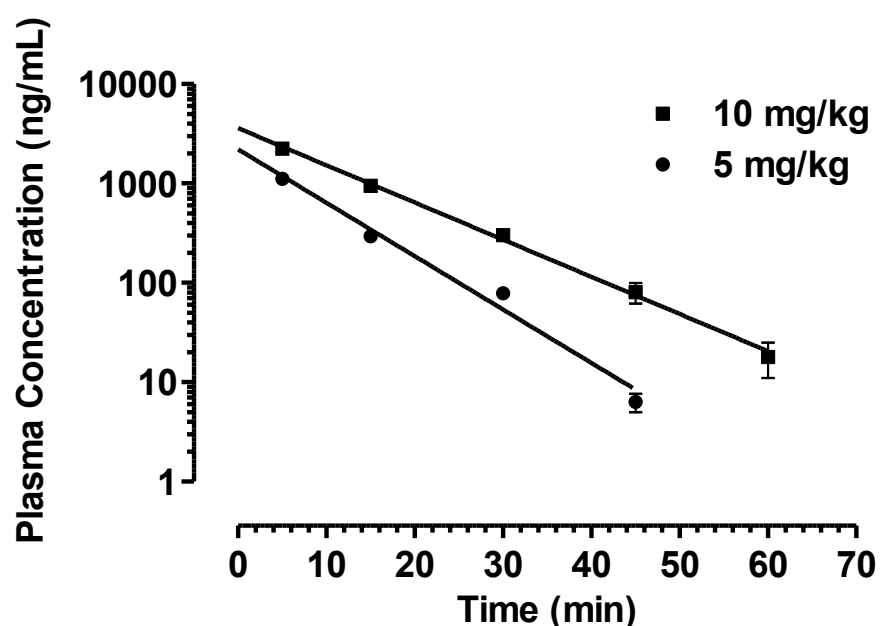


Figure 4.3 Pharmacokinetic profiles of pinosylvlin in Sprague Dawley rats after intravenous administration of pinosylvlin solubilised in 0.3 M HP- β -CD at the doses of 5 mg/kg and 10 mg/kg. Symbols represent mean concentrations and error bars represent SD. n = 5 except at 60 min for 10 mg/kg where n = 4.

4.3.4 Tissue distribution of pterostilbene

The tissue distribution of pterostilbene was profiled in Sprague Dawley rats after single oral administration at a dose of 40 mg/kg. The tissue concentration-time profiles are shown in Figure 4.4 (a) while the exposures of pterostilbene in various tissues are shown in Figure 4.4 (b). Similar to a previous study (Yeo et al., 2013), pterostilbene showed abundant plasma exposure. Interestingly, the tissue levels were much higher than plasma levels, with the exposure of PTS in the heart, lung, brain, kidney and liver approximately 4.6-, 5.2-, 6.7-, 8.9- and 26.1-fold higher than that of plasma respectively. The results demonstrated that pterostilbene was widely distributed into the major organs after a single oral dose.

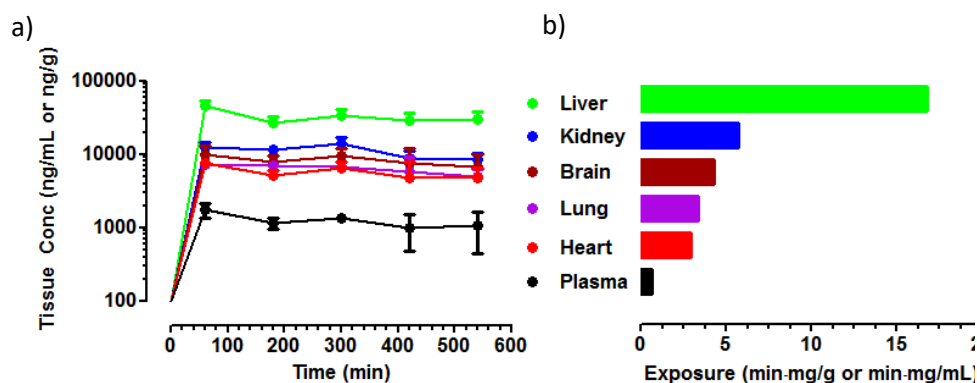


Figure 4.4 Tissue distribution profiles of pterostilbene in Sprague Dawley rats after oral administration of pterostilbene solubilised in 0.3 M HP- β -CD (40 mg/kg). a) Tissue concentrations in various tissues at different times ($n = 3$) and b) Tissue exposures calculated from the mean value of the three rats at each time point in the tissue concentration-time profiles.

4.4 Discussion

In all the pharmacokinetic studies conducted, both intravenous and oral administrations were investigated using at least one group of rats for each administration route. This fulfilled the primary objective of determining the basic pharmacokinetic parameters for each compound such as plasma clearance which can be obtained from the profile of the intravenous group and oral exposure and bioavailability which can be calculated from the profile of the oral group. Oral exposure and bioavailability are particularly important parameters since the oral route is the most preferred route of administration due to its convenience. Based on preliminary studies, additional groups such as different doses or formulations were studied to reveal interesting aspects of their pharmacokinetic characteristics.

When the intravenous dose of pinosylvin was increased from 5 mg/kg to 10 mg/kg, capacity-limited elimination was observed as the $t_{1/2}$ and $MTT_{0 \rightarrow \text{last}}$ became longer. Since pinosylvin is mainly eliminated through non-renal route (Roupe et al., 2005), it is likely that this phenomenon was due to increased saturation of metabolic enzymes. Comparing the intravenous profiles of pinosylvin with DHS (data from this study) and resveratrol (data from previous publication in our laboratory (Chen et al., 2016)) obtained using the same animal model (Table 4.5) offers some clues on the structural-pharmacokinetic relationship. The structure of resveratrol consists of two meta-hydroxyl moieties with one para-hydroxyl group. On the other hand, pinosylvin is a dihydroxystilbene with only the meta-hydroxyl moieties of resveratrol

while DHS is a dihydroxystilbene with two para-hydroxyl groups. Comparison shows that DHS has the lowest V_c and CI while its $MTT_{0 \rightarrow last}$ is the longest. On the other hand, pinosylvin has the highest V_c and CI while its $MTT_{0 \rightarrow last}$ is one of the shortest. It appears that the meta-hydroxyl groups are associated with faster CI and shorter $MTT_{0 \rightarrow last}$ since DHS which has only the para-hydroxyl group has a slower clearance and longer $MTT_{0 \rightarrow last}$ compared to pinosylvin and resveratrol. The absence of the para-hydroxyl groups in pinosylvin may have enhanced its clearance by mechanisms such as increased affinity to metabolic enzymes or reduced binding to plasma proteins (greater fraction unbound) which can result in higher metabolism and excretion. The high V_c of pinosylvin is further evidence of its increased fraction unbound. Phase II conjugation reactions (glucuronidation and sulphation) of the hydroxyl groups have been identified as the major biotransformation pathways of resveratrol (Wenzel and Somoza, 2005). Despite the reduced number of hydroxyl groups in both pinosylvin and DHS, only DHS showed lower susceptibility to metabolic clearance. As such, the location of the hydroxyl group plays a more pertinent role in determining the clearance instead of the number of hydroxyl groups. This is supported by the identification of meta-hydroxyl group as the main site for metabolism in resveratrol (Wenzel and Somoza, 2005). The additional hydroxyl groups in other locations, such as the para-hydroxyl group in resveratrol, may serve to impede the binding of metabolic enzymes which could have resulted in the reduced clearance of resveratrol compared to pinosylvin. Furthermore, oxyresveratrol,

which has an additional hydroxyl group at the ortho-position compared to resveratrol, has a slower clearance than resveratrol (67.5 ± 22.8 mL/min/kg vs 107.4 ± 18.5 mL/min/kg; two-tailed t test: $p < 0.05$) (Chen et al., 2016). The clearance of isorhapontigenin was similar to resveratrol (86.7 ± 13.3 mL/min/kg vs 107.4 ± 18.5 mL/min/kg; two-tailed t test: $p > 0.05$) and not higher as its additional methylated hydroxyl (methoxy) group at the meta-position is less susceptible to metabolism compared to hydroxyl groups. This resistance of methoxy moiety against phase II metabolism is well demonstrated by the significantly lower clearance of pterostilbene, a resveratrol analogue methylated at both the meta-hydroxyl groups (Yeo et al., 2013).

The oral bioavailability of a xenobiotic is dependent on factors such as its physicochemical properties, pre-systemic metabolism and physiological conditions. Physicochemical properties including aqueous solubility and membrane permeability are critical factors determining the absorption of the compound (Veber et al., 2002).

Aqueous solubility has been shown to affect the absorption of several stilbene analogues (Lin and Ho, 2011; Yeo et al., 2013). In the present study, the oral absorption of DHS was also highly dependent on its solubility as evident from the improved oral pharmacokinetic properties such as $AUC_{0 \rightarrow \text{last}}$ and F of DHS when it was administered as solution instead of suspension. For other stilbene analogues, administration as a solution formulation did not improve the extent of absorption due to their better intrinsic solubility (Chen et al., 2016; Das et al., 2008).

Poor membrane permeability is not a likely barrier to the oral absorption of the stilbene analogues investigated in this study. This is because of their favourable physicochemical properties such as low molecular weight, moderate lipophilicity (measured using xLogP3), low polar surface area, low number of hydrogen bond donors and acceptors and low number of rotatable bonds (Table 4.6) (NCBI, 2016)

Assuming that the physiological characteristics of the animals used in different studies were largely consistent, the oral bioavailability and exposure of the stilbene analogues are essentially affected by their pre-systemic metabolism if solubility is not an issue. Comparing the oral pharmacokinetics of DHS (in the optimised solution formulation), pinosylvin and resveratrol, the F and $MTT_{0 \rightarrow last}$ of pinosylvin were the lowest while that of DHS were the highest. Similarly, the dose-normalised $AUC_{0 \rightarrow last}$ and dose-normalised C_{max} showed the same trend (Table 4.5). These observations can also be adequately explained by the reasoning described earlier that the location of hydroxyl groups at the meta-positions were more susceptible to metabolic clearance. This increased first-pass metabolism resulted in the reduced oral bioavailability and exposure of the stilbene compounds. Another possible mechanism behind these observations is increased binding of compounds with the meta-hydroxyl groups to the efflux transporters. These prominent barriers to oral bioavailability which are widely distributed in the intestinal membrane have been found to hinder the oral absorption of resveratrol (Henry et al., 2005). The structural similarities between resveratrol and these analogues

suggest the possible involvement of this mechanism. Despite having similar intravenous pharmacokinetic profiles, the oral pharmacokinetics of isorhapontigenin was better than resveratrol. Its F and dose-normalised $AUC_{0 \rightarrow last}$ were significantly higher than resveratrol (F : $32.6 \pm 6.0\%$ vs $22.3 \pm 4.3\%$; dose-normalised $AUC_{0 \rightarrow last}$: $(3.74 \pm 0.69) \times 10^3$ min·mg/mL vs $(2.08 \pm 0.40) \times 10^3$ min·mg/mL; Mann Whitney test: $p < 0.05$) while its $MTT_{0 \rightarrow last}$ and dose-normalised C_{max} were higher but statistical significance was not achieved ($MTT_{0 \rightarrow last}$: 246.3 ± 19.3 min vs 184.6 ± 48.8 min; dose-normalised C_{max} : 14.9 ± 1.3 g/mL vs 11.0 ± 4.1 g/mL; Mann Whitney test: $p > 0.05$). The additional meta-methoxy group in isorhapontigenin could have facilitated the oral absorption by hindering the binding with efflux transporters or pre-systemic intestinal metabolic enzymes.

The tissue distribution of pterostilbene was investigated to identify potential target organs and obtain a general idea of the distribution of stilbene analogues. In addition to its excellent pharmacokinetics which has been observed in an earlier study performed in our laboratory (Yeo et al., 2013), high tissue penetration of pterstilbene was observed after a single oral dose where the concentrations required for *in vitro* anti-cancer and neuroprotective activities were achievable at the target organs (Carey et al., 2013; Hou et al., 2014; Yang et al., 2013). Such observation corroborated with the findings of a recent study which administered a lower dosage (22.5 mg/kg) of pterostilbene solution orally. However, the dose-normalised tissue exposures of pterostilbene obtained in this study were approximately 1.5-fold as high as the

values reported in that study. This is reasonable since pterostilbene has been shown to exhibit non-linear pharmacokinetics (Yeo et al., 2013) and the higher dosage used in the present study would have further saturated the elimination of pterostilbene to result in higher exposures. Although resveratrol also show distribution into the major organs also (Juan et al., 2010; Liang et al., 2013), pterostilbene was clearly more extensively distributed, likely due to its higher lipophilicity. With such knowledge, prediction of the tissue distribution of the other stilbene analogues can be made from our understanding of their physicochemical properties (Øie, 1986).

Table 4.5 Comparison of the pharmacokinetic parameters of DHS, pinosylvin and resveratrol

Parameters (Administration route)	Rank		
	1 (highest)	2	3
V _c (IV) (L/kg)	Pinosylvin (2.67 ± 0.64)	Resveratrol (1.63 ± 0.89)	DHS (0.89 ± 0.33) **
Cl (IV) (mL/min/kg)	Pinosylvin (250.9 ± 49.7)	Resveratrol (107.4 ± 18.5)	DHS (44.7 ± 5.1) **
MTT _{0→last} (IV) (min)	DHS (24.1 ± 8.8)	Pinosylvin (11.4 ± 1.3)	Resveratrol (11.1 ± 1.8) **,##
F (PO) (%)	DHS (36.3 ± 4.8)	Resveratrol (22.3 ± 4.3)***	Pinosylvin (< 1)***,###
MTT _{0→last} (PO) (min)	DHS (255.6 ± 25.1)	Resveratrol (184.6 ± 48.8)*	Pinosylvin (48.4 ± 10.7) ***,###
Dose-normalised AUC _{0→last} (PO) (min·mg/mL)	DHS ((7.71 ± 1.02) × 10 ³)	Resveratrol ((2.08 ± 0.40) × 10 ³)	Pinosylvin (17.7 ± 31.9) ***
Dose-normalised C _{max} (PO) (g/mL)	DHS (35.6 ± 10.6)	Resveratrol (11.0 ± 4.1)	Pinosylvin (0.56 ± 1.1) ***

Values are presented as mean ± SD

*p < 0.05, **p < 0.01, ***p < 0.001 between this group and rank 1 group

##p < 0.01, ###p < 0.001 between this group and rank 2 group

Mann Whitney test with Dunns post test was used to compare between groups except for MTT_{0→last} and F where one way ANOVA with Tukey post test was used.

Table 4.6 Physicochemical properties of stilbene compounds

Parameter	Compound			
	Resveratrol	DHS	Pinosylvin	Isorhaponti-genin
Molecular weight (g/mol)	228	212	212	258
xLogP3	3.1	3.5	3.5	3.2
Polar surface area (Å ²)	60.7	40.5	40.5	69.9
No. of H bond donors	3	2	2	3
No. of H bond acceptors	3	2	2	4
No. of rotatable bonds	2	2	2	3

4.5 Conclusion

This chapter investigated the intravenous and oral pharmacokinetic profiles of three stilbene analogues, DHS, isorhapontigenin and pinosylvin using the Sprague Dawley rat pharmacokinetic model. In addition to being the first study that comprehensively characterise the important pharmacokinetic parameters of these compounds, comparison with resveratrol also revealed that the position but not number of hydroxyl groups are important determinants of their clearance and bioavailability. DHS and isorhapontigenin have been identified as orally available analogues with higher bioavailability compared to resveratrol while pinosylvin is only suitable for topical application due to its extensive first-pass and systemic clearance. The

tissue distribution of pterostilbene was also assessed after a single oral dose using the same rat model. Compared to resveratrol, pterostilbene exhibited more extensive distribution into the major target organs such as brain and lungs. This warrants further development of pterostilbene as a therapeutic agent and provides a basis for the prediction of the tissue distribution for the other stilbene analogues.

CHAPTER 5
METABOLITE IDENTIFICATION AND
PHARMACOKINETICS OF NOVEL
SYNTHETIC STILBENE ANALOGUE
***TRANS*-2,6-DIFLUORO-4'-(N,N-**
DIMETHYLAMINO)STILBENE (DFS)

5.1 Introduction

The growing interest in stilbene analogues has extended to attempts to synthesise derivatives with improved potential for clinical use (Kondratyuk et al., 2011; Szekeres et al., 2010). Recently, *trans*-2,6-difluoro-4'-(N,N-dimethylamino)stilbene (DFS) has emerged as a drug candidate that not only possessed potent biological activity but was also an orally available compound with better pharmacokinetic properties than resveratrol (Lin et al., 2013; Zhang et al., 2013). A better understanding of its pharmacokinetics, including its metabolism, will be useful for its further development including application in other therapeutic areas such as inflammation.

Studies in the past few years have facilitated a decent grasp of the metabolism of resveratrol and its other naturally occurring analogues with hydroxyl and methoxy moieties (Huang et al., 2010; Remsberg et al., 2008; Shao et al., 2010). Generally, phase II metabolism involving glucuronidation and sulphation of the hydroxyl groups are the predominant biotransformations of these compounds (Setoguchi et al., 2014; Yu et al., 2002). Other than forming conjugated metabolites which are often pharmacologically inactive and excreted rapidly, a small proportion of resveratrol is converted to piceatannol which has been found to exert more potent effects in some studies (Potter et al., 2002). While much progress has been made for these naturally-occurring stilbene analogues which have similar functional groups, the metabolic pathways for synthetic stilbene analogues with different functional groups such as DFS are still unclear.

In addition to identification, quantitative assessment of the pharmacokinetic profiles of metabolites following parent drug administration will reveal the potential contribution of active metabolites to the observed efficacy of the parent drug. Furthermore, independent investigations of the drug metabolites themselves might uncover additional, promising drug candidates with superior potency and pharmacokinetics relative to the parent drug (Fura, 2006).

The aim of this chapter is to first identify the metabolites formed from DFS in mouse, rat and human microsomes using LC-MS/MS. Next, a sensitive LC-MS/MS multiple reaction monitoring (MRM) method will be developed for the simultaneous quantification of DFS and a key active metabolite in rat plasma to study their pharmacokinetic profiles following DFS administration. Finally, the pharmacokinetics of DFS and this metabolite will be compared through separate profiling in Sprague-Dawley rats following respective intravenous and oral administration of each compound. This study will deliver a novel insight into the metabolism of DFS and enable comparison of the pharmacokinetics of DFS with its major active metabolite.

5.2 Methods

5.2.1 Chemical syntheses

The putative metabolites (M1-M10) used in this study were synthesised by the laboratory of Professor David S. Watt (University of Kentucky, KY. USA).

5.2.2 Liver microsomal metabolism

A liver microsomal incubation system was prepared by mixing 100 mM potassium phosphate buffer (pH = 7.4), NADPH regenerating system solutions, and liver microsomes. Pooled mouse, rat and human liver microsomes were separately used to investigate the metabolites of DFS in different species. These mixtures were first equilibrated for 5 min in a shaking water bath maintained at 37 °C. Subsequently, the metabolic study was initiated by adding a DMSO solution of DFS (final concentration = 20 µM, ≤ 0.2 % DMSO). An aliquot of the sample was withdrawn at 60 min, and the reaction was terminated using an equal volume of ice cold acetonitrile to precipitate the proteins. Blank samples were prepared in a similar manner except that DMSO was added in place of DFS solution. Negative control samples were also prepared in the same way except that immediate termination of the reaction was carried out using ice cold acetonitrile. All experiments were carried out in triplicate. The samples were vortexed and centrifugation was carried out at 10 000 g for 15 min. Finally, the clear supernatant was transferred into a HPLC vial and subjected to LC-MS/MS analysis.

5.2.3 Sample preparation

A protein precipitation procedure was utilised to clarify plasma samples before LC-MS/MS analysis. Five volumes of IS (*trans*-3,5,4'-trimethoxystilbene (TMS)) in methanol (final concentration = 200 ng/mL) was added to one volume of rat plasma. Following vigorous vortex, the samples were centrifuged at 10 000 g for 10 min at 4 °C.

The clear supernatant was transferred into a HPLC vial and subjected to LC-MS/MS analysis.

5.2.4 Instrumental analysis

Metabolite identification analysis was carried out using the QTRAP® 5500 mass spectrometer described in Chapter 2.2.2. Chromatographic separation was performed using a reversed-phase HPLC column (Agilent Poroshell 120 EC-C18: 75 × 3.0 mm, 2.7 µm) maintained at 30 °C and protected by a guard column (Agilent Fast Guard Column, 3.0 × 5 mm, 2.7 µm). The mobile phase consisted of acetonitrile and 0.1% formic acid in milliQ water delivered at a flow rate of 0.3 mL/min with the following gradient schedule: a) 0.0 – 12.0 min, acetonitrile 10 → 90%; b) 12.0 – 15.0 min acetonitrile 90%; c) 15.0 – 15.1 min acetonitrile 90 → 10%; d) 15.1 – 20.0 min acetonitrile 10%. A divert valve directed the eluent to waste from 0.0 min to 3.0 min and 15.0 min to 20.0 min. The injection volume was 5 µL and the auto-sampler was set to maintain the temperature at 4 °C.

The eluent from the chromatographic column was introduced directly to the mass spectrometer via ESI ion source operating in the positive ionization mode. Following optimisation as described in Chapter 2.2.2, the compound parameters for DFS are shown in Table 5.1. The source parameters, consisting of curtain gas, gas 1, gas 2, temperature and ion spray voltage were set at 20 psi, 40 psi, 30 psi, 600 °C and 4500 V, respectively.

The MRM-IDA-EPI (multiple reaction monitoring-information dependent acquisition-enhanced product ion) method was used to identify the metabolites of DFS in the different species. In this method, MRM channels of the predicted metabolites were generated by the analyst software based on the most common Phase I biotransformation pathways and the three original MRM transitions of the parent compound. MRM was used as the survey scan to trigger the dependent EPI scan of the metabolites, which allowed acquisition of structural information about the metabolites. A total of 69 MRM survey channels, including three channels for the parent compound, were used with a dwell time of 10 ms for each transition. Interpretation of the MS/MS spectra and comparison with standards where available were then carried out to identify the metabolites formed from the list of theoretically possible metabolites.

The same declustering potential (DP), entrance potential (EP), and collision exit potential (CXP) values, which were optimised for DFS, 173.1 V, 11.8 V and 14.1 V, respectively, were used for the MRM transitions of the predicted metabolites and rolling collision energy was used. A threshold of 1000 counts per second was used for the MRM survey scan, above which the EPI scan would be triggered. The EPI scan rate was 20 000 Da/s over the m/z range of 100 to 300. The CE values were set at 40 eV with CE spread of 20 eV. The dynamic fill time was used to prevent overfilling of the ion trap.

For the analysis of plasma samples, the same HPLC-MS/MS instrument was used. A reversed-phase HPLC column (Agilent

Poroshell 120 EC-C18: 3.0 × 50 mm, 2.7 µm) protected with a guard column (Agilent Fast Guard Column, 3.0 × 5 mm, 2.7 µm) was utilized for chromatography. Gradient elution was employed using methanol and milliQ water at a flow rate of 0.6 mL/min. The gradient elution condition was: a) 0.0 - 1.5 min methanol 65 %; b) 1.5 – 3.0 min methanol 65 → 90 %; c) 3.0 – 5.0 min 90 % methanol; d) 5.0 – 5.5 min methanol 90 → 65 % e) 5.5 – 7.0 min methanol 65 %. This chromatographic method yielded the most desirable peak shape and separation out of the different conditions tested. The injection volume was 1 µL, and the temperature of the column and auto-sampler were maintained at 50 °C and 4 °C respectively.

The optimization of the MS operating parameters for DMDFS and TMS were carried out in a similar manner as DFS. The compound parameters were optimized by sequential ramping, and the most intense precursor-to-product ion transitions were used for quantification (Table 5.1). The dwell time for all three compounds was set at 100 ms.

Table 5.1 Optimised MS compound parameters for DFS, DMDFS and TMS

Compound	Q1 mass/Q3 mass	DP (V)	EP (V)	CE (eV)	CXP(V)
DFS	260.2/245.2	173.1	11.8	28.2	14.1
DMDFS	246.1/231.2	183.3	8.9	28.9	9.6
TMS	271.0/152.0	10.0	5.0	58.2	5.16

DP = Declustering potential; EP = Entrance potential;
CE=Collision energy; CXP=Collision exit potential

5.2.5 Method validation

The LC-MS/MS method for DFS and DMDFS quantification was validated by evaluating several parameters as described in Chapter 2.2.3. The concentration of calibration standards were 2, 10, 25, 50, 100, 300 and 500 ng/mL while the concentration of QC samples were 5, 250 and 400 ng/mL.

The stability of the stock solutions for DFS, DMDFS and IS was evaluated after storing at room temperature for three days. The stability of the plasma samples was assessed using five replicates of the three QC concentrations under conditions encountered during sample handling, namely short-term stability on ice for 3 h, long-term stability at -80 °C for 2 months, freeze thaw stability after three cycles (-80 °C to 25 °C) and post-preparative stability at 4 °C for 20 h.

5.2.6 Pharmacokinetic study

The pharmacokinetic study was conducted as described in Chapter 2.2.4. The study design consisted of 21 rats allocated into four different groups. Group 1 (n = 5) and Group 2 (n = 6) received DFS through bolus intravenous injection at 4 mg/kg and oral gavage at 10 mg/kg, respectively; Group 3 (n = 5) and Group 4 (n = 5) received DMDFS through bolus intravenous injection at 4 mg/kg and oral gavage at 10 mg/kg, respectively. The dosing vehicle was 0.01 M HCl-0.3 M HP- β -CD which aided in the complete solubilisation of the DFS and DMDFS (Lin et al., 2013). Serial blood samples were collected before dosing and at 5, 15, 30, 60, 90, 120, 180, 300, 480 and 720 min after intravenous administration and at 15, 30, 45, 60, 90, 120, 180, 300,

480 and 720 min after oral administration. The blood samples were centrifuged at 5000 g for 5 min and the plasma layer was retrieved and stored at -80 °C until analyzed.

5.2.7 Pharmacokinetic calculation

Pharmacokinetic calculation was completed using WinNonlin standard version 1.0 (Scientific Consulting, Apex, NC, USA). Since the logarithmic plasma DFS and DMDFS concentration–time curves for all rats which received intravenous administration showed a bi-exponential decline, a two-compartment first-order open model ($C = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t}$) was chosen to represent the intravenous pharmacokinetic profiles. Plasma exposure (area under the plasma concentration–time curve from 0 min to last measurable point ($AUC_{0 \rightarrow \text{last}}$)), clearance (Cl), mean transit time (MTT) and terminal elimination half-life ($t_{1/2}$) were calculated using non-compartmental analysis without extrapolation to infinity. The value $1/y^2$ was used as the weighting factor in all pharmacokinetic modelling (Lin et al., 2013; Yeo et al., 2013). The absolute oral bioavailability (F) of DFS and DMDFS in Group 2 and Group 4, respectively, were calculated using the following equation:

$$F (\%) = \frac{AUC (\text{individual values of Group 2 or 4})}{10 \text{ mg/kg}} \div \frac{AUC (\text{mean value of Group 1 or 3})}{4 \text{ mg/kg}} \times 100\%$$

Assuming linear intravenous pharmacokinetics, the fraction of DFS converted to DMDFS (f_m) after intravenous administration of DFS was determined using the following equation (Rowland and Tozer, 1995):

$$f_m = \frac{Cl_{DMDFS}(\text{mean value of Group 3}) \times AUC_{DMDFS}(\text{individual values of Group 1})}{Cl_{DFS}(\text{individual values of Group 1}) \times AUC_{DFS}(\text{individual values of Group 1})}$$

All statistical calculations were performed as described in Chapter 2.16.

5.3 Results

5.3.1 Metabolite identification

The liver microsomal incubation systems generated Phase I metabolites of DFS in the three species, which were identified by harnessing the capability of the QTrap system in performing the MRM-IDA-EPI analysis. Parallel investigation of the blank, negative control and DFS-dosed samples using the same instrumental conditions allowed the identification of unique peaks in the DFS-dosed samples that could be designated as metabolites. The negative control also provided the EPI and chromatographic retention time of the parent compound, which formed the basis of metabolite identification. The metabolic pathways of DFS shown in Figure 5.1 are based on the mass shift of metabolites and fragments in the EPI spectra and was confirmed using the fragmentation patterns and retention times of authentic standards, where available. An inter-species comparison of the metabolic profiles of DFS is presented in Table 5.2.

5.3.1.1 MRM transition 246.2 → 231.1 (M1)

One of the major metabolites displayed a $[M+H]^+$ ion with m/z 246.2 and a retention time of approximately 11.4 min. This corresponded to trans-2,6-difluoro-4'-(N-methylamino)stilbene (DMDFS), an analogue

that has shown more potent chemotherapeutic effects compared to DFS (Zhang et al., 2011a). The major fragment ion was m/z 231.0 which was 14 Da lower than the major fragment ion in DFS. This indicated that the metabolite could have formed from a loss of a methylene group from DFS. The MS/MS spectrum of DMDFS (M1) is shown in Figure 5.2 (a). DMDFS was abundant in all three species and its identity confirmed was using an authentic standard.

5.3.1.2 MRM transition 276.2 → 261.2 (M2 and M3)

Two metabolites with $[M+H]^+$ ion at 16 Da higher than DFS eluted at 6.2 min and 9.8 min, indicating that they are mono-oxidation products. The difference in retention times of these two metabolites suggested that oxidation occurred at structurally unrelated positions. The MS/MS spectra are presented in Figure 5.2 (b) and (c). By comparing with authentic standards, the position of oxidation for the metabolites at 6.2 min (M2) and 9.8 min (M3) were found to be at position C-2' (*trans*-2,6-difluoro-4'-(N,N-dimethylamino)-2'-hydroxystilbene) and position C-3 (*trans*-2,6-difluoro-4'-(N,N-dimethylamino)-3-hydroxystilbene) respectively. M3 was found in all three species while M2 was found in human and mouse samples only.

5.3.1.3 MRM transition 274.2 → 259.2 (M4)

A biotransformation product with m/z 14 Da higher than DFS eluted at 9.8 min. Based on the retention time, it is likely to be formed from the oxidation of an N-methyl group in DFS to an N-formyl group. The MS-MS spectrum is shown in Figure 5.2 (d). Both mouse and rat

microsomes formed this product, and it was confirmed using authentic standard.

5.3.1.4 MRM transition 262.2 → 247.2 (M5)

An increase by 2 Da relative to DFS was detected for one metabolite with retention time of 8.3 min. This could be a metabolite formed from a combination of two sequential biotransformation reactions (i.e., demethylation and oxidation). This conjecture was supported by its short retention time and a shift of +16 Da for some of the major fragment ions compared to DMDFS. The MS/MS spectrum is shown in Figure 5.2 (e). This metabolite was present in all three species.

5.3.1.5 MRM transition 258.2 → 243.2 (M6 and M7)

Two metabolites with $[M+H]^+$ ions that are 2 Da lower than DFS were observed at 6.4 min and 8.9 min. Based on their relatively short retention times, these metabolites were presumed to have undergone oxidation and defluorination. The MS/MS spectra are shown in Figure 5.2 (f) and (g). Both metabolites were present in mouse microsomes while only the metabolite at 6.4 min was present in rat microsomes and only the metabolite at 8.9 min was present in human microsomes.

5.3.1.6 MRM transition 292.2 → 277.2 (M8 and M9)

Two metabolites at different retention times were found with the above MRM transition. Since the m/z of the parent ion is 32 Da higher than that of DFS, these metabolites were presumed to result from bis-oxidation. The MS-MS spectra are presented in Figure 5.2 (h) and (i).

The retention times were 4.7 min and 5.2 min. These two metabolites were present in mouse while only the latter one was present in rat.

5.3.1.7 MRM transition 260.2 → 245.2 (M10)

One metabolite with similar $[M+H]^+$ as DFS eluted at 11.7 min. Due to the similarity in fragmentation pattern of this metabolite compared to DFS, it is likely that this metabolite is the *cis*-isomer of DFS. The MS/MS spectra of M10 is shown in Figure 5.2 (j).

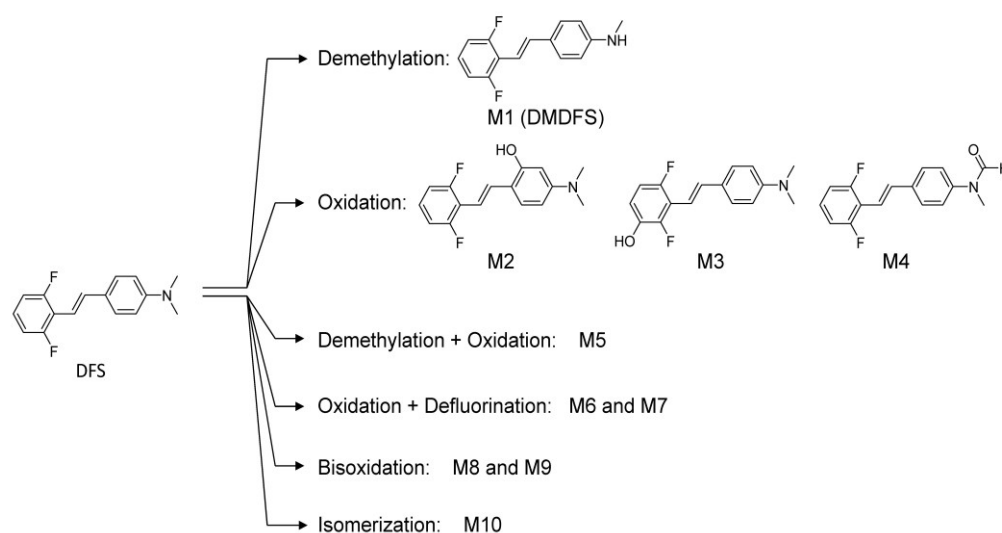
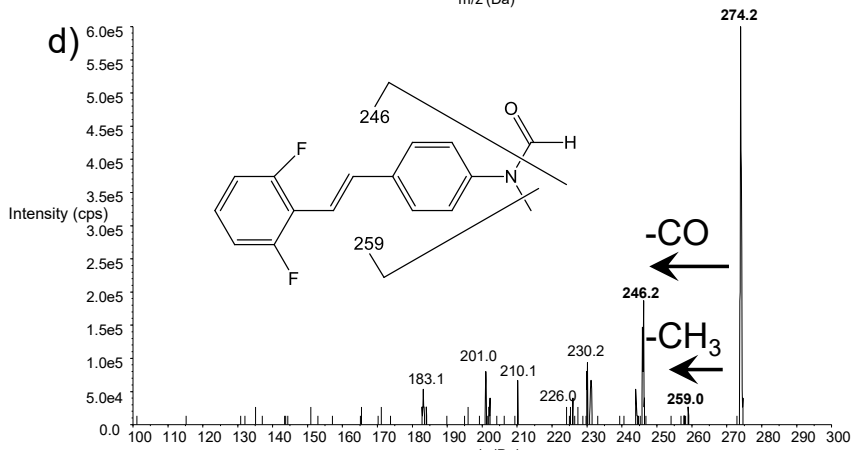
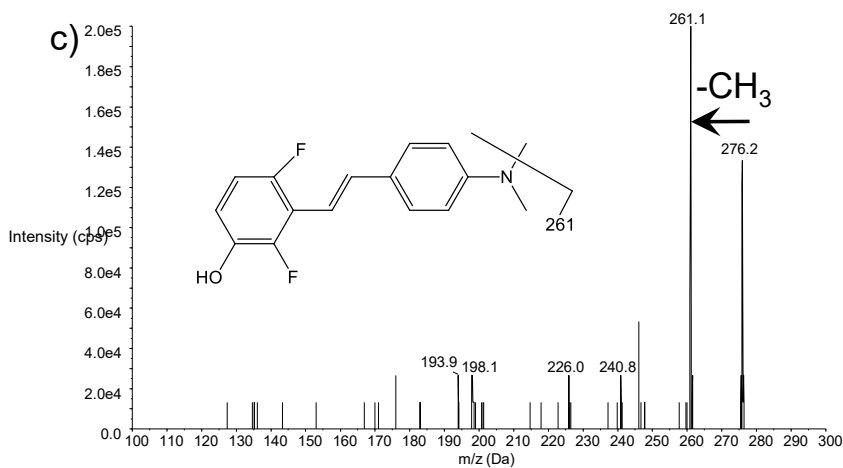
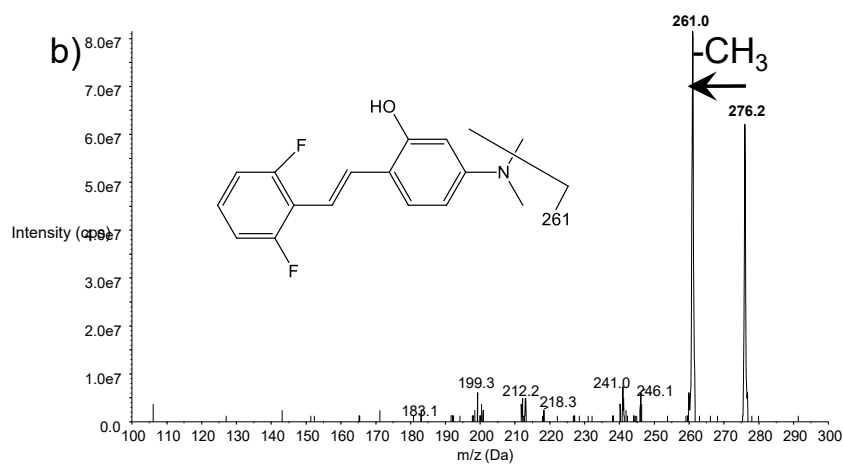
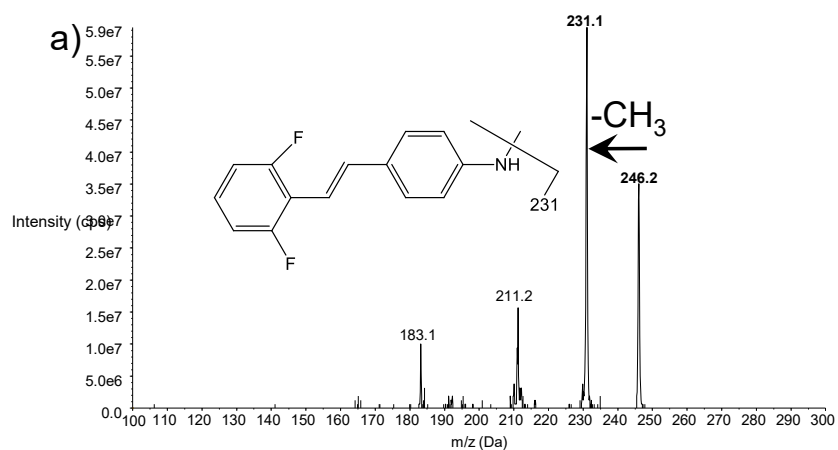
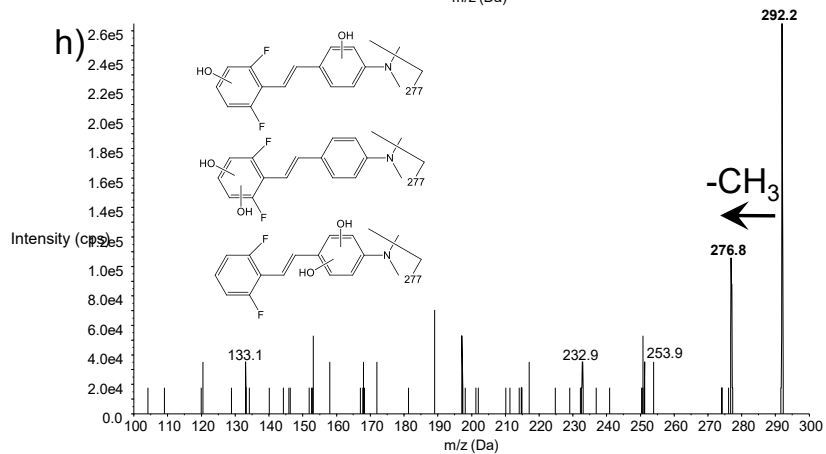
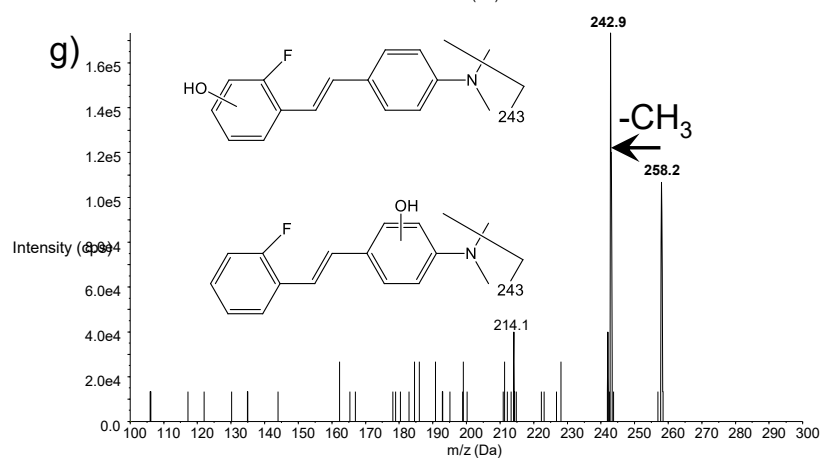
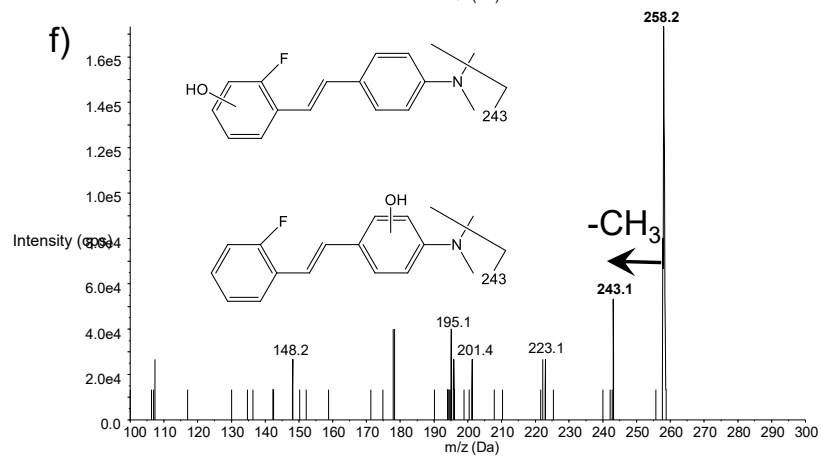
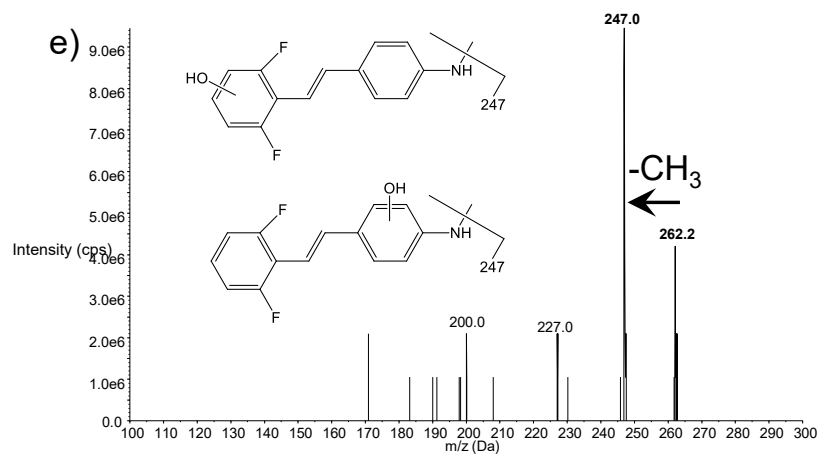


Figure 5.1 Metabolic pathways of DFS. The proposed identity of the metabolites are based on the mass shift of metabolites and fragments in the EPI spectra and confirmed using the fragmentation patterns and retention times of authentic standards, if available.

Table 5.2 Mass change and retention times of putative metabolites present in the liver microsomal samples of different male species

Metabolite	Mass Change (Da)	Retention time (min)	Species		
			Mouse	Rat	Human
M1	-14	11.4	✓	✓	✓
M6	-2	6.4	✓	✓	-
M7	-2	8.9	✓	-	✓
M10	0	11.7	✓	✓	✓
M5	+2	8.3	✓	✓	✓
M4	+14	9.8	✓	✓	-
M2	+16	6.2	✓	-	✓
M3	+16	9.8	✓	✓	✓
M8	+32	4.7	✓	-	-
M9	+32	5.2	✓	✓	-





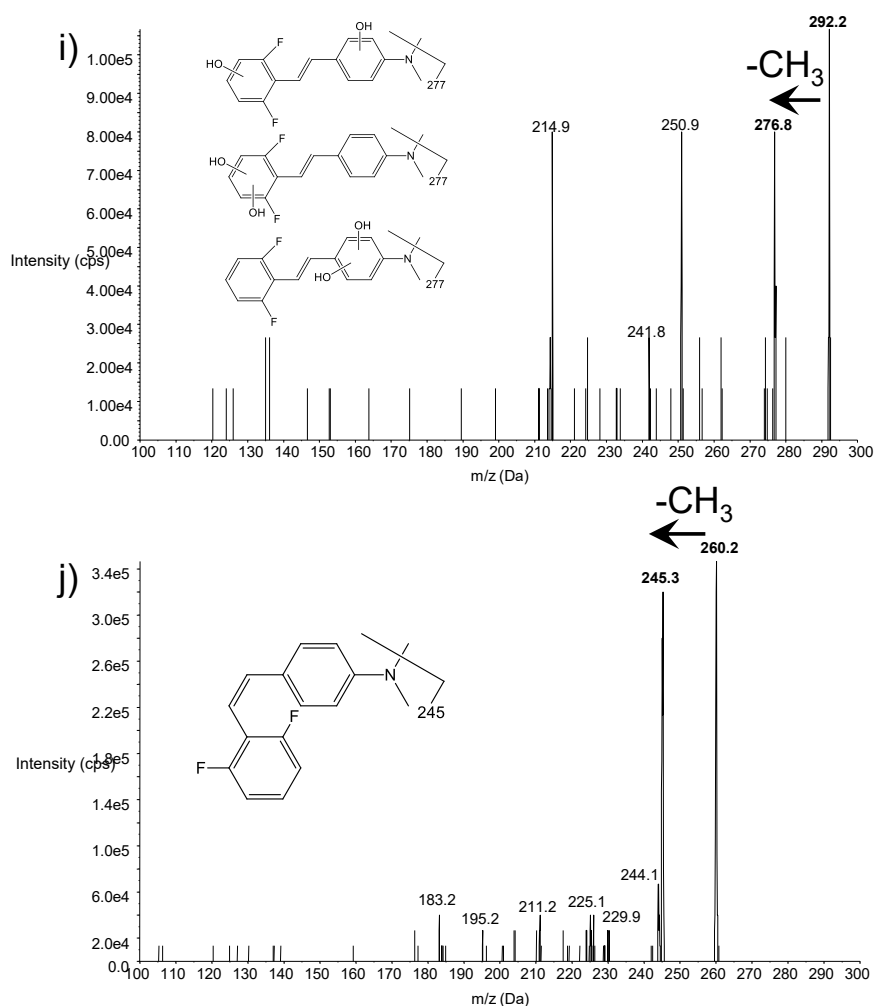


Figure 5.2 MS/MS spectra and structures of putative DFS metabolites a) M1, b) M2, c) M3, d) M4, e) M5, f) M6, g) M7, h) M8, i) M9 and j) M10. Liver microsomes from mouse, rat and human were incubated separately with DFS for 60 min before the reactions were stopped and the metabolites were identified using MRM-IDA-EPI method on the QTRAP® 5500 mass spectrometer. Diagrams are representative of $n = 3$ independent experiments.

5.3.2 Method validation

In order to study the pharmacokinetic profiles of DFS and that of its major metabolite DMDFS, following administration of DFS or DMDFS, a LC-MS/MS for simultaneous quantification of both compounds using TMS as internal standard was developed and validated. The MS/MS spectra and proposed major fragments of DFS, DMDFS and TMS are

shown in Figure 5.3. The fragmentation behavior of TMS observed in this study is similar to other methoxy-substituted stilbenes (Güsten et al., 1974).

The blank rat plasma samples from six different rats and pre-dosing plasma samples ($n = 21$) did not show significant endogenous interference at the retention times of DFS, DMDFS and TMS (IS), demonstrating the selectivity of the assay (Figures 5.4 (a), (b) and (c)). Moreover, analysis of pharmacokinetic samples confirmed the absence of co-eluting metabolite peaks in the same MRM channels (Figures 5.4 (d) and (e)). An important parameter to consider in the use of LC-MS/MS platform is the matrix effect since ion suppression or enhancement by different matrices could result in an inaccurate analysis. Matrix effects did not significantly affect these assays since the average matrix factor in six different sources of rat plasma were consistent at the QC concentration (5 ng/mL) for DFS and DMDFS and at the working concentration for IS at 1.16 ± 0.12 (CV = 10.4%), 0.929 ± 0.10 (CV = 8.6%) and 1.01 ± 0.11 (CV = 9.9%), respectively.

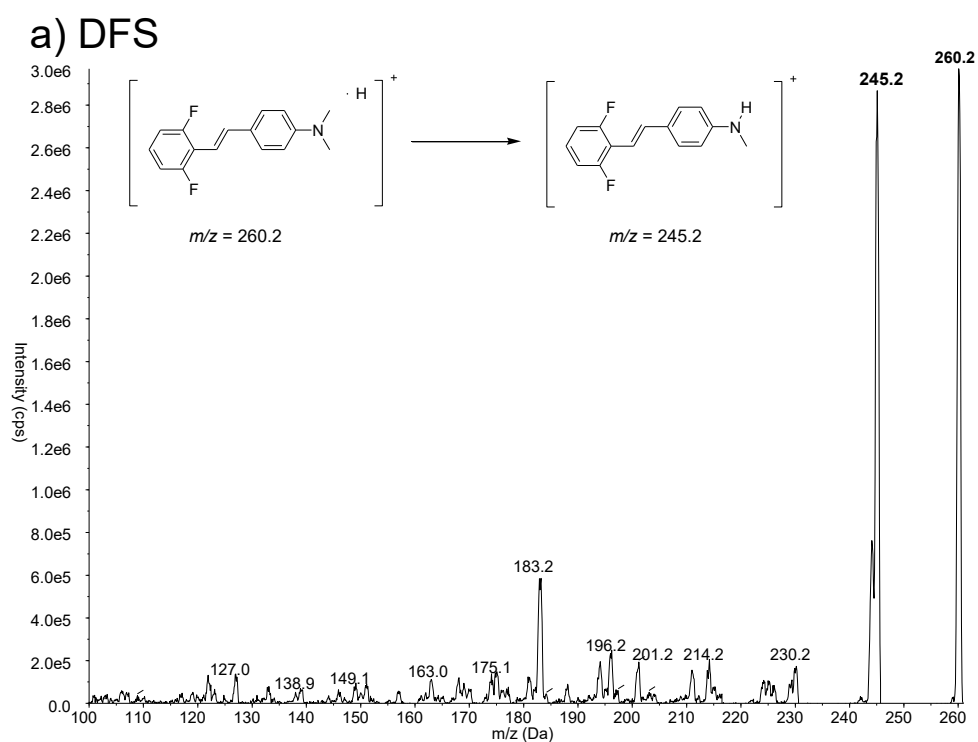
The present assay was a sensitive method for quantification of DFS and DMDFS, with LLOQ of 2 ng/mL. Calibration curves constructed in the intra-day and inter-day assays showed excellent linearity ($R^2 > 0.99$). Good accuracy and precision were also achieved as shown in the analytical recovery and CV of QC samples in Table 5.3.

The protein precipitation procedure employed yielded a high recovery for DFS and DMDFS at the three QC concentrations ($n = 5$) as shown

in Table 5.4. Similarly, the IS showed a high recovery of $105.1 \pm 5.5 \%$ (Table 5.4). These corroborated the use of this simple protein precipitation method for sample preparation

Investigations into the stability of samples exposed to conditions commonly encountered during sample handling confirmed that they were not subjected to significant degradation (Table 5.4).

In summary, a simple and sensitive LC-MS/MS method was developed and validated for the simultaneous quantification of DFS and DMDFS in rat plasma, and this method was suitable for their pharmacokinetic investigation in rats.



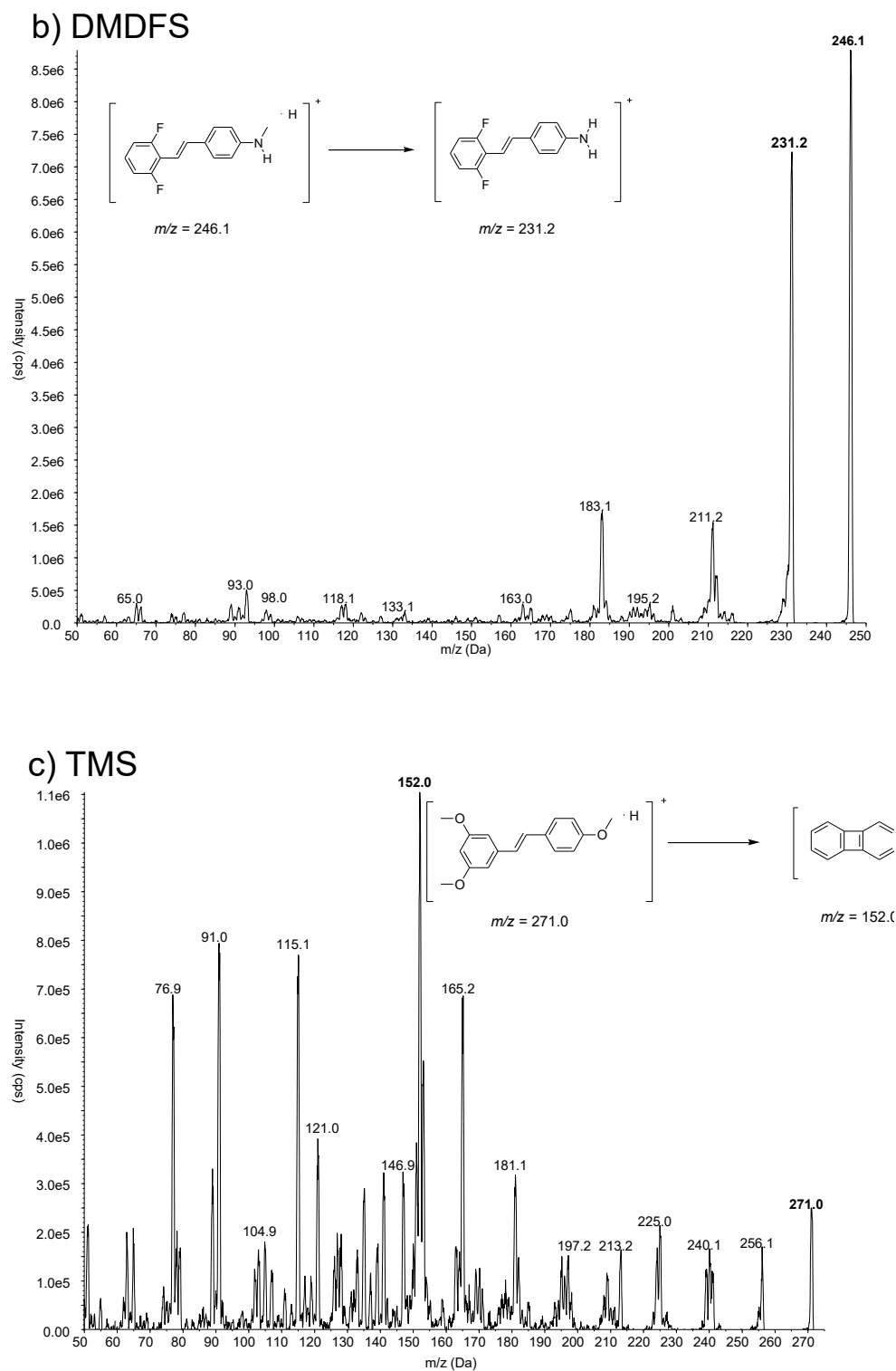


Figure 5.3 MS/MS spectra and possible fragment ion structures of a) DFS, b) DMDFS and c) TMS.

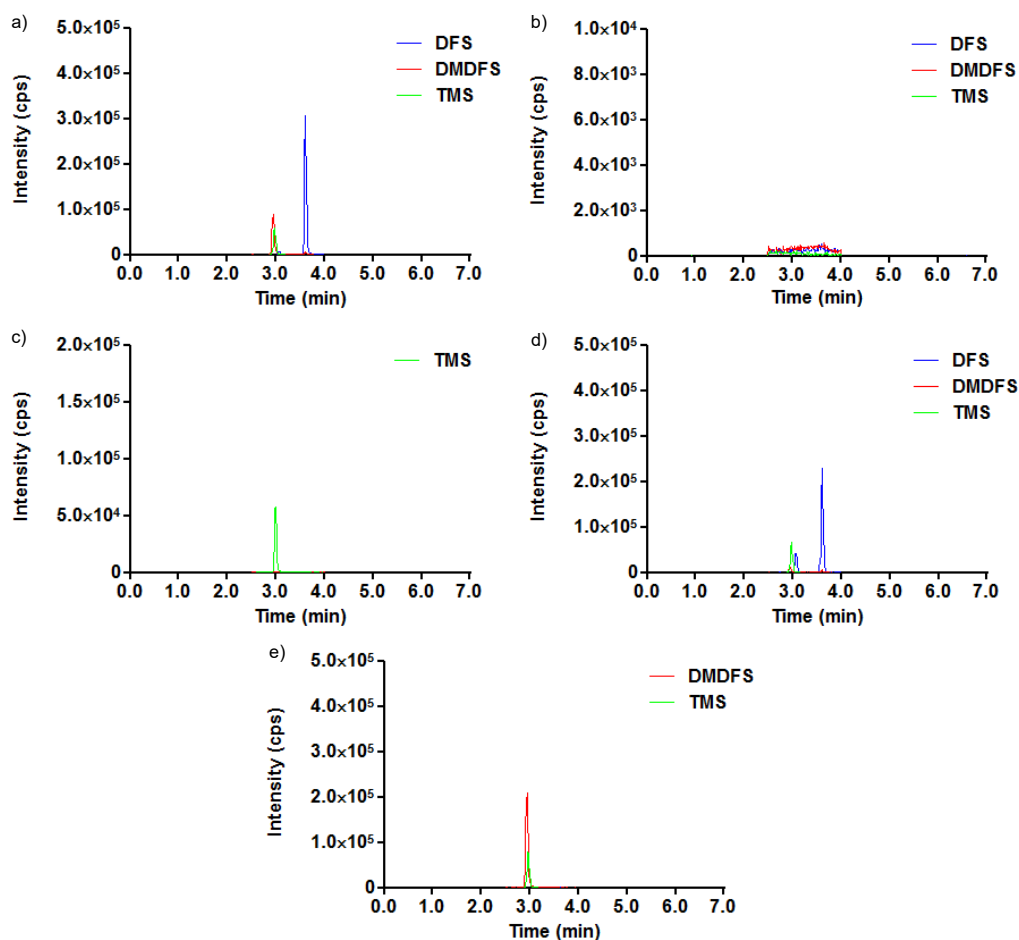


Figure 5.4 LC-MS/MS chromatograms of DFS rat plasma samples a) blank rat plasma spiked with DFS (100 ng/mL), DMDFS (100 ng/mL) and TMS (1 μ g/mL), b) pre-dosing rat plasma sample, c) blank rat plasma spiked with TMS (1 μ g/mL), d) rat plasma collected 90 min after 4 mg/kg intravenous DFS administration, e) rat plasma collected 90 min after 10 mg/kg oral DMDFS administration. DFS (blue), DMDFS (red) and TMS (green) eluted at approximately 3.6 min, 3.0 min and 3.0 min respectively.

Table 5.3 Intra- day and inter-day accuracy (analytical recovery) and precision (CV) of DFS and DMDFS

Parameters	Nominal Concentration (ng/mL)		
	5	250	400
DFS			
<u>Intra-day</u>			
Analytical recovery (%)	102.2 ± 1.5	88.4 ± 2.2	93.0 ± 4.8
CV (%)	1.4	2.5	5.1
<u>Inter-day</u>			
Analytical recovery (%)	103.1 ± 7.8	97.8 ± 2.5	105.7 ± 1.1
CV (%)	7.6	2.6	1.1
DMDFS			
<u>Intra-day</u>			
Analytical recovery (%)	102.5 ± 2.9	91.3 ± 4.5	94.6 ± 6.5
CV (%)	2.9	4.9	6.9
<u>Inter-day</u>			
Analytical recovery (%)	114.5 ± 3.1	97.2 ± 5.7	107.8 ± 4.9
CV (%)	2.7	5.8	4.6

Data is expressed as mean ± SD (n = 5 for intra-day and n = 3 for inter-day assays).

Table 5.4 Stability and absolute recovery of DFS, DMDFS and TMS

Conditions	Nominal Concentration (ng/mL) (% remaining)		
	5	250	400
DFS			
Short term (3 h on ice)	90.1 ± 8.9	88.8 ± 10.0	91.0 ± 4.2
Freeze thaw (3 cycles)	110.0 ± 7.6	101.7 ± 12.5	99.9 ± 3.4
Post preparative (20 h at 4 °C)	89.0 ± 7.8	106.7 ± 8.4	99.3 ± 8.9
Long term (-80°C for 2 months)	100.8 ± 4.1	101.1 ± 5.1	102.3 ± 6.0
Stock solution (3 days at room temperature)			100.2 ± 4.1
Absolute recovery (%)	105.5 ± 5.4	98.4 ± 4.7	105.8 ± 3.2
DMDFS			
Short term (3 h on ice)	92.9 ± 4.7	87.7 ± 10.1	92.6 ± 5.5
Freeze thaw (3 cycles)	102.2 ± 9.3	99.0 ± 11.4	106.6 ± 2.8
Post preparative (20 h at 4 °C)	98.8 ± 9.2	112.1 ± 8.2	101.5 ± 8.8
Long term	95.8 ± 10.5	95.3 ± 3.3	102.4 ± 2.6
Stock solution (3 days at room temperature)			103.7 ± 7.3
Absolute recovery (%)	110.8 ± 7.2	97.0 ± 4.8	94.3 ± 5.5
TMS			
Post preparative (20 h at 4 °C)		104.8 ± 6.0	
Stock solution (3 days at room temperature)		103.7 ± 7.3	
Absolute recovery (%) (n =15)		105.1 ± 5.5	

Data is expressed as mean ± SD (n = 5 unless otherwise stated).

5.3.3 Pharmacokinetics of DFS and its major metabolite DMDFS

The pharmacokinetic profiles of DFS and DMDFS were assessed separately following single bolus intravenous administration (Group 1 and 3) at 4 mg/kg. The intravenous plasma concentration-time profiles of DFS and DMDFS are shown in Figures 5.5 (a) and (b), and the major intravenous parameters are presented in Table 5.5.

The plasma concentrations of DFS and DMDFS declined with a rapid distribution phase followed by a slower elimination phase, which is a bi-exponential decline that corresponded to the two-compartment, first-order open model (Lin et al., 2013). DFS displayed a moderate volume of distribution of the central compartment ($V_c = 3.93 \pm 1.09$ L/kg), clearance ($Cl = 62.7 \pm 23.2$ mL/min/kg), terminal elimination half-life ($t_{1/2} = 299 \pm 73$ min) and mean transit time ($MTT = 123 \pm 14$ min). Similarly, after intravenous administration, DMDFS showed moderate V_c (3.22 ± 0.72 L/kg), Cl (49.9 ± 4.8 mL/min/kg), $t_{1/2}$ (320 ± 27 min) and MTT (116 ± 12 min) that were comparable to DFS (two tailed unpaired t-test: $p > 0.05$).

Upon intravenous administration of DFS, DMDFS was consistently detected as a metabolite (Figure 5.5 (a)). The plasma exposure ratio between metabolite and parent compound (AUC_{DMDFS}/AUC_{DFS}) was 0.102 ± 0.024 while the fraction of DFS metabolized to DMDFS (f_m) after intravenous administration was 0.0919 ± 0.0406 (i.e. approximately 10% of the elimination of DFS after intravenous dosing was attributed to demethylation). Interestingly, the apparent half-life of DMDFS as metabolite ($t_{1/2} = 233 \pm 138$ min) was comparable to the $t_{1/2}$

of DFS or DMDFS after intravenous administration (two tailed unpaired t-test: $p > 0.05$).

Since oral dosing is the most convenient and preferred form of drug administration, solutions of DFS and DMDFS were separately administered at 10 mg/kg, and their oral pharmacokinetics were profiled (Figures 5.5 (c) and (d) and Table 5.6). DFS was absorbed rapidly with a moderate maximal plasma concentration ($C_{\max} = 321 \pm 99$ ng/mL) and time to maximal plasma concentration ($t_{\max} = 30 - 120$ min). Moreover, it had abundant plasma exposure ($AUC_{0 \rightarrow \text{last}} = 67.3 \pm 20.5$ min· μ g/mL) and fairly good oral bioavailability ($F = 42.1 \pm 12.8\%$). Similarly, upon oral administration, DMDFS was quickly absorbed with $t_{\max}$ comparable to DFS (Mann Whitney test: $p > 0.05$). However, its $C_{\max}$ tended to be lower than that of DFS (204 ± 87 ng/mL vs 321 ± 99 ng/mL, two tailed unpaired t-test: $p = 0.0695$) while its $AUC_{0 \rightarrow \text{last}}$ (34.7 ± 7.2 min· μ g/mL vs 67.3 ± 20.5 min· μ g/mL, two tailed unpaired t-test: $p < 0.01$) and F ($18.7 \pm 3.9\%$ vs $42.1 \pm 12.8\%$, two tailed unpaired t-test: $p < 0.01$) were lower than that of DFS.

After oral dosing, DFS was so extensively metabolized to DMDFS (Figure 5.5 (c)) that the plasma exposure of DMDFS following oral administration of DFS was comparable to that after oral administration of DMDFS (29.6 ± 10.3 min· μ g/mL vs 34.7 ± 7.2 min· μ g/mL, $p > 0.05$) (Figure 5.5 (d)). The $AUC_{\text{DMDFS}} / AUC_{\text{DFS}}$ after oral dosing was higher than that after intravenous injection (0.440 ± 0.075 min· μ g/mL vs 0.102 ± 0.024 min· μ g/mL, $p < 0.0001$). This difference in $AUC_{\text{DMDFS}} / AUC_{\text{DFS}}$ between intravenous and oral dosing route can be explained by the

first-pass effect whereby after absorption in the gastrointestinal tract, some of the DFS is converted to DMDFS before it goes into systemic circulation. Therefore, more DFS was metabolized to DMDFS following oral administration, leading to higher level of AUC_{DMDFS} / AUC_{DFS} .

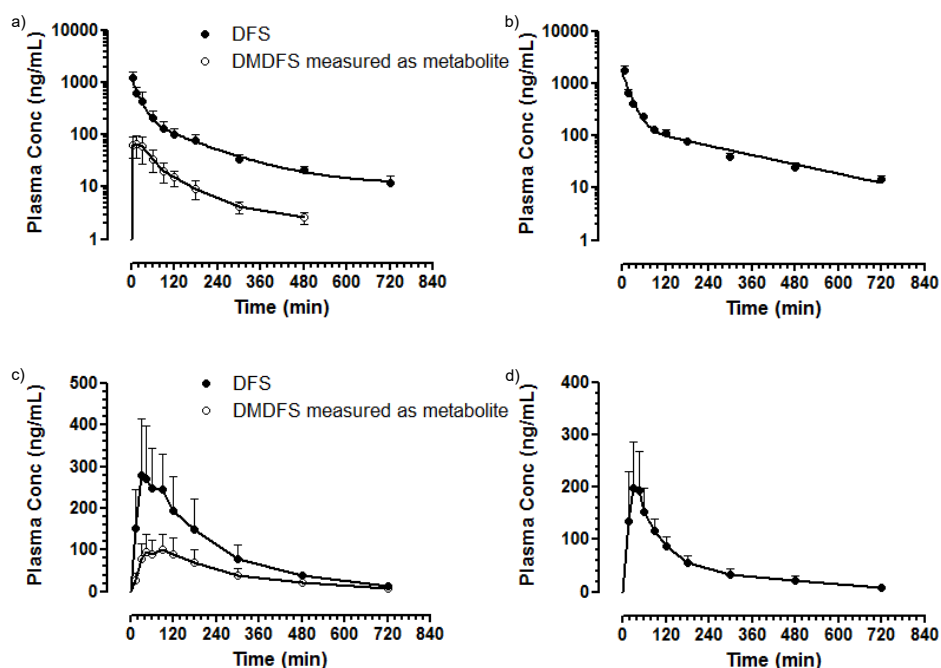


Figure 5.5 Pharmacokinetic profiles of DFS in Sprague Dawley rats after a) intravenous DFS administration (4 mg/kg), b) intravenous DMDFS administration (4 mg/kg), c) oral DFS administration (10 mg/kg) and d) oral DMDFS administration (10 mg/kg). Symbols represent mean plasma concentrations and error bars represent SD (n = 5 except for oral DFS administration where n = 6)

Table 5.5 Intravenous pharmacokinetic parameters of DFS and DMDFS

Compound Parameters	DFS		DMDFS
	DFS	DMDFS measured as metabolite	DMDFS
Dose (mg/kg)	4	-	4
V _c (L/kg)	3.93 ± 1.09	-	3.22 ± 0.72
t _{max} (min)	-	5 – 30	-
C _{max} (ng/mL)	-	72.8 ± 33.1	-
AUC _{0→last} (min·µg/mL)	64.0 ± 19.5	6.75 ± 2.96	74.0 ± 6.8
Cl (mL/min/kg)	62.7 ± 23.2	-	49.9 ± 4.8
MTT (min)	123 ± 14	110 ± 19	116 ± 12
t _{1/2} (min)	299 ± 73	233 ± 138	320 ± 27
AUC _{DMDFS} /AUC _{DFS}	-	0.102 ± 0.024	-
f _m	-	0.0919 ± 0.0406	-

Data expressed as mean ± SD (n = 5).

Table 5.6 Oral pharmacokinetic parameters of DFS and DMDFS

Compound Parameters	DFS		DMDFS
	DFS	DMDFS measured as metabolite	DMDFS
$t_{\max}$ (min)	30 – 120	30 – 180	30 – 45
$C_{\max}$ (ng/mL)	321 ± 99	109 ± 35	204 ± 87
$AUC_{0 \rightarrow \text{last}}$ (min·µg/mL)	67.3 ± 20.5	29.6 ± 10.3	34.7 ± 7.2**
F (%)	42.1 ± 12.8	-	18.7 ± 3.9**
MTT (min)	202 ± 15	227 ± 14	193 ± 32
$t_{1/2 \lambda Z}$ (min)	181 ± 35	183 ± 33	212 ± 29
$AUC_{\text{DMDFS}}/AUC_{\text{DFS}}$	-	0.440 ± 0.075	-

Data is expressed as mean ± SD (DFS, n=6; DMDFS, n=5).

**p < 0.01 between the DFS group and this group.

Two tailed unpaired t-test was used to compare between groups.

5.4 Discussion

In the present study, metabolite identification of DFS utilised a QTrap system capable of conducting IDA experiments such as simultaneous MRM experiments and EPI spectra acquisition for structural elucidation (Xia et al., 2003). Survey scans for IDA experiments include enhanced Q3 single MS (EMS), precursor ion and / or neutral loss (PI / NL) and MRM. Although MRM is a ‘targeted’ approach that may overlook metabolites that are unexpected or have radically different fragmentation patterns (Li et al., 2005a), utilization of their ‘non-targeted’ counterpart, namely EMS and PI/NL, routinely prove inferior in the number of detected metabolites (Huang et al., 2011; Shou et al., 2005). The superior performance of the MRM survey scan results from its high sensitivity and selectivity, which reduces background noise

(Huang et al., 2011). As such, the MRM-IDA-EPI method was applied as a preliminary screen to identify the metabolites of DFS in this study.

Inter-species differences in the metabolite profiles of DFS were investigated using liver microsomes from mice, rats and humans. Discerning the differences was essential since although rodents are widely used in pre-clinical drug testing, variation of expressions and activities of the metabolic enzymes in rodents compared to humans necessitates the study of metabolism of DFS in each species individually before extrapolation of these pre-clinical data to humans (Martignoni et al., 2006). The search for active metabolites was focused on the Phase I biotransformations in liver microsomes since the conjugation reactions in Phase II usually render the molecule less active than metabolites from Phase I transformations (Obach, 2013). N-demethylation and mono-oxidation reactions were the dominant biotransformations of DFS, findings that were consistent with the known major metabolic pathways of drugs with similar functional groups (Obach, 2013).

Prior to further development of the potent novel synthetic stilbene analogues DFS and DMDFS (Zhang et al., 2011a), their pharmacokinetic characteristics will have to be studied in greater detail. Upon intravenous administration, both DFS and DMDFS displayed favourable pharmacokinetic profiles including fairly abundant plasma exposure and moderate Cl , $t_{1/2}$ and MTT. Interestingly, both DFS and DMDFS possessed similar intravenous pharmacokinetic profiles and comparable major pharmacokinetic parameters ($p > 0.05$). However,

their oral pharmacokinetic profiles were different. Although both DFS and DMDFS were absorbed rapidly, the plasma exposure and oral bioavailability of DFS was at least two-fold higher than those of DMDFS ($p < 0.01$). Clearly, the oral pharmacokinetics of DFS was more favourable than those of DMDFS. Moreover, its demethylation metabolism was so extensive that the DMDFS plasma exposure after oral dosing of DFS was comparable to that seen after DMDFS administration ($p > 0.05$).

Aqueous solubility, membrane permeability and metabolic stability are the key determinants of oral bioavailability (Hurst et al., 2007). When solubilized with HP- β -CD, the solubility barrier to the oral bioavailability of DFS and DMDFS were removed. Therefore, the difference in oral bioavailability between DFS and DMDFS could be attributed to their different membrane permeability and/or metabolic stability.

The pharmacokinetic profiles of DFS have also been examined in Sprague Dawley rats with same formulation but at different doses (Lin et al., 2013). Bringing together available data allows the dose-pharmacokinetic relationship to be elucidated. When the intravenous dose was increased from 2 to 4 mg/kg, the major pharmacokinetic parameters including CI, $t_{1/2}$ and MTT remained unchanged (two tailed unpaired t-test: $p > 0.05$). When the oral dose was adjusted from 6 to 10 mg/kg, the dose normalised $C_{\max}$ ($C_{\max}/\text{Dose}$), plasma exposure ($\text{AUC}_{0 \rightarrow \text{last}}/\text{Dose}$) and MTT increased from 14.7 ± 6.3 g/mL, 1.80 ± 1.55 min·g/mL and 88.5 ± 37.8 min to 32.1 ± 9.9 g/mL (two tailed unpaired t-test: $p < 0.01$), 6.73 ± 2.05 min·g/mL (two tailed unpaired t-test: $p <$

0.001) and 202 ± 15 min (two tailed unpaired t-test: $p < 0.001$), respectively, indicating dose-dependent oral pharmacokinetics.

The dose-dependent oral pharmacokinetics of DFS could be rationalised by non-linear first-pass metabolism (saturable first-pass metabolism) (Braddy and Jackson, 2010). At relatively low doses (e.g., 6 mg/kg) where first-pass metabolism was not saturated, the hepatic/intestinal extraction ratio was relatively high. Therefore, an increased percentage of DFS was metabolised before it entered systemic circulation, resulting in a relatively low DFS plasma exposure and oral bioavailability. On the other hand, at relatively high doses (e.g., 10 mg/kg) where the first-pass metabolism became increasingly saturated, the hepatic/intestinal extraction ratio was reduced, leading to relatively higher DFS plasma exposure and oral bioavailability.

5.5 Conclusion

In the present study, the liquid chromatography-quadrupole ion trap system was successfully utilized for the identification of the phase I metabolites of DFS in mouse, rat, and human microsomes. Ten putative metabolites (M1-M10) were identified and several of the most important metabolites were confirmed using authentic standards that were synthesised by unambiguous chemical methods. The metabolic profiles of the three species showed some differences, but the major metabolites were consistent across the different microsomal systems, thereby justifying the use of rodents in pre-clinical studies. DMDFS was identified as the major metabolite in all species.

Both DFS and DMDFS displayed similar intravenous pharmacokinetic profiles, and approximately 10% of DFS clearance was attributed to demethylation. After oral dosing, DFS was rapidly absorbed with a fairly good oral bioavailability. As DFS was extensively converted to DMDFS especially after oral administration, oral dosing of DFS (10 mg/kg) led to a plasma exposure of DMDFS comparable to that seen after direct oral dosing of DMDFS (10 mg/kg). Since DFS displayed favourable pharmacokinetic profiles and formed significant amount of the active metabolite, DMDFS, further investigation into its therapeutic application is warranted.

CHAPTER 6
EFFECTS OF INFLAMMATORY STIMULI ON
CYTOKINE RELEASE IN BEAS-2B, A549
AND PRIMARY HUMAN AIRWAY
EPITHELIAL CELLS

6.1 Introduction

The use of *in vitro* models has been crucial in understanding the pathogenesis of COPD and evaluating the therapeutic potential of new compounds (Krimmer and Oliver, 2011). As COPD is a multifaceted disease, no single model will be adequate to replicate the entire pathology. Lung epithelial cells are commonly employed in COPD inflammatory studies as they are the first point of contact in the lungs for environmental insults such as cigarette smoke and are known to produce inflammatory mediators that drive inflammation (Barnes, 2004; Thorley and Tetley, 2007). Immortalised cell lines including A549 (type II alveolar epithelial cell line derived from human pulmonary adenocarcinoma) and BEAS-2B (adenovirus-12 SV40 hybrid virus transformed human bronchial epithelial cell) are well-characterised models with high reproducibility and ease of use but primary cells remain the most relevant model.

A wide range of stimuli can elicit inflammatory response in airway epithelial cells. For instance, IL-1 β , which is increased in sputum and lungs of COPD patients (Botelho et al., 2011; Sapey et al., 2009), activates a network of pro-inflammatory proteins (Lappalainen et al., 2005; Rosenwasser, 1998). Similarly, CSE, a complex mixture containing thousands of chemicals and reactive compounds such as nicotine, acrolein and polycyclic aromatic hydrocarbons (Lu et al., 2003), is capable of initiating and perpetuating various inflammatory signal cascades (Dye and Adler, 1994).

The production of pro-inflammatory mediators is the converging point for the multiple inflammatory signaling pathways. Many of these mediators such as IL-6 and CXCL8 are secreted into the extracellular environment resulting in propagation of inflammation. IL-6 is a pleiotropic cytokine linked to the systemic manifestations of COPD and is elevated in plasma and exhaled breath condensate of COPD patients (Bucchioni et al., 2003; Debigare et al., 2003; Hageman et al., 2003). Similarly, CXCL8 is a potent neutrophil chemoattractant implicated in lung inflammatory disease and has been widely used a marker for inflammatory status in preclinical studies (Barnes, 2009; Hollander et al., 2007; Pease and Sabroe, 2002; Zhang et al., 2011b).

The aim of this chapter is to establish models of airway epithelial cell inflammation using both cell lines and primary cells that can be used to evaluate the anti-inflammatory effects of stilbene analogues. Inflammatory stimuli relevant to COPD, namely IL-1 β and CSE, will be used to induce inflammation which can be measured by determining the release of IL-6 and/or CXCL8. It is hypothesised that these stimuli will be able to induce increased release of IL-6 and CXCL8 after a 24 h exposure.

6.2 Methods

6.2.1 Subject selection

Primary lung epithelial cells were isolated from the lung samples of healthy non-smokers, healthy smokers and COPD patients. The

characteristics of the subjects used in this study are shown in Table 6.1.

Table 6.1 Patient demographics

Demographic	Value
Age (years)	60 $\pm$ 2.6
Gender (M:F)	3:2
Smoking history (pack years)	37.3 $\pm$ 12.6
FEV ₁ (L)	1.9 $\pm$ 0.7
FEV ₁ % predicted	60.9 $\pm$ 18.8
FVC (L)	3.0 $\pm$ 0.7
FVC % predicted	78.8 $\pm$ 12.3
FEV ₁ : FVC	0.5 $\pm$ 0.1

Data are presented as mean $\pm$ SEM. 1 pack year represents 20 cigarettes per day for 1 year.

6.2.2 Cell culture

BEAS-2B, A549 and primary human airway epithelial cells were cultured as described in Chapter 2.2.6. The cells were grown to approximately 90% confluency in 96 well plates before the media were changed to serum free DMEM supplemented with 2 mM L-glutamine (for A549) or K-SFM (for BEAS-2B) or bronchial epithelial growth medium (BEGM) without hydrocortisone (for primary cells) overnight.

6.2.3 Cell treatment

For CSE stimulation, CSE was prepared as described in Chapter 2.2.7 and diluted using the respective medium to a range of concentrations from 0% to 300%. IL-1 β was diluted in the respective cell culture medium and used at 1 ng/mL. After stimulation with the respective stimuli for 24 h, the cell culture supernatant was collected for

measurement of IL-6 and/or CXCL8 using ELISA. Cell viability was monitored using MTT assay.

6.2.4 Statistics

In order to facilitate statistical comparison, concentrations of cytokines below the lower limit of quantification were designated as 32 pg/mL. Statistical analyses were carried out as described in Chapter 2.2.16.

6.3 Results

6.3.1 Effect of CSE on BEAS-2B airway epithelial cells

Initial experiments were performed to determine the optimal concentrations of stimuli to test the inhibitory effects of stilbene analogues on inflammatory indices. Firstly, experiments were performed using the airway epithelial cell line, BEAS-2B. Low concentrations of CXCL8 were released by BEAS-2B cells under basal conditions (< 31.3 pg/mL) and when these cells were exposed to CSE there was a trend of increased CXCL8 release in a concentration-dependent manner (Figure 6.1 (a)). There was a 16-fold increase in CXCL8 release over baseline in the presence of 200% CSE and 20-fold increase with 250% CSE ($p < 0.05$). However, 300% CSE led to a decrease in release of CXCL8. It was possible that CSE was cytotoxic at high concentrations; therefore, cell viability assays were performed. CSE had little effect on cell viability at concentrations up to 200% CSE, however, at 300% CSE there was an approximately 60% decline in cell viability which was not statistically significant (Figure 6.1 (b)).

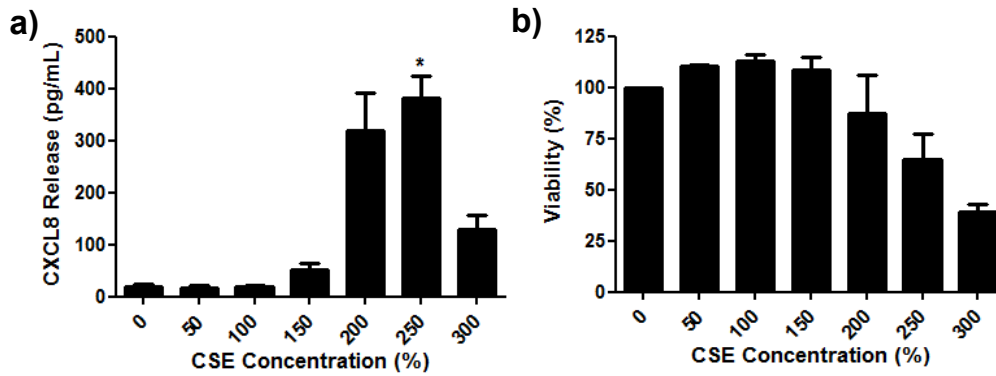


Figure 6.1 Effects of different concentrations of CSE on the release of CXCL8 and cell viability of BEAS-2B cells. BEAS-2B cells were cultured in the presence or absence of increasing concentrations of CSE for 24 h. a) Cell media were harvested and CXCL8 measured by ELISA. b) Viability of cells was measured by MTT assay. Data are mean $\pm$ SEM for $n = 4$ independent experiments where $*p < 0.05$ compared to non-stimulated cells.

6.3.2 Effects of CSE on A549 type II alveolar cells

In A549 cells, the basal level of CXCL8 release was 71.3 ± 24.7 pg/mL. CSE exposure led to a concentration-dependent increase in the release of (Figure 6.2 (a)). The highest level of CXCL8 release occurred at a stimulation of 300% CSE, with a 3-fold increase over baseline. The cytotoxic effect of CSE was reduced in A549 cells compared with BEAS-2B cells with no significant effect on cell viability at concentrations up to 300% CSE (Figure 6.2 (b)).

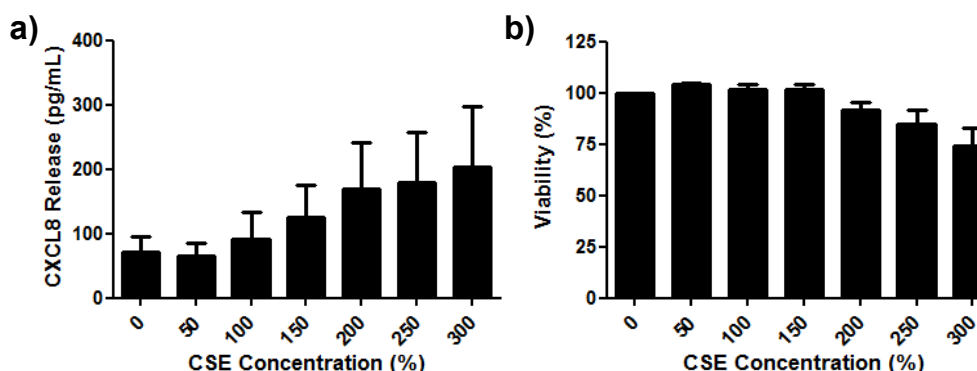


Figure 6.2 Effects of different concentrations of CSE on the release of CXCL8 and cell viability of A549 cells. A549 cells were cultured in the absence or presence of increasing concentrations of CSE for 24 h. a) Cell media were harvested and CXCL8 measured by ELISA. b) Viability of cells was measured by MTT assay. Data are mean $\pm$ SEM for $n = 8$ independent experiments.

6.3.3 Effects of CSE on primary human airway epithelial cells

Similar to A549 cells, CSE also had minimal effect on CXCL8 release in primary human airway epithelial cells (Figure 6.3 (a)). Basal CXCL8 release by these cells (4.7 ± 1.9 ng/mL) increased by approximately 2-fold with 150% CSE (8.6 ± 3.4 ng/mL), but decreased when cells were exposed to 200% CSE. IL-6 release was not significantly altered by CSE exposure too (Figure 6.3 (b)). Similar to A549 cells, these primary cells appeared resistant towards CSE as there was no significant decline in cell viability ($> 85\%$) when the CSE concentration was escalated (Figure 6.3 (c)).

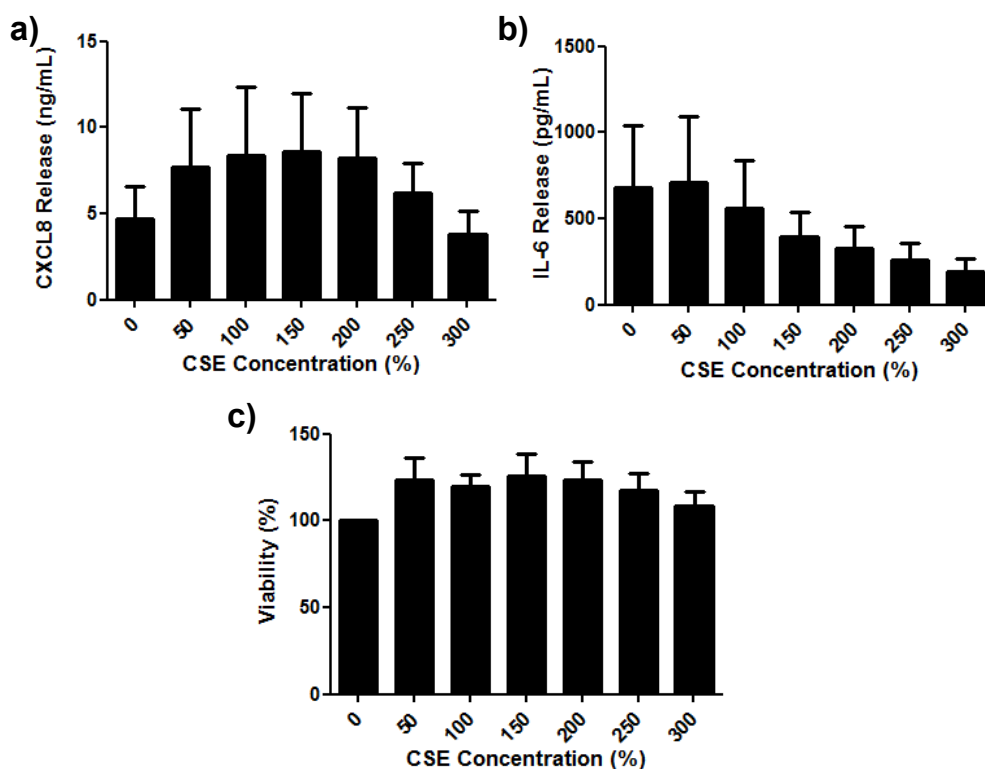


Figure 6.3 Effects of different concentrations of CSE on the release of CXCL8, IL-6 and cell viability of primary human airway epithelial cells. Cells were cultured in the absence or presence of increasing concentrations of CSE for 24 h. Cell media were harvested and a) CXCL8 and b) IL-6 were measured by ELISA. c) Viability of cells was measured by MTT assay. Data are mean $\pm$ SEM for $n = 5$ independent experiments.

6.3.4 Effect of IL-1 β on cytokine release in BEAS-2B, A549 and primary human airway epithelial cells

Having shown that CSE had modest effects on the release of CXCL8 from airway epithelial cells, a second stimulus was considered. IL-1 β is a key mediator that is released from cells including macrophages, under inflammatory conditions. Therefore, the effects of IL-1 β on cytokine release from airway epithelial cells were examined. IL-1 β at 1 ng/mL elicited a significant increase in the release of both IL-6 and CXCL8 ($p < 0.01$) in BEAS-2B cells (IL-6: 2.2 ± 0.3 ng/mL; CXCL8: 3.4

± 1.0 ng/mL respectively) (Figure 6.4 (a)). Similarly, A549 cells released significantly higher amount of IL-6 (3.0 ± 0.8 ng/mL) ($p < 0.01$) and CXCL8 (315 ± 70 ng/mL) ($p < 0.001$) compared to baseline when stimulated by IL-1 β (Figure 6.4 (b)). For both cell lines, IL-1 β had no effect on cell viability.

In primary airway epithelial cells, stimulation with 1 ng/mL IL-1 β brought about approximately 2-fold increase in IL-6 and CXCL8 release (1.8 ± 0.6 ng/mL and 10.1 ± 3.0 ng/mL for IL-6 and CXCL8 respectively), which were not significantly different from baseline (Figure 6.4 (c)). Such subdued effects from IL-1 β stimulation contrasts with that of the transformed cell lines. Cell viability was also not significantly altered by IL-1 β .

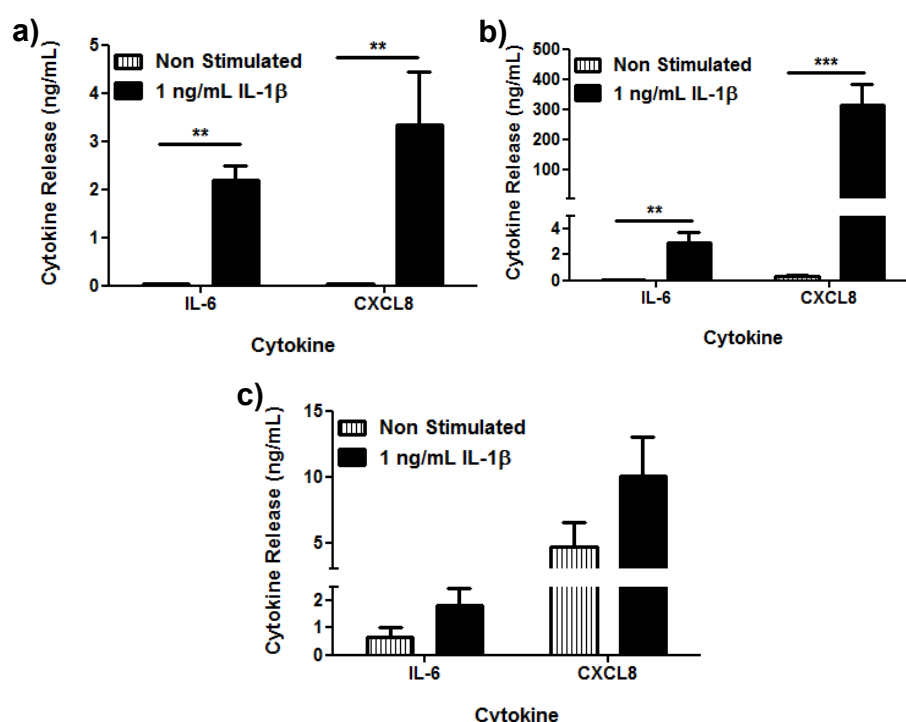


Figure 6.4 Effects of 1 ng/mL IL-1 β on IL-6 and CXCL8 release in a) BEAS-2B cells, b) A549 cells and c) primary human airway epithelial cells. Cells were stimulated with 1 ng/mL IL-1 β for 24 h. Cell media were harvested and CXCL8 and IL-6 were measured by ELISA. Data are mean $\pm$ SEM for $n = 5$ independent experiments where ** $p < 0.01$ and *** $p < 0.001$.

6.3.5 Effect of IL-1 β in combination with CSE on cytokine release in primary human airway epithelial cells

In order to establish a primary cell airway inflammation model with suitable window of cytokine release between the basal and stimulated states, 1 ng/mL IL-1 β and CSE in combination were examined..

The maximum CXCL8 output was from a combination of 150% CSE and 1 ng/mL IL-1 β where it was approximately 3.5-fold compared to baseline (Figure 6.5 (a)). A similar trend was observed for IL-6 release where the maximum release which occurred at 50% CSE was approximately 3.5-fold higher than baseline (Figure 6.5 (b)), but this then decreased back to baseline with increasing CSE concentration.

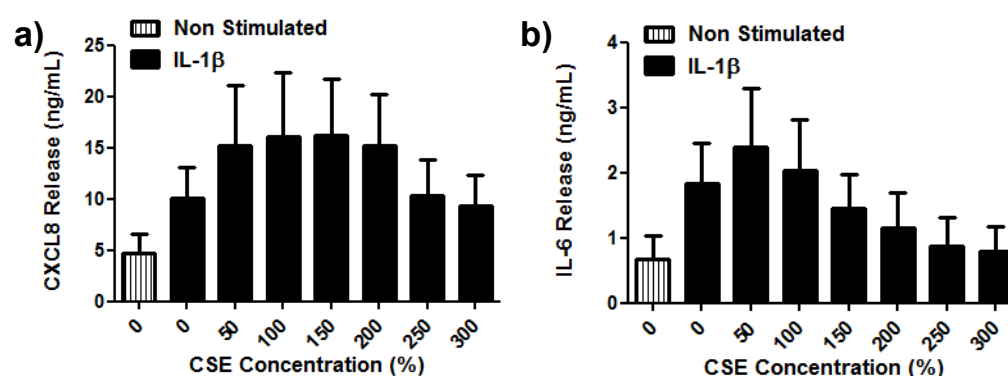


Figure 6.5 Effects of 1 ng/mL IL-1 β in combination with different concentrations of CSE on CXCL8 and IL-6 release in primary human airway epithelial cells. Cells were stimulated with 1 ng/mL IL-1 β with different concentrations of CSE for 24 h. Cell media were harvested and a) CXCL8 and b) IL-6 measured by ELISA. Data are mean $\pm$ SEM for n = 5 independent experiments.

6.4 Discussion

CSE was found to have differential effects on the cytokine output of the different cells. BEAS-2B cells appeared to be more sensitive to CSE

compared to A549 cells and primary airway epithelial cells. Over the same concentration range of CSE used, the maximum CXCL8 release was 20-fold over baseline for BEAS-2B while it was less than 2-fold for A549 and primary cells. This contrasting sensitivity was also reflected in cell viability where only BEAS-2B showed reduced viability at the highest concentration of CSE tested. Previous studies have also shown that BEAS-2B cells had increased CXCL8 output in response to CSE stimulation (Hudy et al., 2010; Moon et al., 2013) . One study, however, failed to observe any increase in CXCL8 release (Laan et al., 2004). The use of only a short 15 min CSE exposure in this reported study could explain the disparity in observations.

There has also been conflicting observations in A549 cells. While some studies have reported enhanced CXCL8 release in CSE-stimulated A549 cells (Li et al., 2013; Masubuchi et al., 1998), several others, including the present study, have shown marginal effects on CXCL8 release (Kode et al., 2006; Moodie et al., 2004; Victoni et al., 2014). Likewise, although it has been shown that CSE augmented CXCL8 release in primary bronchial epithelial cells (Hudy et al., 2010; Kode et al., 2006; Mio et al., 1997), these observations are consistent with the absence of clear CXCL8 increase seen in primary type II alveolar epithelial cells (Witherden et al., 2004). It is well-recognised that inconsistencies in observations between various studies of the same cell line stems from the highly diversified protocols used in the preparation and use of CSE (Krimmer and Oliver, 2011). The type and number of cigarettes used and duration of stimulation are some of the

variables which can affect the exposure of cells to the numerous chemicals in CSE. Despite this, CSE is still widely used as a relevant model for cigarette smoke-induced inflammation as its effects correlates well with *in vivo* exposure (Pierrou et al., 2007).

IL-1 β stimulation markedly increased the release of IL-6 and CXCL8 from BEAS-2B and A549 cells at 1 ng/mL, which is consistent with earlier reports (Donnelly et al., 2004; Durham et al., 2013). A study on A549 cell has shown that further increase in concentration of IL-1 β beyond 1 ng/mL did not significantly enhance CXCL8 release (Coulter et al., 1999) . As such, 1 ng/mL IL-1 β was a suitable stimulus for BEAS-2B and A549 cells which provided sufficient stimulation window while not impacting cell viability considerably. On the other hand, the response of primary cells was relatively subdued (Coulter et al., 1999). When CSE was added to IL-1 β to provide additional inflammatory stimulation, the cytokine release window between baseline and stimulated cells was increased.

For these assays measuring the release of inflammatory cytokines, measurement of cytokine levels was only performed 24 h after stimulation as it is known that the levels only increase substantially at approximately 24 h following the processes of transcription and translation (Coulter et al., 1999; Relja et al., 2014).

6.5 Conclusion

Airway inflammatory cell models using BEAS-2B, A549 and primary airway epithelial cells have been established. CSE was a suitable

inflammatory stimulus for BEAS-2B while IL-1 β sufficiently stimulated the release of cytokines from BEAS-2B and A549 cells. For primary cells, a combination of IL-1 β and 50% CSE was required to elicit a sufficient stimulation window for the screening of anti-inflammatory therapeutics.

CHAPTER 7

**EFFECTS OF STILBENE ANALOGUES ON
CYTOKINE RELEASE IN BEAS-2B, A549
AND PRIMARY HUMAN AIRWAY
EPITHELIAL CELLS STIMULATED WITH
DIFFERENT INFLAMMATORY STIMULI**

7.1 Introduction

The anti-inflammatory and anti-oxidative properties of stilbene analogues have been extensively described as listed in Chapter 1.8.2. Many of these studies were focused on the biological effects of resveratrol in inflammatory models. Examples include the inhibition of prostaglandin E₂ (PGE₂) release in human mammary epithelial cells (Subbaramaiah et al., 1998), inhibition of LPS-induced release of TNF- α , IL-6 and iNOS in murine microglia and astrocytes (Lu et al., 2010) and repression of LPS/IFN γ -induced PGE₂ in human peripheral blood leukocytes (Richard et al., 2005). Such effects have been also observed in *in vivo* animal models and human clinical trials (Bo et al., 2013; Csiszar, 2011; Pearson et al., 2008; Timmers et al., 2011). In particular, there has been accumulating evidence of the therapeutic value of resveratrol in airway inflammatory processes. Studies have shown its cytokine inhibitory effects in lung epithelial cells and alveolar macrophages from COPD patients and its radical scavenging effects in CSE-stimulated lung epithelial cells (Culpitt et al., 2003a; Donnelly et al., 2004; Kode et al., 2008). Resveratrol also reduced the production of steroid resistant CXCL8 from TNF- α -stimulated human airway smooth muscle cells (Knobloch et al., 2010). Furthermore, the induction of cytokines in *in vivo* models such as LPS-challenged rats and mice with ovalbumin-induced allergic inflammation were reduced with resveratrol administration (Birrell et al., 2005; Chen et al., 2015).

Although not well-studied, promising stilbene analogues with better anti-inflammatory and anti-oxidative effects have emerged in recent

years. In several screening studies, analogues of resveratrol showed improved effects including inhibition of nitric oxide production in LPS-stimulated murine macrophage and microglial cells, reduction of TNF- α release in LPS-stimulated microglial cells, free radical scavenging in 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and inhibition of CXCL8 and TNF- α in human mononuclear cells stimulated with LPS/IFN- γ (Kondratyuk et al., 2011; Meng et al., 2008; Richard et al., 2005). However, only a limited number of studies have investigated the effects of these analogues in airway inflammation. The positive effect of one analogue, desoxyrhapontigenin, in inhibition of LPS-induced neutrophil infiltration and another analogue, pterostilbene, in suppressing inflammation-related airway remodeling in mice suggest that these analogues may hold promise as alternative anti-inflammatory agents for COPD (Choi et al., 2014; Kuo et al., 2011).

The aim of this chapter is to investigate the anti-inflammatory effects of a number of stilbene analogues (Table 7.1) by measuring their effects on the release of IL-6 and CXCL8 in cellular models of airway inflammation. The transformed cell models established in the earlier chapter will first be used and the more promising compounds will be further evaluated in primary airway epithelial cells. It is hypothesised that some stilbene analogues are more potent than resveratrol in the inhibition of stimulated cytokine release from airway epithelial cells.

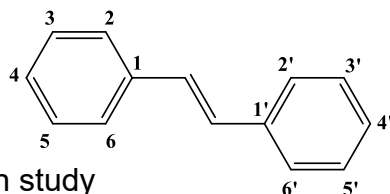


Table 7.1 Substituents in stilbene analogues used in study

Analogue	Substituent (other than H)							
	2	3	4	5	6	2'	3'	4'
Resveratrol	-	OH	-	OH	-	-	-	OH
Pinosylvin	-	OH	-	OH	-	-	-	-
<i>trans</i> -4,4'-dihydroxystilbene (DHS)	-	-	OH	-	-	-	-	OH
Pinostilbene	-	OH	-	OCH ₃	-	-	-	OH
Pterostilbene	-	OCH ₃	-	OCH ₃	-	-	-	OH
<i>trans</i> -3,5,4'-trimethoxystilbene (TMS)	-	OCH ₃	-	OCH ₃	-	-	-	OCH ₃
Oxyresveratrol	-	OH	-	OH	-	OH	-	OH
Piceatannol	-	OH	-	OH	-	-	OH	OH
Rhapontigenin	-	OH	-	OH	-	-	OH	OCH ₃
Isorhapontigenin	-	OH	-	OH	-	-	OCH ₃	OH
<i>trans</i> -2,6-difluoro-4'-(N,N-dimethylamino)stilbene (DFS)	F	-	-	-	F	-	-	N(CH ₃) ₂

7.2 Methods

7.2.1 Subject selection

Primary human airway epithelial cells were isolated from the lung samples of subjects from different groups (Table 7.2).

Table 7.2 Demographics of patients used in study

Demographic	Group	
	Healthy (n = 6)	COPD (n = 8)
Age (years)	68 ± 4	66 ± 3
Gender (M:F)	0:6	4:4
Smoking history (pack years)	0	34.5 ± 5.7
FEV ₁ (L)	2.1 ± 0.2	1.1 ± 0.2
FEV ₁ % predicted	90.7 ± 3.3	53.7 ± 11.8
FVC (L)	2.8 ± 0.2	2.4 ± 0.4
FVC % predicted	102.8 ± 4.3	53.7 ± 11.8
FEV ₁ : FVC	0.77 ± 0.03	0.45 ± 0.06

Data are presented as mean ± SEM. 1 pack year represents 20 cigarettes per day for 1 year.

7.2.2 Cell culture

BEAS-2B, A549 and primary human airway epithelial cells were cultured as described in Chapter 2.2.6. The cells were grown to approximately 90% confluency in 96 well plates before the medium was changed to treatment medium (serum free DMEM supplemented with 2 mM L-glutamine (for A549) or K-SFM (for BEAS-2B) or bronchial epithelial growth medium (BEGM) without hydrocortisone (for primary cells)) overnight.

7.2.3 Cell treatment

Cells were pretreated with the stilbene analogues for 1 h before stimulation. The analogues were first dissolved in DMSO and diluted using the appropriate treatment medium to the desired concentrations. Dexamethasone was used as a control and prepared in the same way. The concentration of DMSO in all compound solutions did not exceed 0.1%. For CSE stimulation, CSE was prepared as described in Chapter 2.2 and diluted down to the stock solution concentration of 2000% (for BEAS-2B cells) and 500% (for primary cells) using the respective media. The final concentrations of CSE used on cells were 200% and 50% for BEAS-2B cells and primary cells respectively. IL-1 β was diluted in the respective cell culture medium or CSE stock solution to a stock concentration of 10 ng/mL (final concentration used on cells = 1 ng/mL). After stimulation with the respective stimuli for 24 h, the cell culture supernatant was collected for measurement of IL-6 and/or CXCL8 using ELISA as described in Chapter 2.2.8. Cell viability was monitored using MTT assay.

7.2.4 Statistics

Comparison between groups and regression were carried out as described in Chapter 2.2.16.

7.3 Results

7.3.1 Effects of stilbene analogues on CSE-stimulated CXCL8 release from BEAS-2B Cells

Initial screening of the anti-inflammatory effects of stilbene analogues was carried out using the CSE-stimulated BEAS-2B inflammation model established in the Chapter 6. In general, most of the stilbene analogues showed concentration-dependent inhibition of CXCL8 release (Figure 7.1) while maintaining cell viability at above 90% compared to untreated cells for concentrations up to 25 μ M. However, DHS enhanced CXCL8 release in the presence of CSE and showed significant cytotoxicity from 10 μ M. Similarly, pterostilbene also caused significant cytotoxicity from 25 μ M and for this reason was only used at concentrations of up to 10 μ M in further experiments. The inhibitory effects of the dehydroxylated resveratrol analogue, pinosylvin, and the fluorinated synthetic analogue, DFS, appeared to be most comparable to resveratrol (Table 7.3). The fully methylated analogue, TMS, on the other hand, tended to be less potent than resveratrol. In contrast, resveratrol analogues with an additional hydroxyl group, oxyresveratrol and piceatannol, showed a trend of increased potency but rhapontigenin, with an additional methylation on the 4'-OH group of piceatannol, showed negligible change in potency compared to piceatannol. Isorhapontigenin, with methylation at the 3'-OH group of piceatannol instead, exhibited significantly better potency compared to resveratrol ($p < 0.01$). Similarly, pinostilbene was significantly more

potent than resveratrol ($p < 0.01$) and was the most potent compound investigated in this study.

Table 7.3 IC₅₀ values of stilbene analogues for inhibition of CSE-stimulated CXCL8 release from BEAS-2B cells

Compound	IC ₅₀ (μM)
Resveratrol	25.2 ± 8.0
trans-2,6-difluoro-4'-(N,N-dimethylamino)stilbene (DFS)	19.5 ± 12.3
trans-4,4'-dihydroxystilbene (DHS)	No inhibition
Isorhapontigenin	2.2 ± 0.9 **
Oxyresveratrol	5.8 ± 2.2
Piceatannol	6.1 ± 3.2
Pinosylvin	18.5 ± 6.7
Pinostilbene	1.2 ± 0.5 **
Pterostilbene	6.7 ± 1.8
trans-3,5,4'-trimethoxystilbene (TMS)	20.4 ± 1.6
Rhapontigenin	6.2 ± 0.7

** $p < 0.01$ compared to resveratrol. Data are mean ± SEM (n = 3-5)

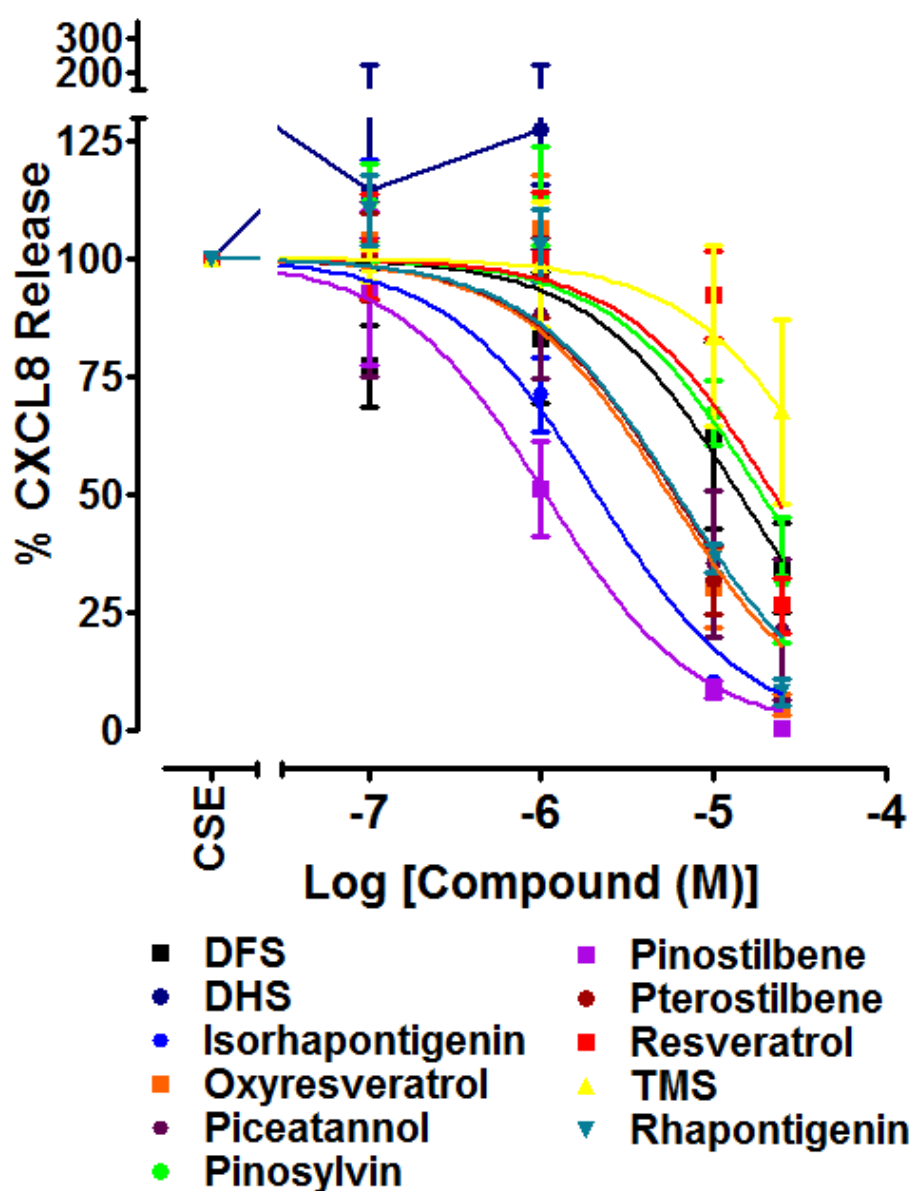


Figure 7.1 Effects of stilbene analogues on CSE-stimulated CXCL8 release from BEAS-2B cells. BEAS-2B cells were treated with various concentrations of stilbene analogues for 1 h before stimulation with 200% CSE for 24 h. Cell media were harvested and CXCL8 measured by ELISA. Data are mean $\pm$ SEM for n = 3-5 independent experiments.

7.3.2 Effects of stilbene analogues on IL-1 β -stimulated IL-6 and CXCL8 release from A549 Cells

Based on the initial screening in BEAS-2B cells and current knowledge of the pharmacokinetic properties of the resveratrol analogues (Chapters 1, 4 and 5), five compounds were selected for further characterisation of their anti-inflammatory effects using A549 cells. These compounds include the top two most potent compounds in the BEAS-2B model, pinostilbene and isorhapontigenin, along with the isomeric compound of isorhapontigenin, rhapontigenin. The other two compounds taken forward were DFS and pterostilbene, both of which showed similar potency as resveratrol but were found to have more favourable pharmacokinetic profiles (Lin et al., 2013; Yeo et al., 2013).

In A549 cells, there was gradual decline in cell viability with increasing concentrations of stilbene analogues. Cell viability was similar for these analogues at each concentration except for pterostilbene which caused a marked decline in cell viability at 100 μ M (Figure 7.2).

Resveratrol showed a concentration-dependent inhibition of IL-6 release with an IC_{50} of 68.6 ± 4.1 μ M. Pinostilbene, an analogue with methylation on one meta-hydroxy group of resveratrol, appeared more potent although statistical significance was not achieved (Table 7.4). The addition of another methylation on the other meta-hydroxy group, as seen in pterostilbene, returned potency to similar level as resveratrol (Figure 7.3 (a); Table 7.4). The fluorinated stilbene analogue, DFS, also showed comparable potency to resveratrol (Figure 7.3 (a); Table 7.4). In contrast, isorhapontigenin, with an additional 3'-OCH₃ group on

the resveratrol structure, was significantly more potent than resveratrol with IC_{50} of $26.6 \pm 2.1 \mu M$ ($p < 0.05$) while its isomeric analogue, rhapontigenin, only showed inhibition at the highest concentration used of $100 \mu M$ (Figure 7.3 (a); Table 7.4).

Having established the effect of selected stilbene analogues on inhibition of IL-6 release, the effect of these compounds on the release of CXCL8 was also investigated. Compared to IL-6, the inhibition of CXCL8 release by resveratrol occurred with a lower potency (Table 7.4). Pterostilbene and rhapontigenin had similar inhibition profiles to resveratrol again (Figure 7.3 (b)). On the other hand, pinostilbene and DFS appeared less potent although it was not statistically significant. Isorhapontigenin was the most potent compound with inhibition profile that tended to be better than the other analogues ((Figure 7.3 (b)); Table 7.4).

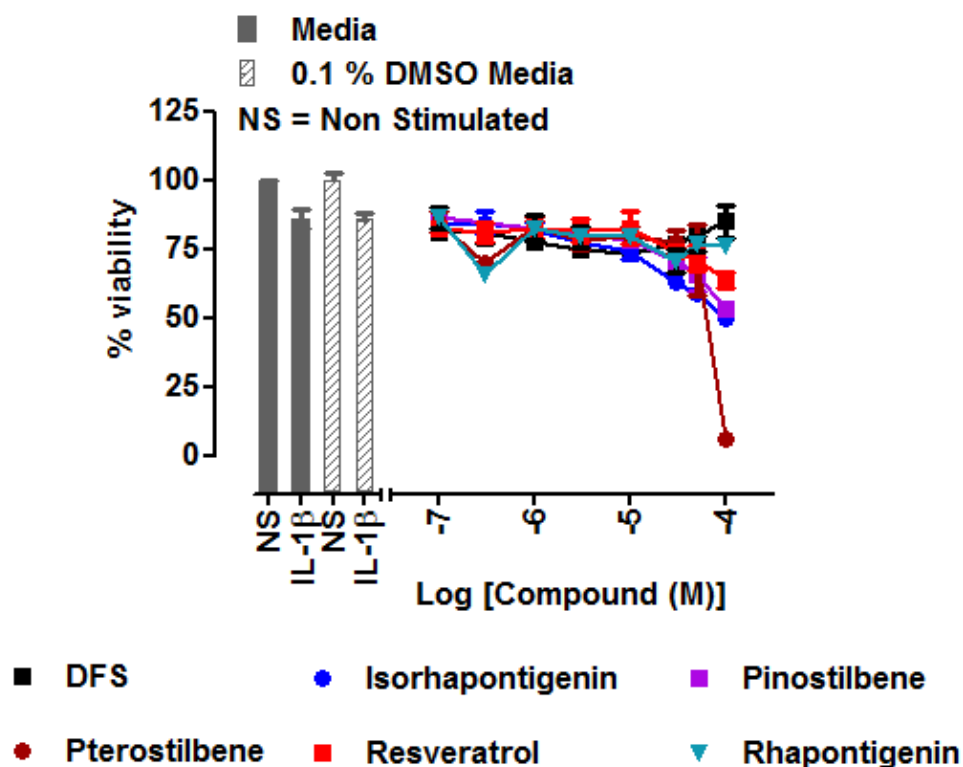


Figure 7.2 Effects of stilbene analogues on cell viability in A549 cells stimulated with 1 ng/mL IL-1 β . A549 cells were treated with various concentrations of stilbene analogues for 1 h before stimulation with 1 ng/mL IL-1 β for 24 h. Control cells were grown in medium or vehicle (0.1 % (w/v) DMSO medium). Cell viability was assessed using MTT assay. Data are mean $\pm$ SEM for n = 4-6 independent experiments.

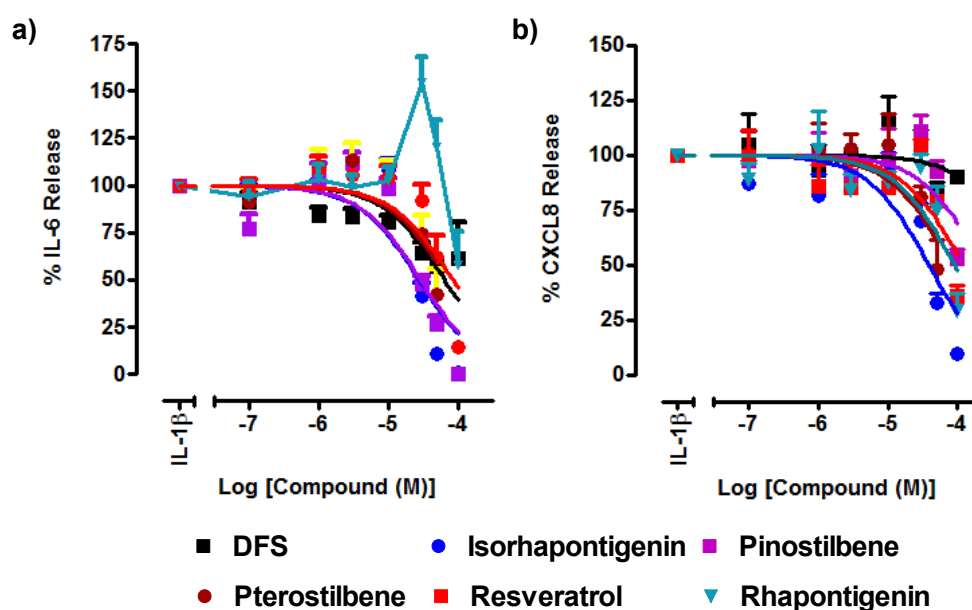


Figure 7.3 Effects of stilbene analogues on cytokine release in IL-1 β stimulated A549 cells. A549 cells were treated with various concentrations of stilbene analogues for 1 h before stimulation with 1 ng/mL IL-1 β for 24 h. Cell media were harvested and a) IL-6 and b) CXCL8 measured by ELISA. Data are mean $\pm$ SEM for $n = 4-6$ independent experiments.

Table 7.4 Estimated IC₅₀ values of stilbene analogues in the inhibition of IL-1 β stimulated IL-6 and CXCL8 release in A549 cells

Compound	IC ₅₀ (μ M)	
	IL-6	CXCL8
Resveratrol	68.6 $\pm$ 4.1	111.5 $\pm$ 13.0
Isorhapontigenin	26.6 $\pm$ 2.1 *	41.3 $\pm$ 6.8
Pinostilbene	27.1 $\pm$ 1.2	227.6 $\pm$ 26.5
Pterostilbene	70.5 $\pm$ 18.6	105.3 $\pm$ 23.9
Rhapontigenin	No inhibition	120.0 $\pm$ 43.8
trans-2,6-difluoro-4'-(N,N-dimethylamino)stilbene (DFS)	118.0 $\pm$ 34.9	499.6 $\pm$ 130.3

*p < 0.05 compared to resveratrol. Data presented as mean $\pm$ SEM ($n = 4-6$).

7.3.3 Effects of stilbene analogues on IL-1 β +CSE stimulated IL-6 and CXCL8 release from primary human airway epithelial cells

Taking into consideration the anti-inflammatory effects of the different stilbene analogues in A549 cells described above, isorhapontigenin and pinostilbene were identified as the most promising analogues. Together with resveratrol as a control, the anti-inflammatory effects of these analogues were investigated using primary human airway epithelial cells. Cells from both healthy subjects and COPD patients were used to assess the effects of these compounds.

IL-6 release from primary cells stimulated with 1 ng/mL IL-1 β and 50% CSE was inhibited by all three compounds in a concentration-dependent manner (Figures 7.4 (a) and (b)). The inhibition profiles of the three compounds appeared similar with isorhapontigenin appearing to be most potent but this was not statistically significant (Table 7.5). Cells derived from different patients did not show any difference in IC₅₀ values (Table 7.5).

CXCL8 release was modulated by the compounds in different ways (Figures 7.4 (c) and (d)). Isorhapontigenin inhibited CXCL8 release from primary cells although its IC₅₀ value appeared to be slightly higher than that for IL-6 release (Table 7.5) with no significant difference between cells from healthy subjects or COPD patients (Figures 7.4 (c) and (d); Table 7.5). This contrasted with the effects of resveratrol and pinostilbene which enhanced the release of CXCL8 in cells from both subject groups.

In order to ensure that cell viability was not contributing significantly to the anti-inflammatory effects observed, MTT assays were performed on the cells after the supernatant was harvested. For isorhapontigenin and resveratrol, concentrations up to 100 μ M did not have significant effect on cell viability ($> 85\%$). However, cell viability decreased to $<60\%$ when pinostilbene was applied at 100 μ M to the cells. As such, these values were excluded from further analysis.

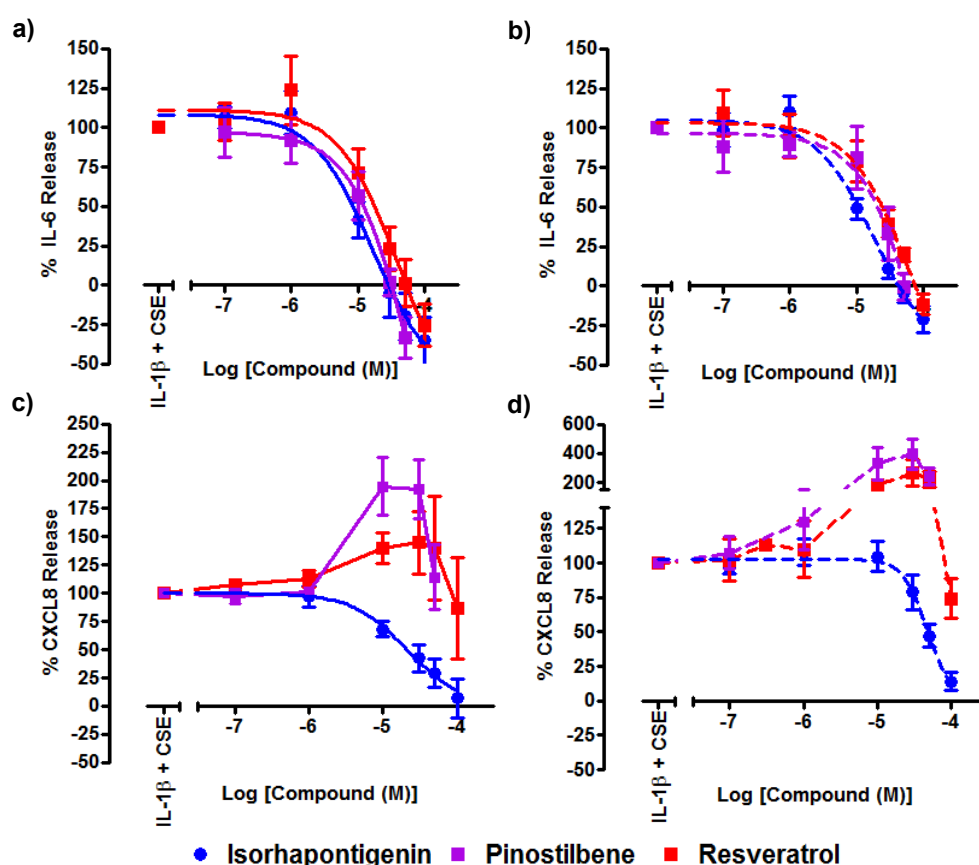


Figure 7.4 Effects of stilbene analogues on cytokine release in primary human airway epithelial cells stimulated by a combination of IL-1 β and CSE. Primary cells from both a,c) healthy and b,d) COPD patients were treated with various concentrations of stilbene analogues for 1 h before stimulation with 1 ng/mL IL-1 β + 50% CSE for 24 h. Cell media were harvested and a,b) IL-6 and c,d) CXCL8 measured by ELISA. Data are mean $\pm$ SEM for $n = 4-8$ independent experiments.

Table 7.5 IC₅₀ values of stilbene analogues in the inhibition of IL-1 β +CSE-stimulated IL-6 and CXCL8 release in primary airway epithelial cells from healthy and COPD patients

Cytokine	Compound	IC ₅₀ (μ M)	
		Healthy	COPD
IL-6	Isorhapontigenin	17.3 $\pm$ 3.4	19.7 $\pm$ 9.1
	Pinostilbene	50.5 $\pm$ 14.2	101.8 $\pm$ 40.2
	Resveratrol	43.5 $\pm$ 17.4	56.5 $\pm$ 23.3
CXCL8	Isorhapontigenin	33.4 $\pm$ 14.3	45.9 $\pm$ 9.4
	Pinostilbene	No inhibition	No inhibition
	Resveratrol	No inhibition	No inhibition

Data presented as mean $\pm$ SEM (n = 4-8)

7.4 Discussion

In this chapter, a series of naturally-occurring stilbene analogues and one synthetic derivative were compared to resveratrol for their cytokine inhibitory effects in human airway epithelial cells. BEAS-2B cells were first used to identify analogues that are more potent. The IL-1 β -stimulated BEAS-2B model was not used in this chapter as preliminary studies found that the stilbene compounds enhanced the release of both IL-6 and CXCL8, which was not congruent with the observations in primary cells. More promising analogues were further verified in A549 cells and the more potent analogues were finally examined in primary airway epithelial cells.

Several studies have investigated the structural activity relationship of stilbene analogues using chemical libraries or commercially available derivatives. These studies sought to identify analogues that are more potent than resveratrol and understand the structural determinants of potency for applications including anti-oxidation, anti-inflammation related activities including cyclooxygenase, NF- κ B and nitric oxide

release inhibition and anti-cancer (Hasiah et al., 2011; Houser et al., 2012; Kang et al., 2009; Kondratyuk et al., 2011; Szekeres et al., 2010). The present study specifically examined the cytokine inhibitory effects of resveratrol and several other stilbene analogues in airway epithelial cells. The comparison of the different analogues provided fresh insights on the important structural features for anti-inflammatory activities in airway inflammation.

In CSE-stimulated BEAS-2B cells, most of the stilbene analogues investigated were at least as potent as resveratrol in inhibiting CXCL8 release. The 4'-OH group in resveratrol does not appear to be critical for inhibitory activity as the dehydroxylated analogue, pinosylvin, retained similar potency compared to resveratrol. This moiety, however, has been shown in some studies to be important for the antioxidant activity of resveratrol (Foti Cuzzola et al., 2011; Stivala et al., 2001), while in another study it was deemed as unfavourable for inhibition of Akt-related androgenic activity (Iguchi et al., 2012). Clearly, the 4'-OH group has varying importance depending on the biological effect examined. In this study, it appears to play a small role in CXCL8 inhibition. Furthermore, DHS which has two para-OH groups enhanced the release of CXCL8 and caused significant cytotoxicity beyond 1 μ M.

Methylation of all the hydroxyl groups did not significantly alter the potency as the fully methylated stilbene analogue, TMS, was as potent as resveratrol. On the other hand, partial methylation of one meta-hydroxyl group significantly enhanced potency as the mono-methylated analogue, pinostilbene, was more potent than resveratrol. Conversely,

additional methylation of the other meta-OH group (pterostilbene) slightly reduced potency and further methylation of the remaining 4'-OH group (TMS) reduced potency back to the same level as resveratrol. Methylation of hydroxyl group increases the lipophilicity of compounds which can help in penetration through the cell membrane. This could partially explain the increased potency of the partially methylated compounds compared to resveratrol. However, when all hydroxyl groups are methylated, the binding of compounds to their targets may be compromised thus resulting in reduced potency.

Stilbene analogues with four substituents tended to be more potent than resveratrol. Oxyresveratrol and piceatannol, which have an additional hydroxyl group at 2' or 3' position respectively compared to resveratrol, were about 3-fold more potent. Similarly, rhapontigenin and isorhapontigenin, which are methylated on the 4'- or 3'-OH moiety of piceatannol respectively, were more potent than resveratrol. It appears that the enhanced potency was not influenced by the position of the additional hydroxyl or methoxy group on the phenol ring of resveratrol.

The 4'-OH group appears to be important for inhibition of IL-6 release by IL-1 β -stimulated A549 cells. This is because rhapontigenin, which is methylated at the 4'-OH group, did not inhibit IL-6 release unlike the other naturally-occurring stilbene analogues. The enhancement of IL-6 release by rhapontigenin could be a result of direct or indirect activation of inflammatory pathways. On the other hand, the OH moiety was less crucial in the inhibition of CXCL8 release. Similar to a previous study using the same A549 model (Donnelly et al., 2004), resveratrol

effectively reduced CXCL8 release with low potency ($111.5 \pm 13.0 \mu\text{M}$ and $72 \pm 11 \mu\text{M}$ in this study and previous study respectively) and the present study further identified isorhapontigenin as a more potent analogue.

In primary human airway epithelial cells, the selected stilbene analogues altered the release of IL-6 and CXCL8 stimulated by a combination of IL-1 β and CSE similarly in both healthy and COPD patients, implying effects through pathways that were not altered by disease state. On the other hand, the stilbene analogues showed slightly reduced potency or complete loss of efficacy in inhibition of CXCL8 release when compared to their effects on IL-6 release. This disparity between inhibition of IL-6 and CXCL8 has also been observed in A549 cells. Such differential effects can be accounted by differences in signalling pathways regulating the release of IL-6 and CXCL8 (Ge et al., 2010).

7.5 Conclusion

The *in vitro* anti-inflammatory effects of stilbene analogues were examined in various airway inflammation cell models. Isorhapontigenin emerged from these assays as the stilbene analogue which consistently demonstrated higher potency than resveratrol in the inhibition of IL-6 and CXCL8 from stimulated cells. Notably, isorhapontigenin maintained this trend of higher potency in primary cells while showing minimal effects on cell viability. Further studies on the anti-inflammatory mechanisms of isorhapontigenin will be conducted in

the next chapter to evaluate if it is a novel anti-inflammatory agent targeting corticosteroid-independent pathways.

CHAPTER 8
MOLECULAR MECHANISMS OF STILBENE
ANALOGUES IN A549 AND PRIMARY
HUMAN AIRWAY EPITHELIAL CELLS

8.1 Introduction

The release of inflammatory cytokines is a multi-step process that is regulated by a complex network of proteins (O'Neill and Greene, 1998; Weber et al., 2010). For example, when IL-1 β binds to the type I IL-1 receptor, a conformational change occurs which recruits the IL-1 receptor accessory protein (Greenfeder et al., 1995). The formation of this complex assembles the myeloid differentiation primary response gene 88 (MyD88) and IL-1 receptor-activated protein kinase (IRAK) 4 through the conserved cytosolic regions known as Toll- and IL-1 receptor-like (TIR) domains (O'Neill, 2000). IRAK4 becomes phosphorylated, leading to phosphorylation of IRAK1, IRAK2 and tumour necrosis factor receptor-associated factor (TRAF) 6. TRAF6 then interacts with NF- κ B inducing kinase (NIK) to activate the NF- κ B pathway through I κ B kinases. Protein kinase phosphorylation is another major event of IL-1 β signalling. Three major MAP kinase cascades are known to be activated by IL-1 β . These are p38, JNK and ERK which becomes activated following phosphorylation of MKK3/6, MKK7 and MEK respectively. In addition, PI3K has also been identified as a downstream effector of IL-1 β signalling (Sizemore et al., 1999). Phosphorylation of these protein kinases induces the activation of transcription factors like NF- κ B and AP-1 that regulate the production of inflammatory cytokines as described in Chapter 1.6.

The signalling pathways elicited by cigarette smoke extract are more convoluted due to the complex nature of the extract and less clearly understood. In bronchial epithelial cells, the stimulation of CXCL8

release by CSE was partly attributed to the activation of TLR4 and TLR9 (Mortaz et al., 2011). Oxidative stress also contributed to this release. In addition, studies in small airway epithelial cells confirmed that CSE induced oxidative stress by reducing the levels of glutathione, an intracellular antioxidant (Kode et al., 2006). CSE also elicited an increase in the nuclear translocation of NF- κ B which was associated with the release of IL-6 and CXCL8 (Kode et al., 2006).

Modulation of these signalling pathways forms the basis of the cytokine inhibitory effects of stilbene analogues reported in Chapter 7. As the best-known analogue, the multi-target nature of resveratrol can be clearly seen from the extensive studies describing its wide array of biological effects. Some examples are its inhibition of MAPKs and the subsequent NF- κ B and AP-1 activation in U937 myeloid and HeLa cells (Manna et al., 2000). In human tenocytes, resveratrol suppressed the activation of PI3K and NF- κ B and this was found to be associated with the activation of the pleiotropic protein deacetylase SIRT 1 (Busch et al., 2012). Specifically in airway epithelial cells, resveratrol inhibited NF- κ B, AP-1 and cAMP-response element-dependent transcription and exerted antioxidant effect by activating Nrf2 to attenuate CSE-induced glutathione depletion and ROS release (Donnelly et al., 2004; Kode et al., 2008). Mechanistic investigations of isorhapontigenin are considerably less exhaustive. In cardiomyocytes, isorhapontigenin inhibited activation of MAPKs, PI3K and the activities of NF- κ B and AP-1 (Li et al., 2005b). It also suppressed the increase in ROS and ERK activation in bovine aortic smooth muscle cells stimulated with oxidized

low density lipoprotein (Liu and Liu, 2004). SIRT1 mRNA was also increased by isorhapontigenin in THP-1 human monocytic cell line (Kawakami et al., 2014).

This chapter aims to examine the mechanism underlying the cytokine inhibitory effects of stilbene analogues observed in the previous chapter. Isorhapontigenin, the most promising analogue identified in Chapter 7, will be the focus of investigation while resveratrol and pinostilbene will be used as controls. Dexamethasone will also be included in the study as a prototypical steroid control to uncover differences between the mechanisms of stilbene analogues and steroid. Firstly, the kinetics of activation of important inflammatory proteins will be characterised in the IL-1 β -stimulated A549 cell model and the effects of compounds will be assessed. Confirmation of effects on important pathways will subsequently be carried out in the primary airway epithelial cell model. It is hypothesised that the stilbene analogues will inhibit activation of multiple inflammatory proteins and show distinct anti-inflammatory mechanisms compared to corticosteroid. Findings from this chapter will enhance our understanding of the mode of action of stilbene analogues in airway epithelial cells which will be crucial in evaluating their therapeutic application as novel anti-inflammatory agents in COPD.

8.2 Methods

8.2.1 Subject selection

Primary human airway epithelial cells were isolated from the lung samples of subjects from different groups and used for the western blot experiments (Table 8.1).

Table 8.1 Demographics of patients used in this study

Demographic	Group		
	Healthy non-smoker (n = 3)	Smoker (n = 3)	COPD (n = 3)
Age (years)	55 ± 9	76 ± 2	70 ± 5
Gender (M:F)	0:3	1:2	2:1
Smoking history (pack years)	0	71 ± 41	72 ± 48
FEV ₁ (L)	2.46 ± 0.02	1.79 ± 0.32	1.33 ± 0.33
FEV ₁ % predicted	91.6 ± 2.3	90.7 ± 14.8	58.3 ± 18.7
FVC (L)	3.12 ± 0.07	1.70 ± 0.07	3.05 ± 0.06
FVC % predicted	99.4 ± 5.8	86.5 ± 8.6	112.0 ± 14.7
FEV ₁ : FVC	0.81 ± 0.03	0.88 ± 0.08	0.54 ± 0.01

Data are presented as mean ± SEM. 1 pack year represents 20 cigarettes per day for 1 year

For the combination treatment assays, cells from a mixture of COPD and healthy patients were used since data obtained in Chapter 7 confirmed that there was no difference in response using cells from different patient groups. Cells from three male COPD patients were used. The mean demographic values for these patients were: age = 70 ± 4 years, smoking history = 94 ± 37 pack years, FEV₁ = 1.38 ± 0.43 L, FEV₁ % predicted = 53.3 ± 18.7%, FVC = 3.14 ± 0.5 L, FVC %

predicted = $90.7 \pm 20.3\%$ and FEV₁: FVC = 0.41 ± 0.10 . In addition, cells from one female non-smoker was also used. The demographics of this patient were: age = 67 years, FEV₁ % predicted and FVC % predicted > 100% and FEV₁: FVC > 0.7.

8.2.2 Cell culture

A549 cells stably transfected with the NF- κ B reporter 6kBtkluc.neo were cultured as described in Chapter 2.2.6. The cells were grown to approximately 90% confluency in white 96 well plates before the medium was changed to serum free DMEM overnight before treatment

A549 and primary human airway epithelial cells were cultured as described in Chapter 2.2. The cells were grown to approximately 80% confluency in 6 well or 12 well plates before the medium was changed to treatment medium (serum free DMEM supplemented with 2 mM L-glutamine (for A549) overnight or BEBM for primary cells for 6 h) before treatment.

8.2.3 Cell treatment

For experiments on A549 cells transfected with 6kBtkluc.neo, cells were pre-treated with the stilbene analogues for 1 h before stimulation. The analogues were first dissolved in DMSO and diluted using treatment medium to the desired concentrations. Dexamethasone was used as a control and prepared in the same way. Concentration of DMSO in all compound solutions did not exceed 0.1%. IL-1 β was diluted in DMEM to a stock concentration of 10 ng/mL (final concentration used on cells = 1 ng/mL). The cells were stimulated with

1 ng/mL IL-1 β for 6 h before cell lysis and luciferase activity were detected as described in Chapter 2.2.13.

For time course experiments investigating the kinetics of protein phosphorylation, cells were stimulated with 1 ng/mL IL-1 β (for A549 cells) or 1 ng/mL IL-1 β + 50% CSE (for primary cells) for the indicated length of time before ice-cold PBS was added to wash the cells and terminate the experiments.

For treatment experiments, cells were pre-treated with the compounds for 1 h before stimulation. The compounds were first dissolved in DMSO and diluted using the appropriate treatment medium to the desired concentrations. Dexamethasone was used as a control and prepared in the same way. Concentration of DMSO in all compound solutions did not exceed 0.1%. IL-1 β was diluted in the respective cell culture medium to a stock concentration of 10 ng/mL (final concentration used on cells = 1 ng/mL). When CSE was used, it was prepared as described in Chapter 2.2.7 and diluted to stock concentration of 500% using BEBM. This solution was further used to dilute IL-1 β to concentration of 10 ng/mL for primary cells stimulation. Cells were stimulated for the indicated length of time before ice-cold PBS was added to wash the cells and terminate the experiments.

To monitor the kinetics of Akt phosphorylation following treatment in A549 cells using stilbene analogues, compound solutions were prepared in treatment medium as described previously. Isorhapontigenin and resveratrol were used at 30 μ M and 100 μ M

respectively. These concentrations showed partial inhibition of Akt activation in previous experiments. LY294002, a specific inhibitor of PI3K, was used as control. Cells were treated for the indicated length of time before ice-cold PBS was added to wash the cells and terminate the experiments.

After washing the cells, protein extraction was carried out as described in Chapter 2.2.9.1 or Chapter 2.2.9.2. Protein quantification was carried out using the method in Chapter 2.2.9.3. Approximately 20 µg of whole cell lysate proteins or 10 µg of nuclear proteins were used for the subsequent gel electrophoresis and western blotting procedures as described in Chapter 2.2.9. RNA was extracted using the method described in Chapter 2.2.15.1 and mRNA levels of c-Jun were quantified using the methods described in Chapter 2.2.15 using the Taqman® gene expression assay.

The effect of stilbene analogues on oxidative stress levels in A549 cells were monitored using the cell permeable redox-sensitive probe, H₂DCF-DA. The compound solutions were prepared as described earlier. Cells were pre-treated using the compound solutions for 1 h prior to addition of 1 ng/mL IL-1β. After 30 min, cells were washed with warm PBS and 10 µM H₂DCF-DA was added to the cells as described in Chapter 2.2.14. Fluorescence of each sample was measured at excitation wavelength of 485 nm and emission wavelength of 520 nm to determine the amount of oxidised product, 2',7'-dichlorodihydrofluorescein, that has accumulated. The background

corrected value of each sample was expressed as a percentage of that of the stimulated cells which were used as controls.

For immunoprecipitation experiments, A549 cells were first treated with vehicle control (0.1% DMSO in serum-free DMEM), 30 μ M isorhapontigenin or 30 μ M resveratrol dissolved in DMSO and diluted using serum free DMEM as described previously for 30 min followed by stimulation using 1 ng/mL IL-1 β as appropriate. After 16 h, SIRT 1 proteins were immunoprecipitated as described in Chapter 2.2.12.

For all cellular experiments, preliminary MTT cell viability experiments were first carried out to ensure that the compound concentrations used did not have significant impact on cell viability (> 90%).

Direct activation of SIRT 1 enzymes by the stilbene analogues was investigated using a fluorometric SIRT 1 enzyme assay as described in Chapter 2.2.11. The stilbene analogues were dissolved in DMSO and diluted to 30 μ M and 100 μ M using assay buffer. Nicotinamide (100 μ M) was prepared similarly and was used as a negative control. The compound solutions were pre-incubated with 1 U SIRT 1 enzymes for 5 min before 25 μ M SIRT 1 substrate and NAD⁺ were added and the reaction was carried out at 37 °C for 45 min.

Investigation of the anti-inflammatory effects of combination treatment using isorhapontigenin and dexamethasone was carried out using the primary airway epithelial cell model described in Chapter 6.3.5. The combination treatment consisted of 5 μ M or 10 μ M isorhapontigenin together with different concentrations (0.1 nM – 10 μ M) of

dexamethasone. Treatment with different concentrations of dexamethasone only was used as control. The concentration of DMSO in all compound solutions did not exceed 0.2%. Cells were pre-treated with the compound solutions for 1 h before stimulation using 1 ng/mL IL-1 β + 50% CSE which was prepared as described in Chapter 6.3.5. After stimulation for 24 h, the cell culture supernatant was collected for measurement of IL-6 and CXCL8 using ELISA as described in Chapter 2.2.8. Cell viability was monitored using MTT assay.

8.2.4 Statistics

All statistical analyses were carried out as described in Chapter 2.2.16.

8.3 Results

8.3.1 Effects of stilbene analogues on the NF- κ B transcription factor pathway

The effects of the stilbene analogues on the NF- κ B pathway were first examined by investigating the activation of I κ B α . When the cells were stimulated with 1 ng/mL IL-1 β , there was an increase in phosphorylation of I κ B α proteins which peaked at 10 min before reducing to basal level and then increasing slightly at 60 min (Figure 8.1). Correspondingly, the total I κ B α levels decreased markedly from 10 min (Figure 8.1). The time point at 10 min was chosen to assess the effects of the compounds on the I κ B α pathway. None of the compounds effected increased phosphorylation of I κ B α even at the highest concentrations used (Figure 8.2 (a)). The total I κ B α protein level that was reduced by IL-1 β stimulation appeared to be increased

slightly at the highest concentration of compounds used (Figure 8.2 (b)). The increase was highest for pinostilbene followed by isorhapontigenin and then dexamethasone and resveratrol. However, the levels were still much lower than cells in the basal state.

As the effects of the compounds on I κ B α phosphorylation were marginal, the nuclear translocation of the major subunits of NF- κ B, p50 and p65, were investigated to determine if there were effects on downstream signalling. IL-1 β elicited a substantial increase in levels of p50 and p65 in the nucleus by 10 min were highly abundant at 60 min (Figure 8.3). The compounds when used at the high concentration of 100 μ M did not appear to have significant effect on this increased nuclear accumulation of p65 or p50 (Figure 8.4 (a) and (b)). Dexamethasone used at 1 μ M was also ineffective at inhibiting this process which is congruent with the observation reported in an earlier study (Newton et al., 1998).

As the stilbene analogues did not appear to inhibit signal transduction processes leading to the nuclear translocation of p65 and p50 subunits, it may be possible that they affected further downstream processes to disrupt NF- κ B activity such as DNA binding. To study the effect of the compounds on NF- κ B activity, A549 cells stably transfected with the NF- κ B luciferase reporter gene, 6kBtkluc.neo, was used. IL-1 β stimulation resulted in approximately 10 fold increase in luciferase expression as measured by the increase in luminescence. Although dexamethasone, the positive steroid control, was highly potent (IC₅₀ = 2.1 ± 0.8 nM), its maximal inhibition was only approximately 60 %

(Figure 8.5 (a)). At the highest concentration (100 μ M) used, isorhapontigenin and pinostilbene inhibited this increase by more than 75 % while resveratrol did not appear to have significant effect (Figure 8.5 (b)). Pinostilbene ($IC_{50} = 49.8 \pm 2.1 \mu$ M) tended to have a higher potency compared to isorhapontigenin ($IC_{50} = 76.2 \pm 5.4 \mu$ M) (Table 8.2).

In summary, isorhapontigenin and pinostilbene were able to reduce the increased NF- κ B activity induced by IL-1 β and this was effected through a pathway independent of I κ B α and the nuclear accumulation of the p50 and p65 subunits.

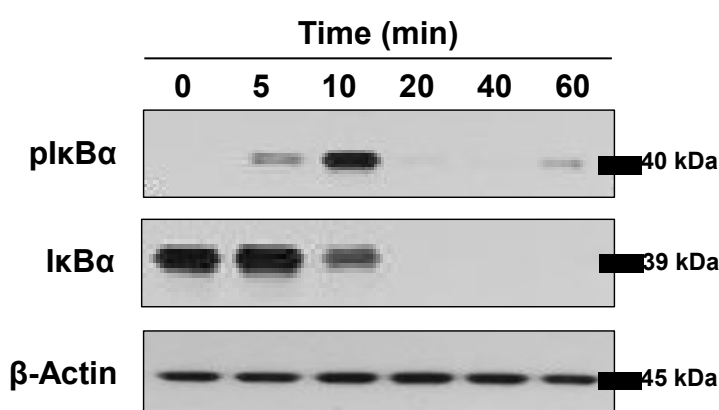


Figure 8.1 Effects of IL-1 β on protein levels of phosphorylated and total I κ B α in A549 cells. Cells were stimulated with 1 ng/mL IL-1 β for the indicated times before lysis to collect cellular proteins that were resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the indicated proteins. β -actin was used as loading control. Blots shown are representative of 3 independent experiments.

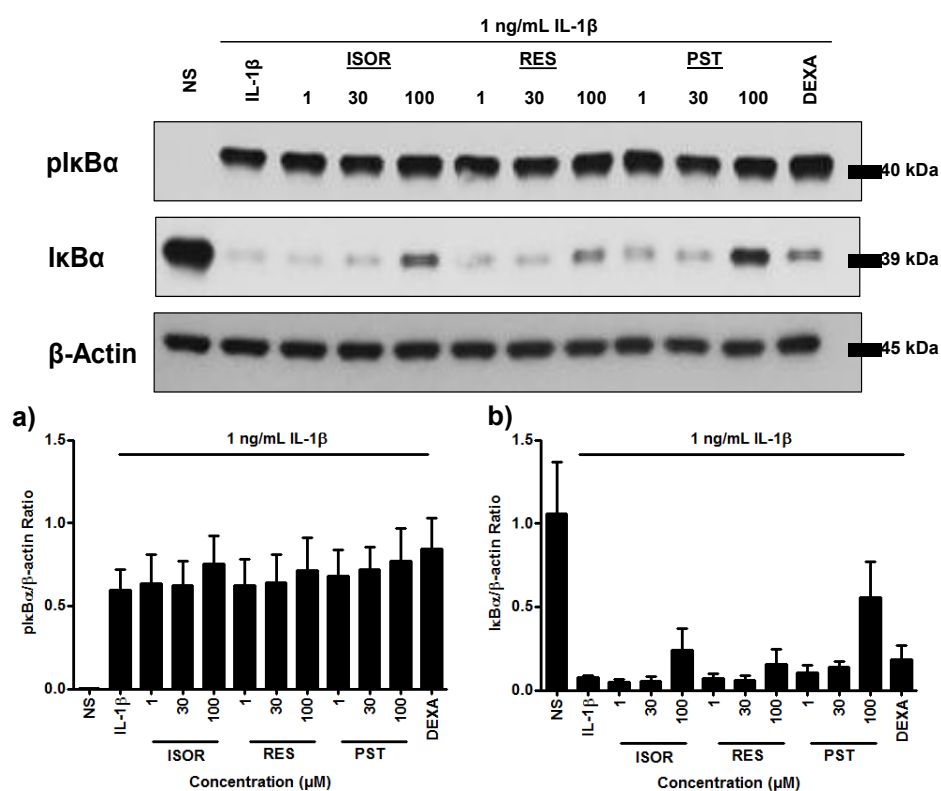


Figure 8.2 Effects of stilbene analogues and dexamethasone on protein levels of a) phosphorylated and b) total IκBα in A549 cells stimulated with IL-1β. Cells were incubated with the indicated concentrations of compounds for 1 h followed by stimulation with 1 ng/mL IL-1β for 10 min before lysis to collect cellular proteins that were resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the indicated proteins (top panel). β-actin was used as loading control. Protein bands were analysed using UVP GelDoc-It imaging system and VisionWorks LS and proteins were normalised to their corresponding β-actin levels (bottom panel). Data are mean ± SEM for n = 3 independent experiments. NS = non-stimulated; ISOR = isorhapontigenin; RES = resveratrol; PST = pinostilbene; DEXA = dexamethasone.

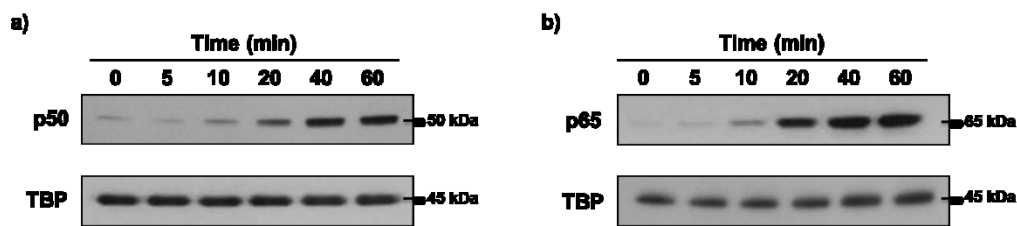


Figure 8.3 Effects of IL-1 β on nuclear protein levels of a) p50 and b) p65 in A549 cells. Cells were stimulated with 1 ng/mL IL-1 β for the indicated times before nuclear proteins were extracted and resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the indicated proteins. TBP was used as loading control. Blots shown are representative of 3 independent experiments.

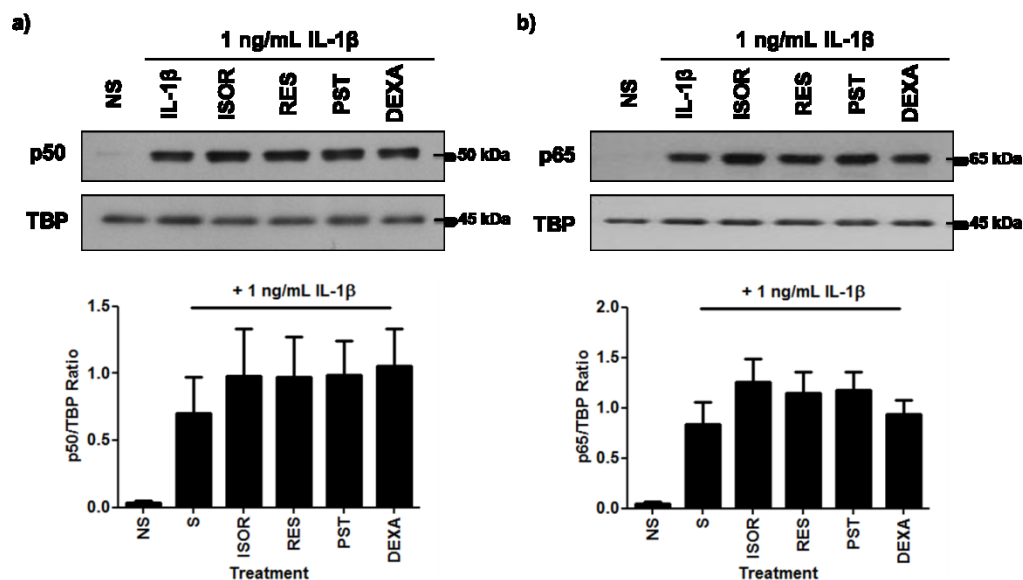


Figure 8.4 Effects of stilbene analogues and dexamethasone on nuclear protein levels of a) p50 and b) p65 in A549 cells stimulated with IL-1 β . Cells were incubated with the indicated concentrations of compounds for 1 h followed by stimulation with 1 ng/mL IL-1 β for 60 min before nuclear proteins were extracted and resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the p50 or p65 (top panel). TBP was used as loading control. Protein bands were analysed using UVP GelDoc-It imaging system and VisionWorks LS and proteins were normalised to their corresponding TBP levels (bottom panel). Data are mean $\pm$ SEM for $n = 3$ independent experiments. NS = non-stimulated; ISOR = isorhapontigenin; RES = resveratrol; PST = pinostilbene; DEXA = dexamethasone.

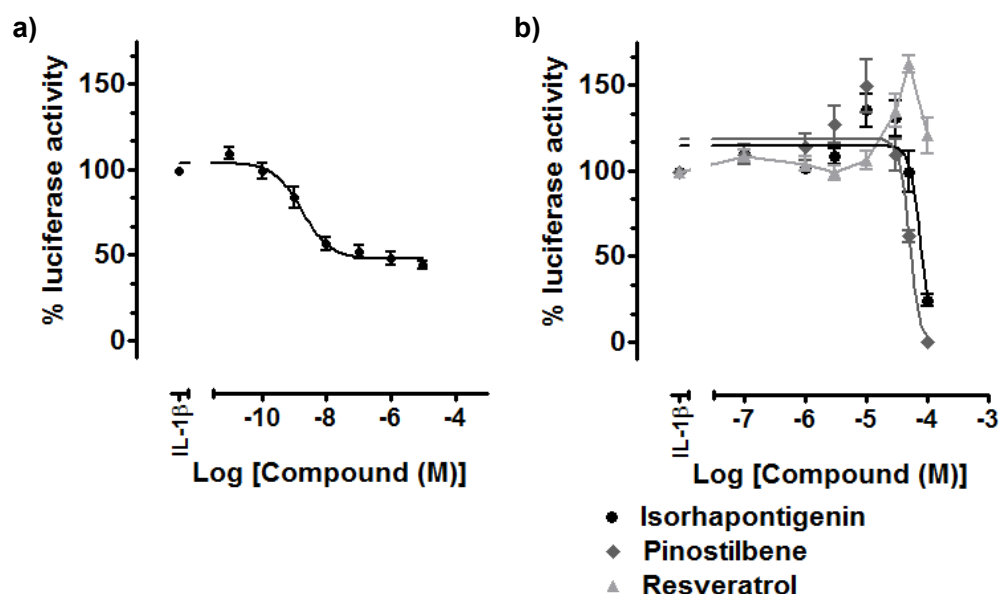


Figure 8.5 Effects of a) dexamethasone and b) stilbene analogues on NF-κB dependent transcription. A549 cells stably transfected with NF-κB reporter gene were incubated with various concentrations of compounds for 1 h before stimulation with 1 ng/mL IL-1β for 6 h. Cells were lysed and luciferase activity was measured. Data are mean ± SEM for n = 4-5 independent experiments.

Table 8.2 Effects of stilbene compounds and dexamethasone on NF-κB reporter gene activity in A549 cells (n = 4-5)

Compound	IC ₅₀
Dexamethasone	2.1 ± 0.8 nM
Isorhapontigenin	76.2 ± 5.4 μM**
Pinostilbene	49.8 ± 2.1 μM
Resveratrol	No inhibition

**p < 0.01 compared to dexamethasone. Kruskal-Wallis test with Dunns post test was used to compare between treatment groups

8.3.2 Effects of stilbene analogues on AP-1 transcription factor pathway

Having observed inhibitory effects on the NF- κ B transcription factor binding, another group of important inflammatory transcription factors, AP-1, was examined. The AP-1 transcription factors are a critical group of inflammatory mediators with rapid induction. To study the effect of the stilbene analogues on this pathway, the protein levels of the major members, c-Jun and c-Fos, were investigated. The total cellular levels of c-Jun decreased slightly after IL-1 β stimulation but increased gradually over time, showing clearly elevated levels from baseline at 60 min (Figure 8.6 (a)). As c-Fos could not be detected in the total cellular extract, its nuclear levels were studied instead. IL-1 β similarly elicited a marked increase in c-Fos at 60 min (Figure 8.6 (b)). As such, 60 min was chosen as the time point to investigate the effects of the compounds.

When A549 cells were treated with stilbene analogues in this model for 1 h before IL-1 β stimulation, the elevation of c-Jun protein levels brought about by IL-1 β stimulation were diminished (Figure 8.7). Isorhapontigenin at the highest concentration (100 μ M) reduced c-Jun protein levels to baseline levels while pinostilbene and resveratrol were less effective. The steroid control, dexamethasone, used at 1 μ M, suppressed c-Jun levels to approximately baseline levels. Since the reduction in protein quantity could be due to alterations at the mRNA or protein levels, c-Jun mRNA levels were studied using qRT-PCR. IL-1 β stimulation increased the expression of c-Jun mRNA significantly to

approximately 3-fold above baseline from 30 min (Kruskal-Wallis test $p < 0.01$) (Figure 8.8 (a)). Isorhapontigenin concentration-dependently repressed this increase, with 100 μM almost completely abrogating the increase (Kruskal-Wallis test: $p < 0.01$) (Figure 8.8 (b)). Similar to the observation at protein levels, resveratrol and pinostilbene appeared less effective than isorhapontigenin. Dexamethasone was weaker at reducing c-Jun mRNA expression compared to its strong effect on c-Jun protein expression, suggesting the involvement of post-transcriptional regulation.

In contrast to their effects on c-Jun, the stilbene analogues enhanced the IL-1 β stimulated increase in amount of nuclear c-Fos levels (Figure 8.9). Dexamethasone brought about a slight reduction in this protein.

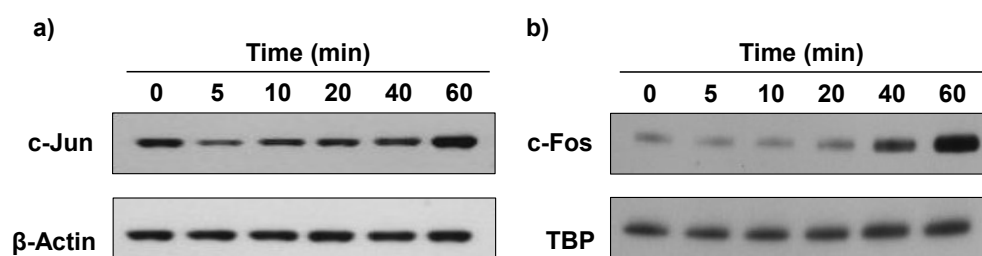


Figure 8.6 Effects of IL-1 β on AP-1 pathway in A549 cells. Cells were stimulated with 1 ng/mL IL-1 β for the indicated times before total cellular proteins (c-Jun) or nuclear proteins (c-Fos) were extracted and resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the a) c-Jun or b) c-Fos proteins. β -actin (c-Jun) or TBP (c-Fos) was used as loading control. Blots shown are representative of 3 independent experiments.

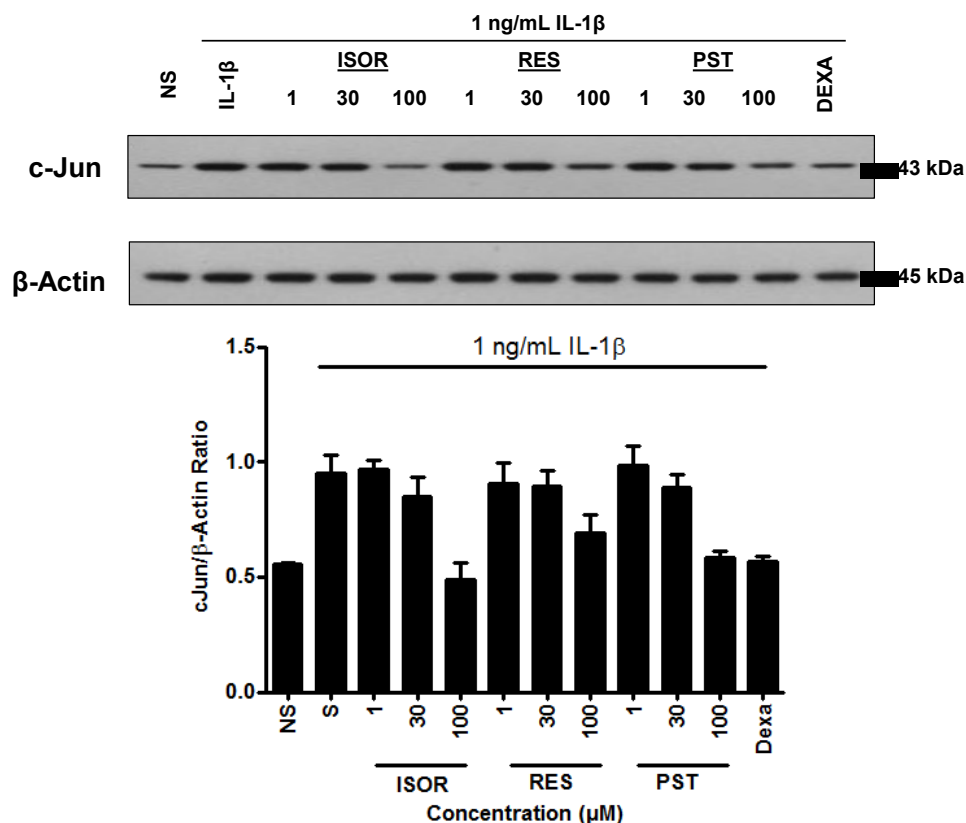


Figure 8.7 Effects of stilbene analogues and dexamethasone on levels of c-Jun protein in A549 cells stimulated with IL-1 β . Cells were incubated with the indicated concentrations of compounds for 1 h followed by stimulation with 1 ng/mL IL-1 β for 60 min before cells were lysed and proteins extracted and resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the c-Jun (top panel). β -actin was used as loading control. Protein bands were analysed using UVP GelDoc-It imaging system and VisionWorks LS and c-Jun proteins were normalised to their corresponding β -actin levels (bottom panel). Data are mean $\pm$ SEM for n = 3 independent experiments. NS = non-stimulated; ISOR = isorhapontigenin; RES = resveratrol; PST = pinostilbene; DEXA = dexamethasone.

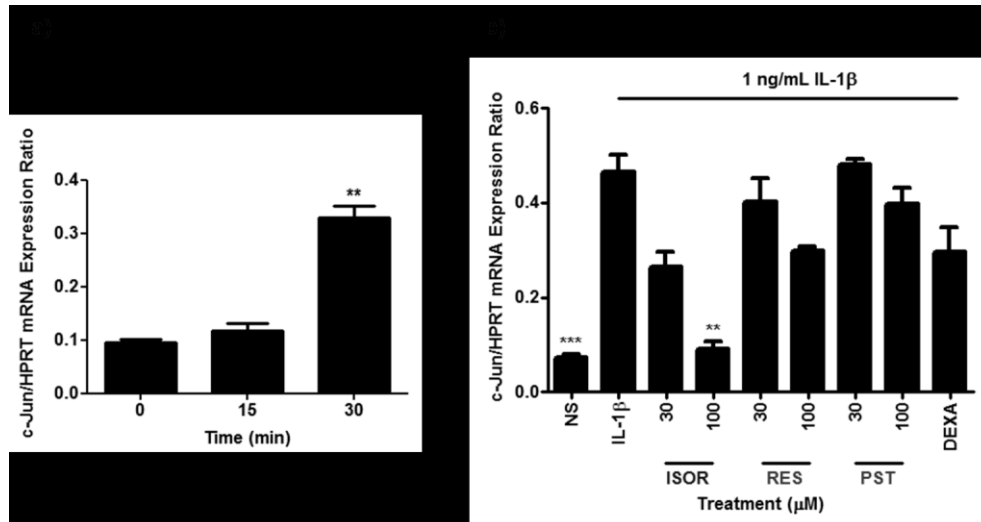


Figure 8.8 Effects of IL-1 β and compounds on c-Jun mRNA levels in A549 cells. Cells were a) stimulated with 1 ng/mL IL-1 β for the indicated times ($n = 4$) or b) treated with compounds for 1 h prior to 1 ng/mL IL-1 β stimulation for 30 min ($n = 5$). After appropriate treatment, RNA was extracted, reverse transcribed to cDNA and c-Jun levels were quantified using TaqMan® qPCR. Data are presented as mRNA expression normalised to housekeeping gene HPRT1 expression. Data are mean $\pm$ SEM. ** $p < 0.01$; *** $p < 0.001$ compared to a) baseline or b) IL-1 β stimulated cells. Kruskal-Wallis test with Dunns post test was used to compare between treatment groups. NS = non-stimulated; ISOR = isorhapontigenin; RES = resveratrol; PST = pinostilbene; DEXA = dexamethasone.

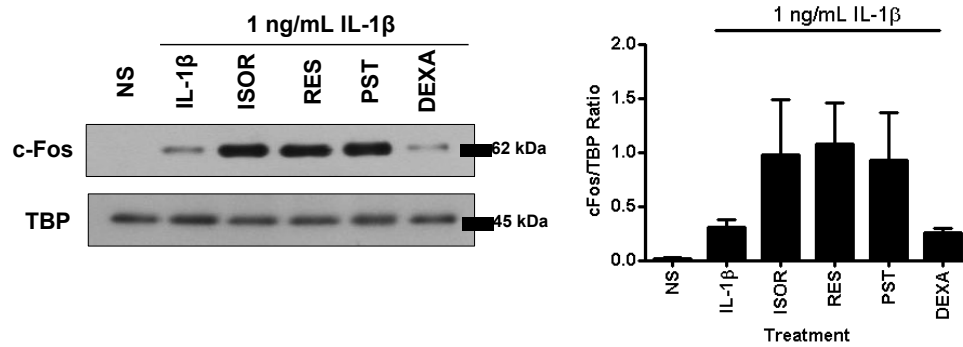


Figure 8.9 Effects of stilbene analogues and dexamethasone on levels of c-Fos nuclear protein in A549 cells stimulated with IL-1 β . Cells were incubated with 100 μ M of stilbene analogues or 1 μ M of DEXA for 1 h followed by stimulation with 1 ng/mL IL-1 β for 60 min before cells were lysed and nuclear proteins extracted and resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the c-Fos (left panel). TBP was used as loading control. Protein bands were analysed using UVP GelDoc-It imaging system and VisionWorks LS and proteins were normalised to their corresponding TBP levels (right panel). Data are mean $\pm$ SEM for $n = 3$ independent experiments. NS = non-stimulated; ISOR = isorhapontigenin; RES = resveratrol; PST = pinostilbene; DEXA = dexamethasone.

8.3.3 Effects of stilbene analogues on MAPK pathway

Since various transcription factors were modulated by the stilbene analogues, it is possible that important upstream inflammatory pathways such as the MAPKs were involved. When stimulated by IL-1 β , the increase in phosphorylation of major MAPK proteins (p38, JNK and ERK) became apparent at 20 min and this was sustained (for ERK) or further enhanced (for p38 and JNK) at 40 min before tapering down at 60 min (Figure 8.10). Levels of total proteins remained similar at all time points. As such, the time point of 40 min was chosen to explore the effects of stilbene analogues on this pathway.

It was found that the stilbene analogues activated the MAPK pathways generally. Pinostilbene activated this pathway to the largest extent while isorhapontigenin had the least effect (Figure 8.11). On the other hand, dexamethasone inhibited the activation of all three proteins. These compounds had negligible effects on the levels of total proteins.

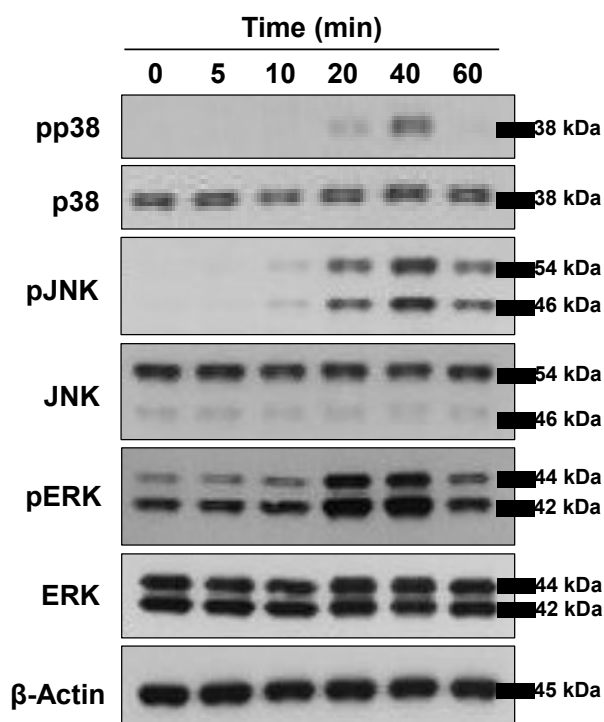


Figure 8.10 Effects of IL-1 β on protein levels of phosphorylated and total MAPK proteins in A549 cells. Cells were stimulated with 1 ng/mL IL-1 β for the indicated times before lysis to collect cellular proteins that were resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the indicated proteins. β -actin was used as loading control. Blots shown are representative of 3 independent experiments.

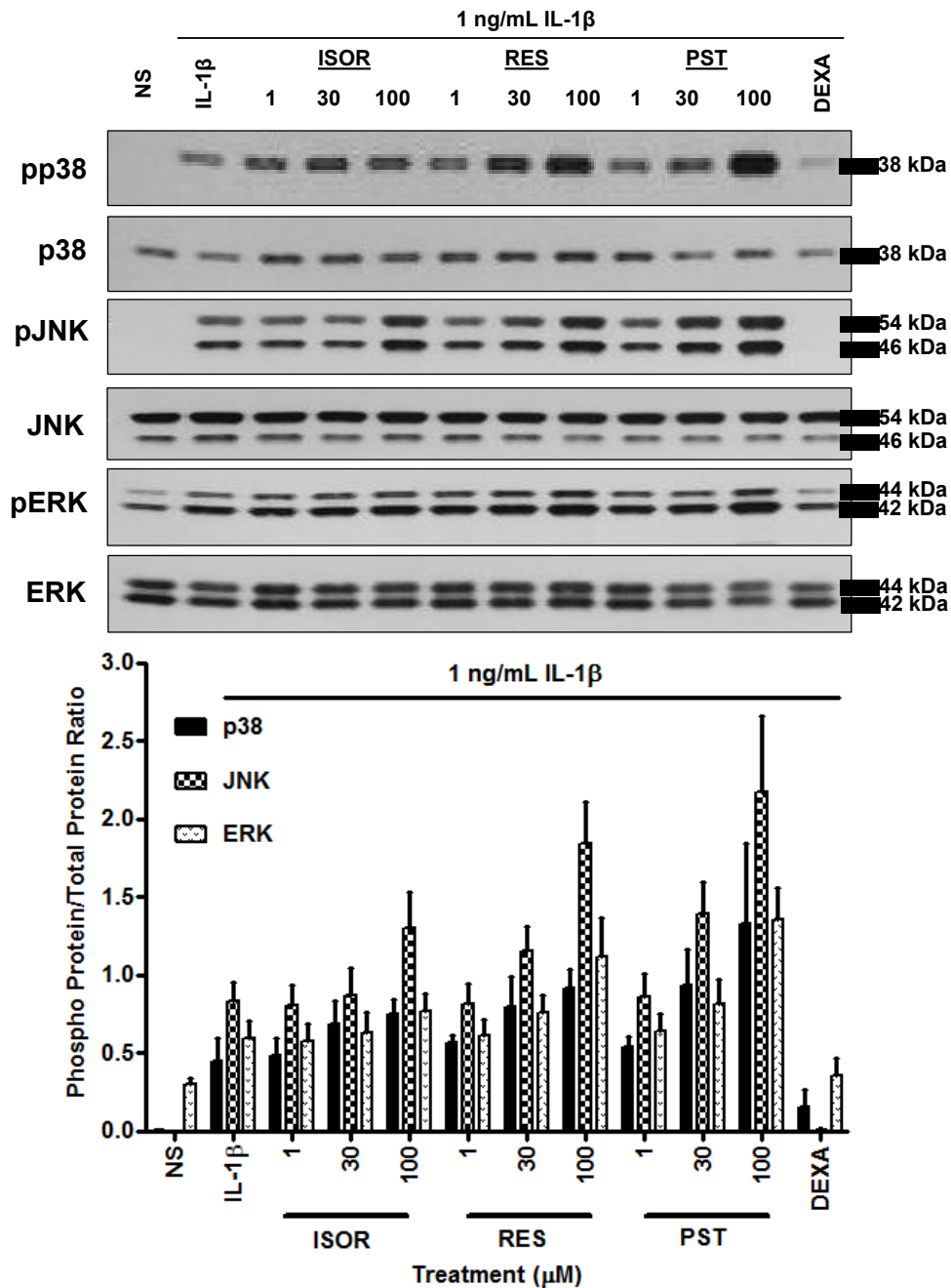


Figure 8.11 Effects of stilbene analogues and dexamethasone on protein levels of phosphorylated and total MAPK proteins in A549 cells stimulated with IL-1 β . Cells were incubated with the indicated concentrations of compounds or 1 μ M DEXA for 1 h followed by stimulation with 1 ng/mL IL-1 β for 1 h before cells were lysed and proteins extracted and resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the indicated proteins (top panel). Protein bands were analysed using UVP GelDoc-It imaging system and VisionWorks LS and phosphorylated proteins were normalised to their corresponding total protein levels (bottom panel). Data are mean $\pm$ SEM for $n = 3$ independent experiments. NS = non-stimulated; ISOR = isorhapontigenin; RES = resveratrol; PST = pinostilbene; DEXA = dexamethasone.

8.3.4 Effects of stilbene analogues on Akt pathway

The PI3k/Akt pathway is another key mediator of inflammation. When A549 cells were stimulated with IL-1 β , the phosphorylation of Akt at both Ser473 and Thr308 increased evidently from 20 min without any changes in total Akt levels (Figure 8.12). The time point at 40 min was chosen to study the effects of the stilbene analogues on this pathway. When treated with the stilbene compounds for 1 h before IL-1 β stimulation, the increased phosphorylation of Akt at both sites were reduced in similar manner without any effect on total Akt levels (Figure 8.13). Isorhapontigenin was clearly the most effective analogue. At 30 μ M, the activation of Akt was reduced to nearly baseline levels while 100 μ M of isorhapontigenin further decreased activation to below baseline levels. Resveratrol and pinostilbene only fully arrested the increased phosphorylation of Akt at 100 μ M. Dexamethasone, on the other hand, when used at the same concentration (1 μ M) which potently reduced MAPK activation, was only marginally effective at suppressing the activation of Akt. This shows that the stilbene analogues and dexamethasone were having distinct effects on inflammatory signalling pathways.

To further understand the nature of Akt inhibition by the stilbene analogues, phosphorylation of Akt at both the Ser473 and Thr308 sites were analysed over 60 min after A549 cells were treated with 30 μ M of isorhapontigenin or 100 μ M of resveratrol. The phosphorylation of Akt was rapidly reduced at 3 min (Figure 8.14 (a) and (b)), similar to the PI3K inhibitor LY294002 (5 μ M) (Figure 8. 14 (c)). This suggested that

the mechanism of stilbene analogues likely involved direct effects on the PI3K/Akt pathway.

In order to determine if the c-Jun inhibition described earlier was associated with Akt inhibition by the stilbene analogues, A549 cells were treated with LY294002 prior to IL-1 β stimulation. Even at the highest concentration (30 μ M) that markedly suppressed the activation of Akt (Ser473), LY294002 did not reduce c-Jun levels significantly (Figure 8.15). This indicates that the stilbene analogues were lowering c-Jun levels through pathways independent of their effects on the PI3K/Akt pathway.

FoxO3A is a well-known downstream effector of PI3K/Akt signalling involved in the release of inflammatory cytokines in airway epithelial cells (Ganesan et al., 2013). To establish that the anti-inflammatory effects of Akt inhibition was partly mediated through FoxO3A in the inflammation model used in this study, nuclear FoxO3A levels were examined (Figure 8.16). IL-1 β reduced nuclear levels of FoxO3A, which is known to be a result of nuclear export following phosphorylation by Akt. As expected, the stilbene analogues attenuated this reduction by IL-1 β and in consonance with their effects on Akt, isorhapontigenin was the most effective stilbene analogue. The involvement of PI3K/Akt signalling was further confirmed by the ability of LY294002 to reverse the effects of IL-1 β . Dexamethasone, on the other hand, only slightly inhibited the IL-1 β -induced reduction in nuclear FoxO3A, which was reflective of its weak effect on the PI3K/Akt pathway discussed earlier.

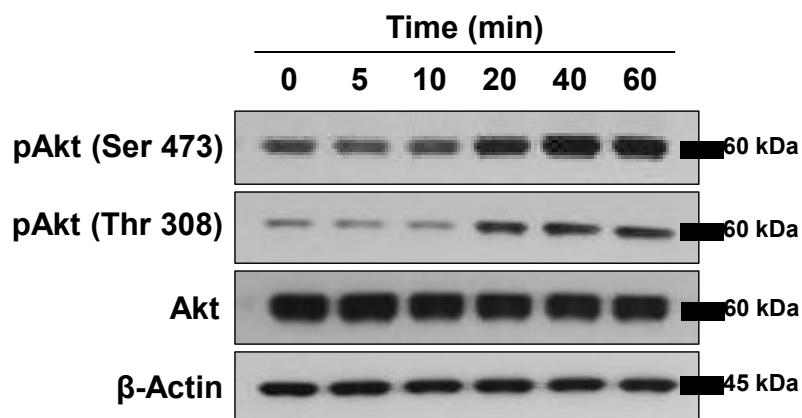


Figure 8.12 Effects of IL-1 β on protein levels of phosphorylated and total Akt proteins in A549 cells. Cells were stimulated with 1 ng/mL IL-1 β for the indicated times before lysis to collect cellular proteins that were resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the indicated proteins. β -actin was used as loading control. Blots shown are representative of 3 independent experiments.

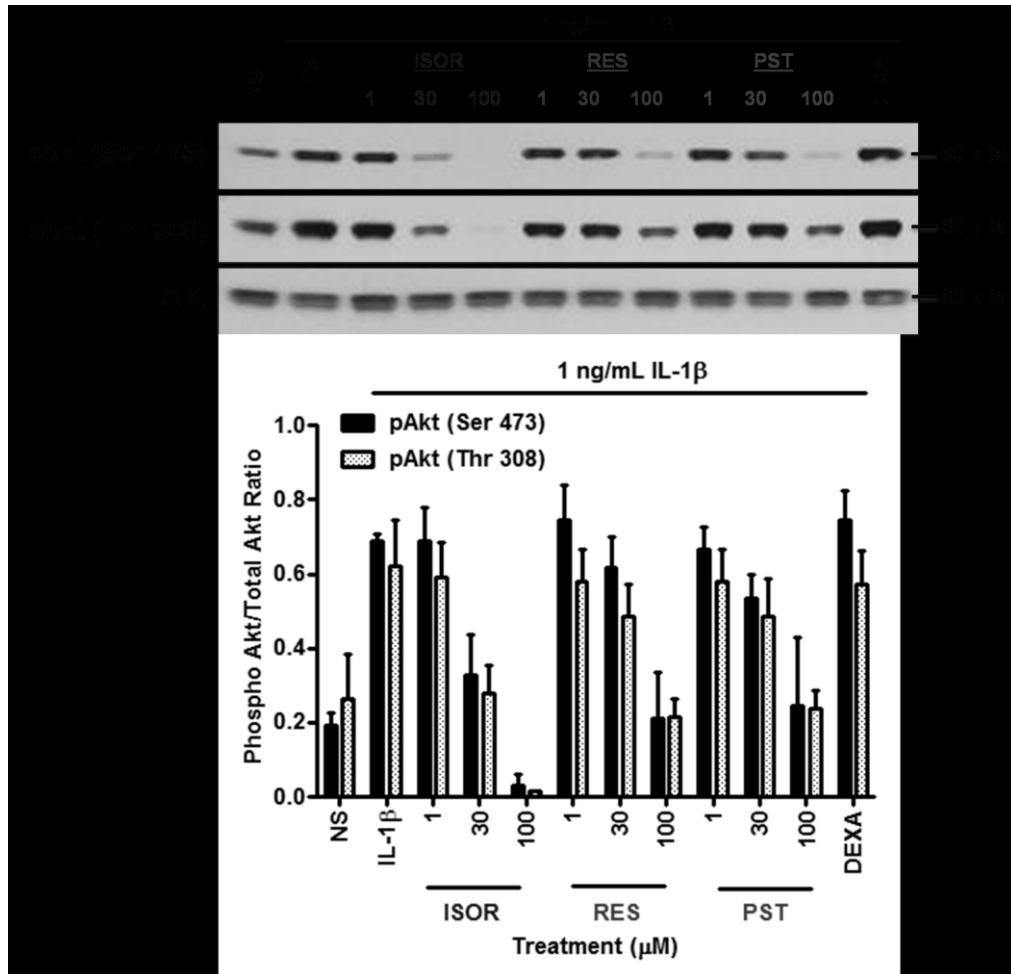


Figure 8.13 Effects of stilbene analogues and dexamethasone on levels of protein levels of phosphorylated and total Akt proteins in A549 cells stimulated with IL-1 β . Cells were incubated with the indicated concentrations of compounds or 1 μ M DEXA for 1 h followed by stimulation with 1 ng/mL IL-1 β for 40 min before cells were lysed and proteins extracted and resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the indicated proteins (top panel). Protein bands were analysed using UVP GelDoc-It imaging system and VisionWorks LS and phosphorylated Akt were normalised to the corresponding Akt protein levels (bottom panel). Data are mean $\pm$ SEM for $n = 3$ independent experiments. NS = non-stimulated; ISOR = isorhapontigenin; RES = resveratrol; PST = pinostilbene; DEXA = dexamethasone.

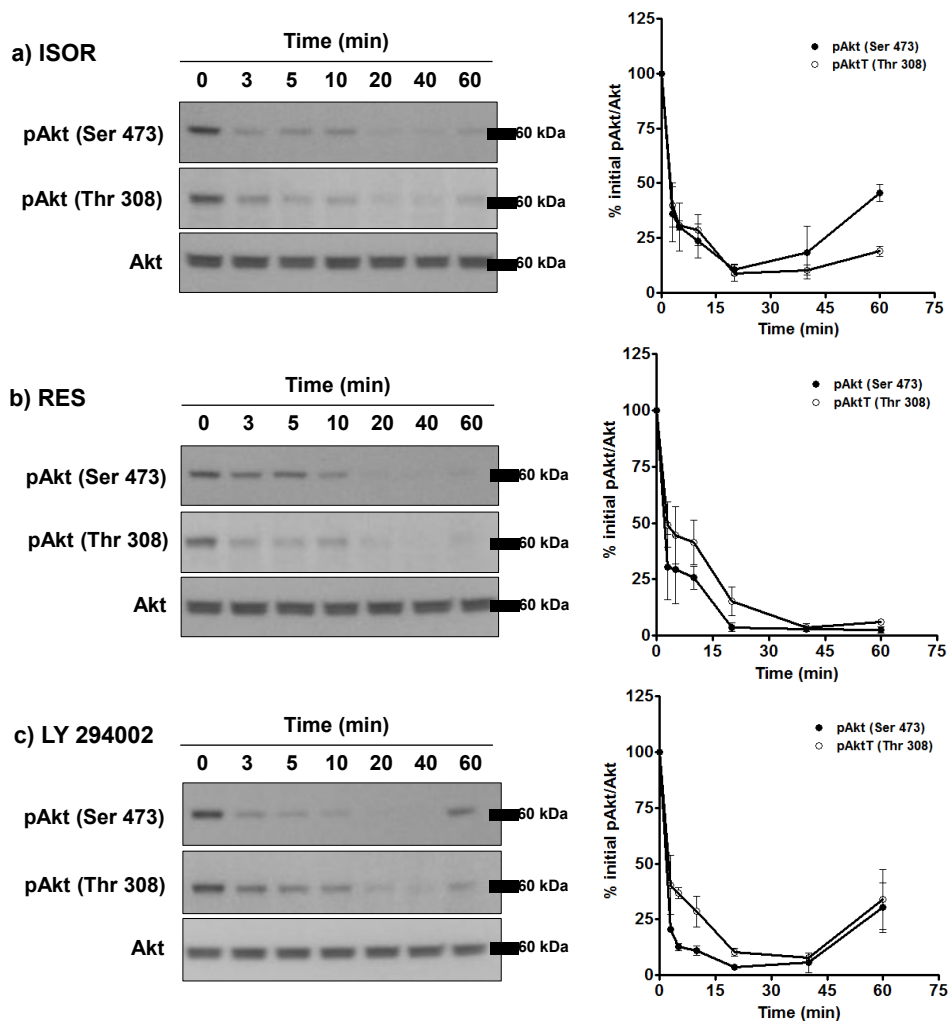


Figure 8.14 Effects of isorhapontigenin, resveratrol and LY294002 on protein levels of phosphorylated and total Akt proteins in A549 cells. Cells were incubated with a) 30 μ M isorhapontigenin, b) 100 μ M resveratrol or c) 5 μ M LY 294002 for the indicated times before lysis to collect cellular proteins that were resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the indicated proteins (left panel). Protein bands were analysed using UVP GelDoc-It imaging system and VisionWorks LS and phosphorylated Akt were normalised to the corresponding Akt protein levels. Normalised data was expressed as a percentage of the corresponding initial normalised data and plotted (right panel). Data are mean $\pm$ SEM for $n = 3$ independent experiments.

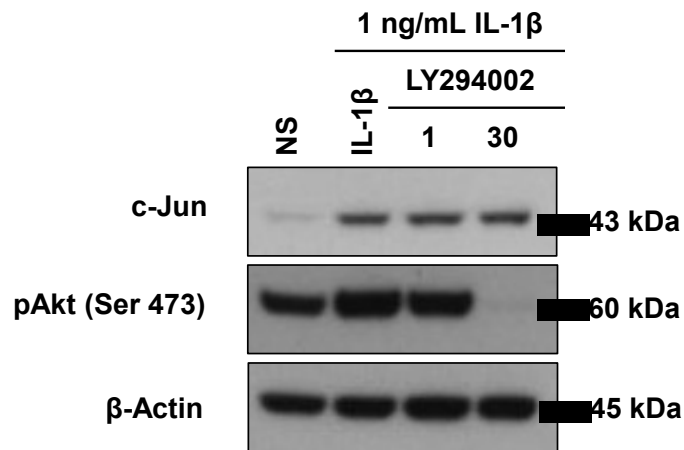


Figure 8.15 Effects of LY294002 on protein levels of c-Jun stimulated by IL-1 β in A549 cells. Cells were incubated with 1 μ M and 30 μ M of LY294002 for 1 h followed by stimulation with 1 ng/mL IL-1 β for 60 min before cells were lysed and proteins extracted and resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the indicated proteins. β -actin was used as loading control. Blots shown are representative of 3 independent experiments.

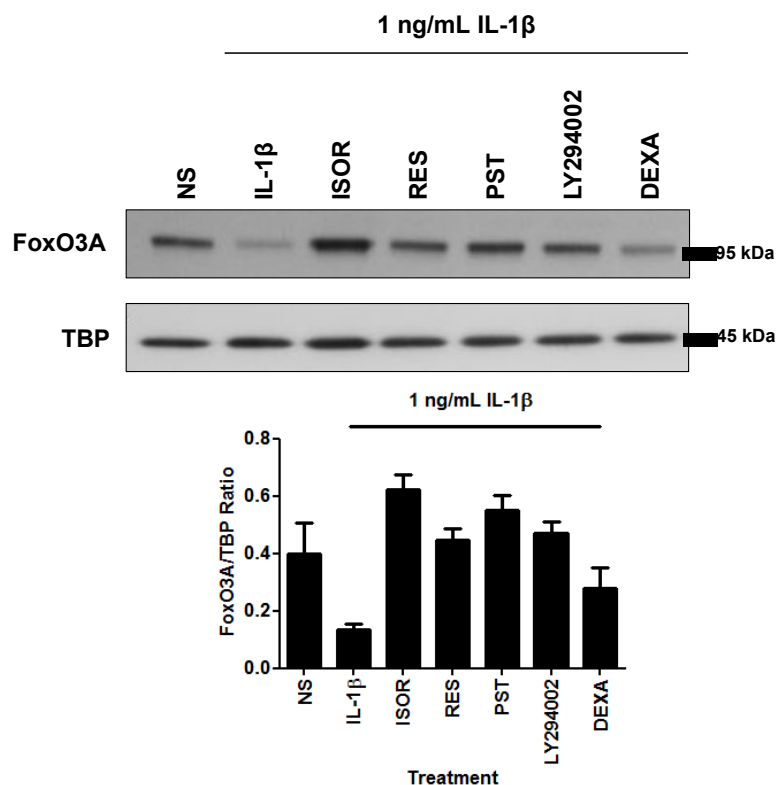


Figure 8.16 Effects of stilbene analogues, LY294002 and dexamethasone on nuclear protein levels of FoxO3A in A549 cells stimulated with IL-1 β . Cells were incubated with 100 μ M stilbene analogues, 30 μ M LY294002 or 1 μ M dexamethasone for 1 h followed by stimulation with 1 ng/mL IL-1 β for 60 min before nuclear proteins were extracted and resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the indicated proteins (top panel). TBP was used as loading control. Protein bands were analysed using UVP GelDoc-It imaging system and VisionWorks LS and FoxO3A levels were normalised to the corresponding TBP protein levels (bottom panel). Data are mean $\pm$ SEM for $n = 3$ independent experiments. NS = non-stimulated; ISOR = isorhapontigenin; RES = resveratrol; PST = pinostilbene; DEXA = dexamethasone.

8.3.5 Effects of stilbene analogues on ROS levels

Intracellular ROS has pleiotropic effects and has been linked to activation of the PI3K/Akt pathway (Ito and Barnes, 2009; To et al., 2010). To investigate if the stilbene analogues modulated the redox status in the A549 inflammatory model, cells were pre-treated with the compounds for 1 h before stimulation with IL-1 β for 30 min (Figure 8.17). IL-1 β stimulation did not significantly alter the ROS levels

compared to baseline. Isorhapontigenin at 30 μM considerably reduced the ROS levels by approximately 50% (Kruskal-Wallis test with Dunns post test: $p < 0.01$) and this was further decreased at 100 μM (Kruskal-Wallis test with Dunns post test: $p < 0.001$). Resveratrol was less effective as it only reduced the ROS levels by approximately 45% when used at 100 μM (Kruskal-Wallis test with Dunns post test: $p < 0.05$). Pinostilbene did not show significant anti-oxidative effects as it only reduced ROS levels by approximately 30%.

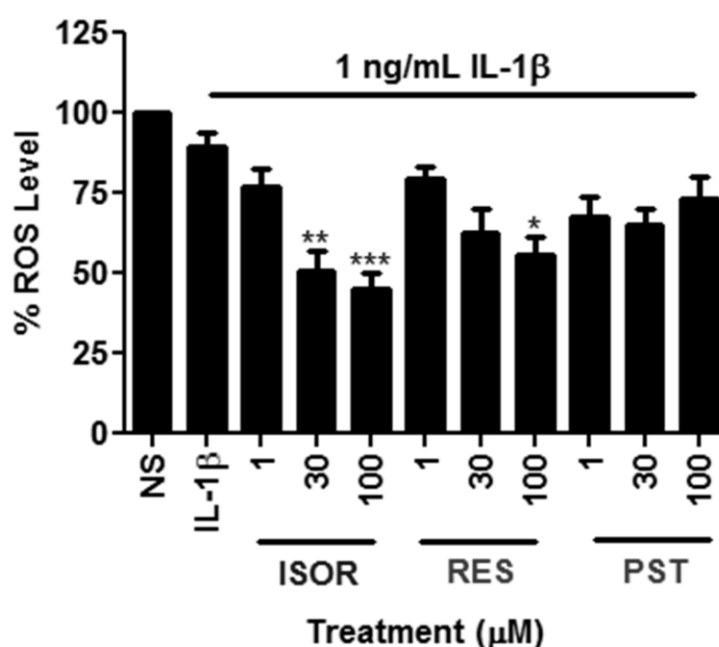


Figure 8.17 Effects of stilbene analogues on ROS levels in A549 cells stimulated with IL-1 β . Cells were incubated with the indicated concentrations of stilbene analogues for 1 h followed by stimulation with 1 ng/mL IL-1 β . After 30 min, 10 μM H₂DCF-DA was loaded and incubated for a further 30 min. Fluorescence was measured at excitation wavelength of 485 nm and emission wavelength of 520 nm. Data are mean $\pm$ SEM for $n = 6$ independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to NS. Kruskal-Wallis test with Dunns post test was used to compare between treatment groups. NS = non-stimulated; ISOR = isorhapontigenin; RES = resveratrol; PST = pinostilbene.

8.3.6 Effects of stilbene analogues on SIRT 1 and SIRT 6

The possible involvement of sirtuins in the anti-inflammatory effects observed for stilbene analogues were investigated by using SIRT1 enzymatic activity assay. When added directly to SIRT1 enzyme, isorhapontigenin at 30 μ M enhanced its activity slightly by about 2-fold while significantly enhancing its activity by approximately 5-fold at 100 μ M (Kruskal-Wallis test with Dunns post test: $p < 0.001$) (Figure 8.18). Resveratrol was more effective as 30 μ M increased the activity by approximately 4-fold (Kruskal-Wallis test with Dunns post test: $p < 0.01$) and 100 μ M augmented the activity by approximately 10-fold (Kruskal-Wallis test with Dunns post test: $p < 0.001$). Pinostilbene showed marginal effects on SIRT1 activity.

The total protein levels of SIRT1 and SIRT6 were examined at 0.5 h, 3 h, 6 h and 16 h to clarify their role in the anti-inflammatory effects of the compounds. SIRT1 levels remained similar to baseline at the various time points following the different treatments (Figure 8.19). SIRT6 levels also appeared comparable to baseline except at 16 h where IL- 1β induced a slight increase which was not affected by isorhapontigenin or resveratrol treatment.

To determine if SIRT1 enzymes were structurally modified in the cells to cause changes in activity, SIRT1 proteins were immunoprecipitated from A549 cells after the different treatments for 16 h and subjected to activity assay. SIRT1 activity was largely similar after the various treatments although resveratrol showed a tendency to increase activity by about 20% above baseline (Figure 8.20). In summary,

isorhapontigenin and resveratrol were able to activate SIRT1 directly but did not produce remarkable changes in protein levels of SIRT1 and SIRT6 or activity of immunoprecipitated SIRT1 in A549 cells.

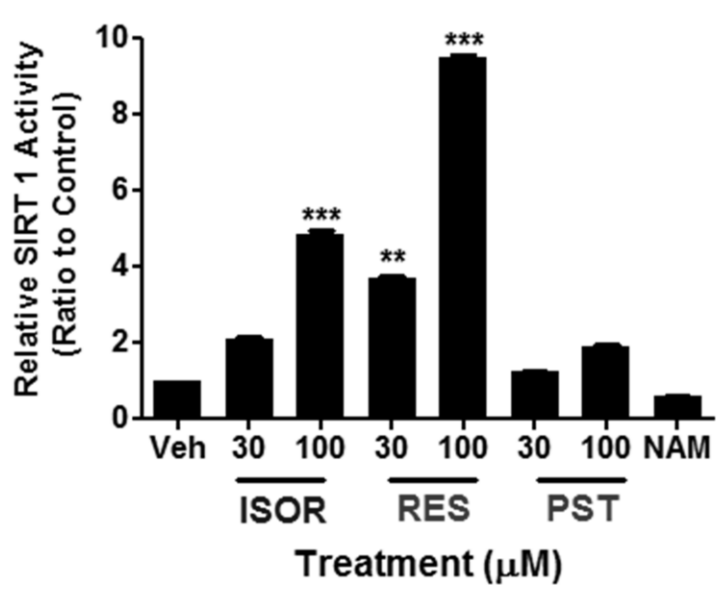


Figure 8.18 Effects of stilbene analogues on the activity of SIRT1 enzyme. Compound solutions were pre-incubated with 1 U SIRT1 enzymes before SIRT1 substrate and NAD^+ were added for 45 min at 37 °C. Nicotinamide (NAM) at 100 μM was used as negative control. The reaction was terminated by addition of Developer solution containing 2 mM of nicotinamide. After incubation at room temperature for 45 min, fluorescence readings at excitation wavelength of 370 nm and emission wavelength of 450 nm were recorded. Data are mean $\pm$ SEM for n = 6 replicates. **p < 0.01 and ***p < 0.001 compared to Veh. Kruskal-Wallis test with Dunns post test was used to compare between treatment groups Veh = vehicle control; ISOR = isorhapontigenin; RES = resveratrol; PST = pinostilbene; NAM = nicotinamide.

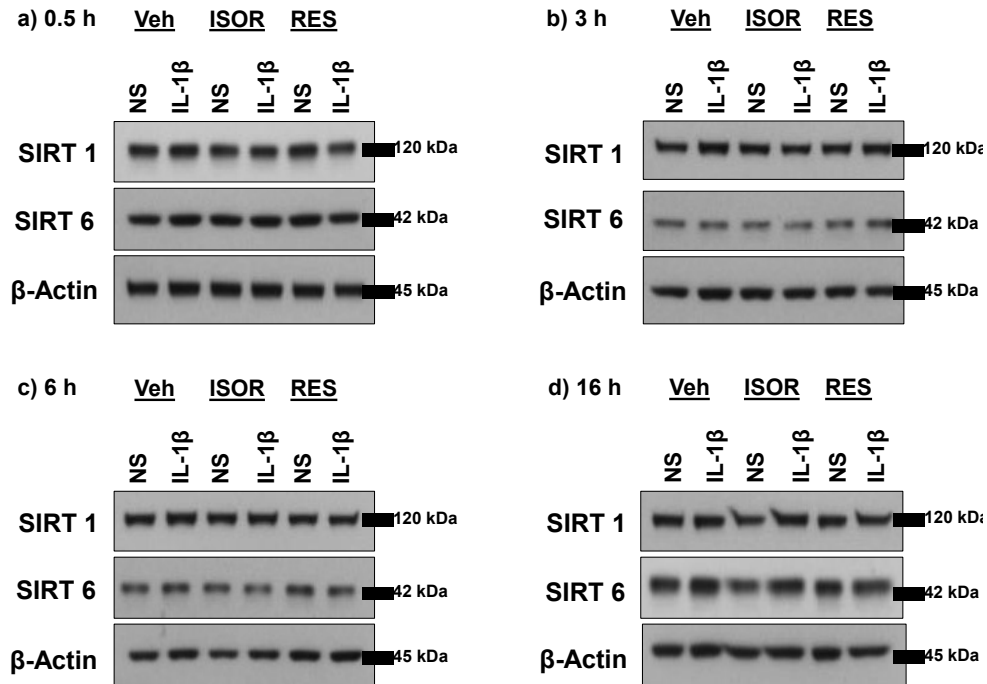


Figure 8.19 Effects of stilbene analogues on protein levels of SIRT1 and SIRT6 proteins in A549 cells with or without stimulation using 1 ng/mL IL-1 β . Cells were incubated with 100 μ M (for 0.5 h, 3 h and 6 h) or 30 μ M (for 16 h) of compounds for 30 min followed by stimulation with 1 ng/mL IL-1 β as appropriate for a) 0.5 h, b) 3 h, c) 6 h and d) 16 h before cells were lysed and proteins extracted and resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the indicated proteins. Protein bands shown are representative of 3 independent experiments. Veh = vehicle control; NS = non-stimulated; ISOR = isorhapontigenin; RES = resveratrol.

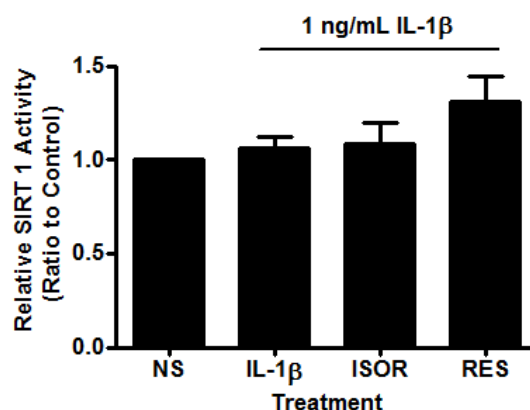


Figure 8.20 Effects of stilbene analogues on activity of SIRT1 immunoprecipitated from A549 cells. Cells were incubated with vehicle control (0.1 % DMSO in serum free DMEM) or 30 μ M of compounds for 30 min followed by stimulation with 1 ng/mL IL-1 β as appropriate for 16 h before cells were lysed and SIRT 1 proteins immunoprecipitated. SIRT1 substrate and NAD⁺ were added to the immunoprecipitated proteins for 45 min at 37 °C. The reaction was terminated by addition of developer solution containing 2 mM of nicotinamide. After incubation at room temperature for 45 min, fluorescence readings at excitation wavelength of 370 nm and emission wavelength of 450 nm were recorded. Data are mean $\pm$ SEM for n = 3 independent replications. NS = non-stimulated; ISOR = isorhapontigenin; RES = resveratrol.

8.3.7 Effects of stilbene analogues on Akt pathway in primary human airway epithelial cells

The key mechanism of stilbene analogues observed in A549 cells involving the inhibition of Akt activation was validated in primary airway epithelial cells. When primary epithelial cells were stimulated with a combination of IL-1 β and 50% CSE, the phosphorylation of Akt at both Ser473 and Thr308 increased from 40 min without any changes in total Akt levels (Figure 8.21). The time point at 60 min was chosen to study the effects of the stilbene analogues on this pathway.

Primary airway epithelial cells from non-smokers, smokers and COPD patients (Figures 8.22 (a), (b) and (c)) were used with the most potent

stilbene analogue, isorhapontigenin, as well as resveratrol and dexamethasone as stilbene and steroid controls respectively. When treated with the stilbene compounds for 1 h before stimulation, the increased phosphorylation of Akt at both sites were reduced in similar manner without any effect on total Akt levels (Figure 8.22 (d) and (e)). Isorhapontigenin was more potent than resveratrol as it reduced the activation state to basal levels at 30 μ M while resveratrol only showed slight reduction. Dexamethasone, at 1 μ M, had limited effect on the activation of Akt. This was consistent with the observations in A549 cells. In addition, IL-1 β was found to be the major driver for Akt activation while CSE had limited effect although it was required for enhanced release of cytokines as reported in Chapter 7. Furthermore, cells from the three subject groups did not show significant differences in response.

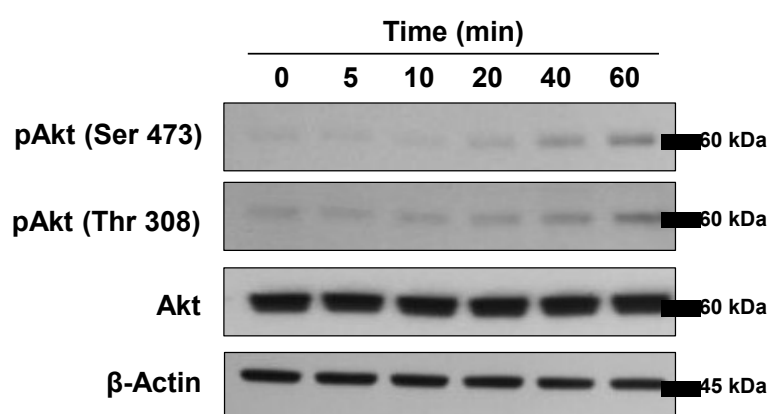
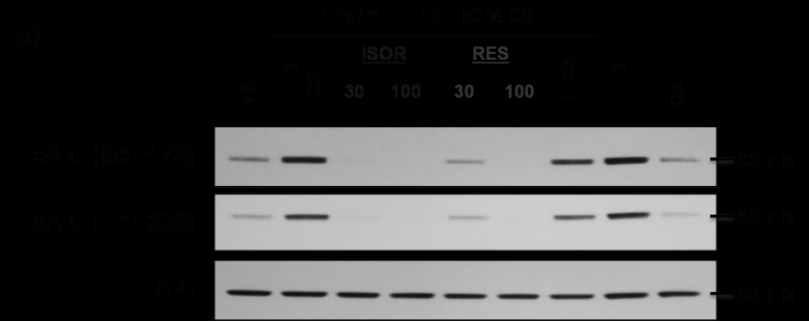
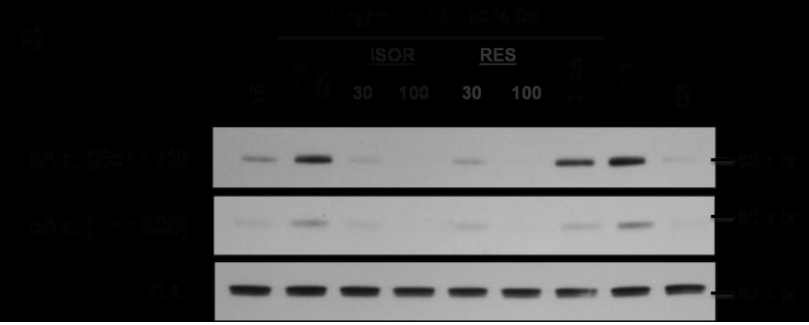
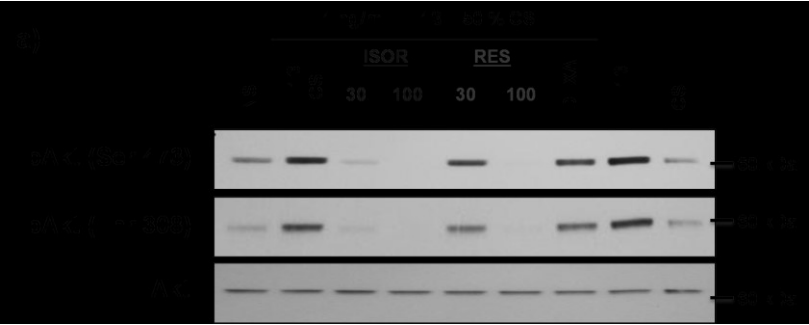


Figure 8.21 Effects of IL-1 β + 50% CSE on protein levels of phosphorylated and total Akt proteins in primary airway epithelial cells. Cells were stimulated with 1 ng/mL IL-1 β + 50% CSE for the indicated times before lysis to collect cellular proteins that were resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the indicated proteins. β -actin was used as loading control. Blots shown are representative of 3 independent experiments.



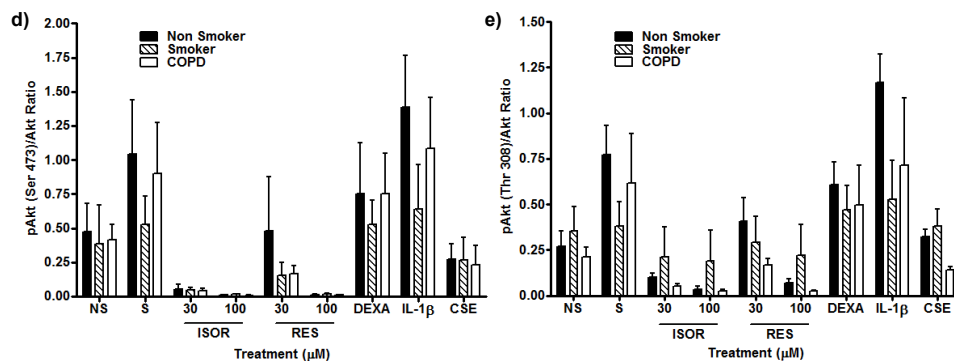


Figure 8.22 Effects of stilbene analogues and dexamethasone on protein levels of phosphorylated and total Akt proteins in primary airway epithelial cells stimulated with IL-1 β + 50 % CSE. Cells from a) non-smokers, b) smokers and c) COPD patients were incubated with the indicated concentrations of compounds or 1 μ M dexamethasone for 1 h followed by stimulation with 1 ng/mL IL-1 β + 50 % CSE for 60 min before cells were lysed and proteins extracted and resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the the indicated proteins (top panel). Protein bands were analysed using UVP GelDoc-It imaging system and VisionWorks LS and d,e) phosphorylated Akt were normalised to the corresponding Akt protein levels. Data are mean $\pm$ SEM for n = 3 independent experiments. NS = non-stimulated; ISOR = isorhapontigenin; RES = resveratrol; PST = pinostilbene; DEXA = dexamethasone.

8.3.8 Effects of stilbene analogues on c-Jun pathway in primary human airway epithelial cells

Another key mechanism of stilbene analogues observed in A549 cells involving the inhibition of cJun protein levels was also validated in primary airway epithelial cells. The total cellular levels of c-Jun showed little changes up to 60 min (Figure 8.23). This differed from the observations in A549 cells. Furthermore, using primary epithelial cells from non-smokers, smokers and COPD patients, no significant inhibition was observed for the compounds possibly due to the lack of stimulatory window (Figure 8.24).

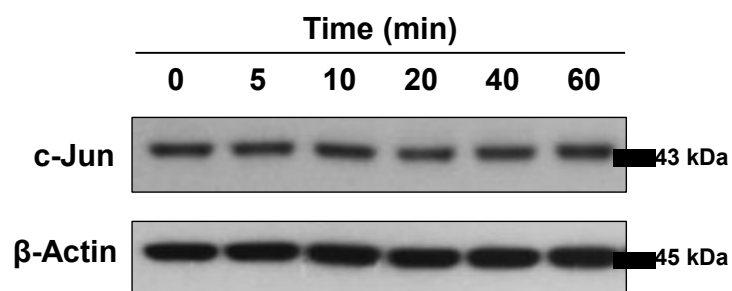


Figure 8.23 Effects of IL-1 β + 50% CSE on c-Jun protein levels in primary airway epithelial cells. Cells were stimulated with 1 ng/mL IL-1 β + 50% CSE for the indicated times before total cellular proteins were extracted and resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the c-Jun. β -Actin was used as loading control. Blots shown are representative of 3 independent experiments.

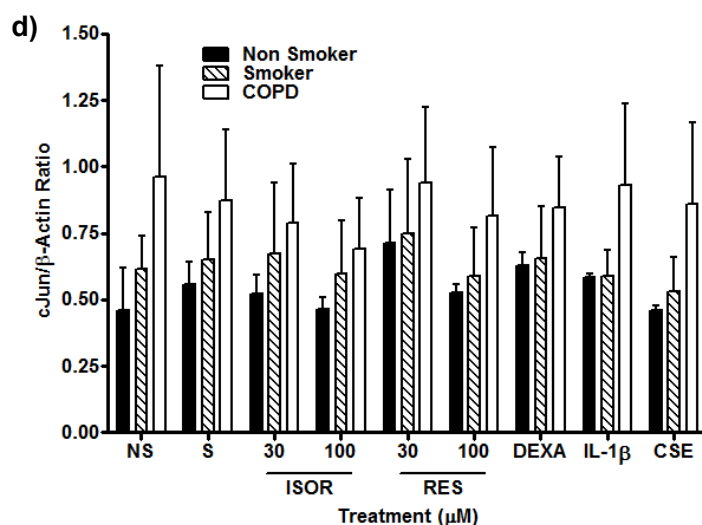
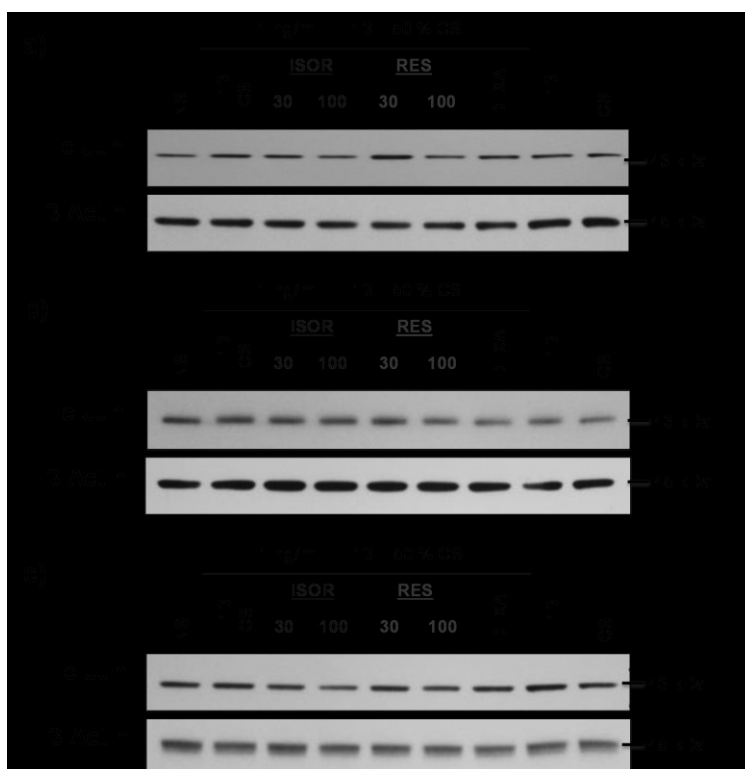


Figure 8.24 Effects of stilbene analogues and dexamethasone on levels of c-Jun protein in primary airway epithelial cells stimulated with IL-1 β + 50% CSE. Cells from a) non-smokers, b) smokers and c) COPD patients were incubated with the indicated concentrations of compounds for 1 h followed by stimulation with 1 ng/mL IL-1 β + 50% CSE for 60 min before cells were lysed and proteins extracted and resolved using SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for the c-Jun. β -actin was used as loading control. Protein bands were analysed using UVP GelDoc-It imaging system and VisionWorks LS and d) c-Jun proteins were normalised to their corresponding β -actin levels. Data are mean $\pm$ SEM for $n = 3$ independent experiments. NS = non-stimulated; ISOR = isorhapontigenin; RES = resveratrol; PST = pinostilbene; DEXA = dexamethasone.

8.3.9 Effects of combination of isorhapontigenin and dexamethasone on the release of IL-6 and CXCL8 in primary human airway epithelial cells

Having identified isorhapontigenin as the most potent stilbene analogue with a distinct mechanism of action compared to dexamethasone, isorhapontigenin was used at 5 μ M and 10 μ M with different concentrations of dexamethasone to observe their combinatory effects on IL-6 and CXCL8 release in primary human airway epithelial cells stimulated with 1 ng/ml IL-1 β + 50% CSE. These two concentrations of isorhapontigenin were selected as they have shown cytokine inhibitory effects close to IC₅₀ in Chapter 7 while not fully suppressing the release of cytokines. Furthermore, these concentrations were also similar to the plasma concentrations achievable through oral dosing (Chapter 4). The treatment conditions used did not show significant cytotoxicity to the cells as cell viability remained > 90%.

Dexamethasone alone resulted in a dose-dependent inhibition of IL-6 and CXCL8 release. As the concentration of isorhapontigenin increased, there was a trend of increased suppression of IL-6 and CXCL8 release (Figure 8.25 (a) and (b)). To assess the role of isorhapontigenin in the cytokine inhibitory effects of dexamethasone, it is necessary to account for the suppressive effects of isorhapontigenin alone. As such, the cytokine release in each combination treatment was expressed as a percentage of the stimuli-induced cytokine release when the corresponding isorhapontigenin treatment was applied

(Figure 8.26). This allows the inhibitory effects of dexamethasone to be evaluated and compared in the presence of different concentrations of isorhapontigenin, which gives an indication of changes in steroid sensitivity (Table 8.3). Combination with isorhapontigenin did not appear to alter the CXCL8 inhibitory effects of dexamethasone but showed a trend of improving its inhibition of IL-6 at higher concentrations (% IL-6 release at 10 μ M dexamethasone: dexamethasone alone = $8.8 \pm 5.6\%$, dexamethasone + 5 μ M isorhapontigenin = $3.3 \pm 9.4\%$, dexamethasone + 5 μ M isorhapontigenin = $-25.4 \pm 14.4\%$; $p > 0.05$) which did not reach statistical significance due to the small sample size in this pilot experiment.

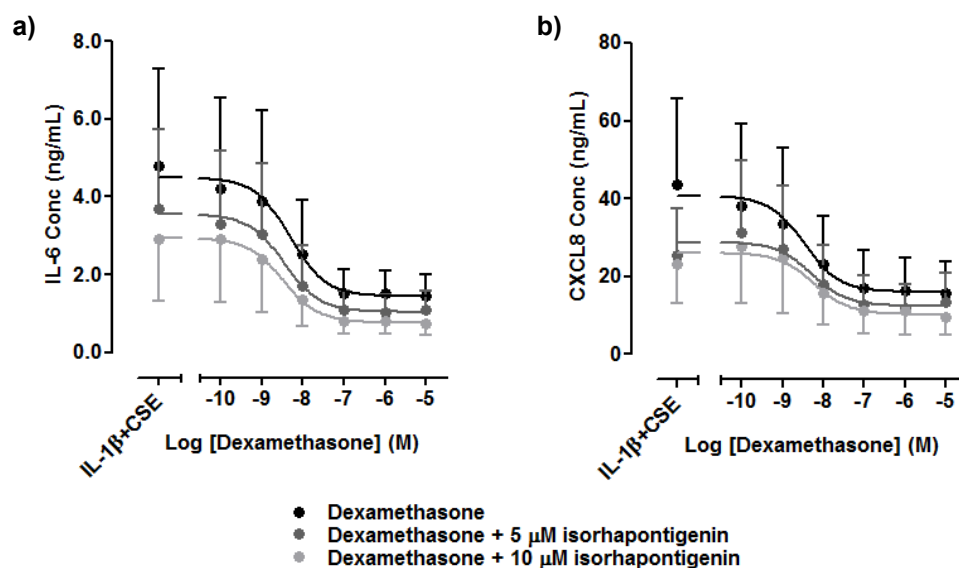


Figure 8.25 Effects of dexamethasone and its combination with isorhapontigenin on the release of a) IL-6 and b) CXCL8 in primary human airway epithelial cells stimulated with IL-1 β + CSE. Primary epithelial cells were treated with compounds for 1 h before stimulation with 1 ng/mL IL-1 β + 50% CSE for 24 h. Cell media were harvested and IL-6 and CXCL8 measured by ELISA. Data are mean $\pm$ SEM for $n = 4$ independent experiments.

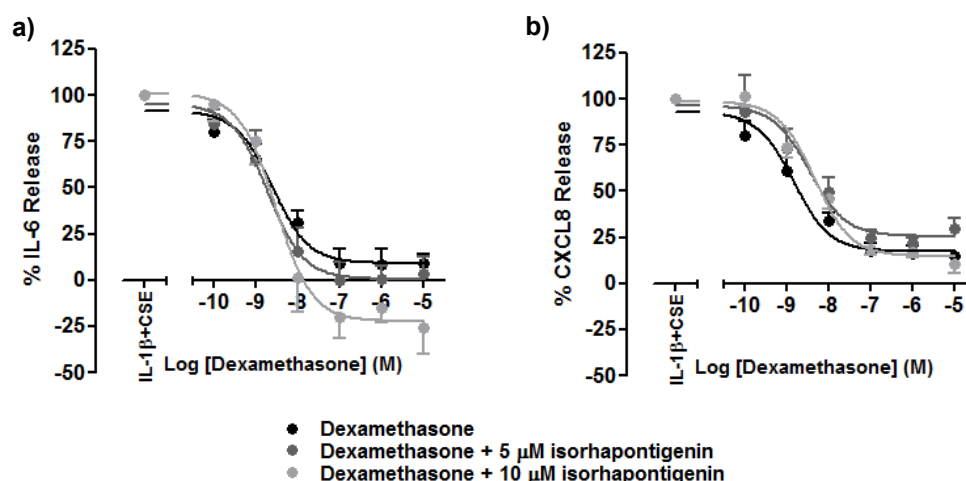


Figure 8.26 Effects of isorhapontigenin on dexamethasone-mediated suppression of a) IL-6 and b) CXCL8 release in primary human airway epithelial cells stimulated with IL-1 β + CSE. Primary epithelial cells were treated with compounds for 1 h before stimulation with 1 ng/mL IL-1 β + 50% CSE for 24 h. Cell media were harvested and IL-6 and CXCL8 measured by ELISA. Data are mean $\pm$ SEM for $n = 4$ independent experiments.

Table 8.3 IC₅₀ and %_{max} for dexamethasone and its combination with isorhapontigenin for the inhibition of IL-6 and CXCL8 release

Parameters		DEXA	DEXA + 5 μ M ISOR	DEXA + 10 μ M ISOR
IL-6	IC ₅₀ (nM)	2.97 $\pm$ 1.49	2.76 $\pm$ 1.17	3.31 $\pm$ 0.87
	% _{max} (%)	8.83 $\pm$ 5.58	3.28 $\pm$ 9.4	-25.36 $\pm$ 14.4
CXCL8	IC ₅₀ (nM)	1.91 $\pm$ 0.74	3.60 $\pm$ 1.44	4.25 $\pm$ 1.38
	% _{max} (%)	14.47 $\pm$ 2.65	29.91 $\pm$ 5.40	10.69 $\pm$ 5.42

*Data presented as mean $\pm$ SEM ($n = 4$)

*DEXA = dexamethasone, ISOR = isorhapontigenin

*%_{max} = % cytokine release observed at the maximum concentration of dexamethasone used (10 μ M)

8.4 Discussion

In this chapter, the effects of isorhapontigenin, pinostilbene and resveratrol on major inflammatory pathways were investigated to uncover the mechanism underlying their suppressive effects on IL-6 and CXCL8 observed in Chapter 7 (Figure 8.27). Dexamethasone was used as a corticosteroid control to discern differences in the mechanisms of stilbene analogues and corticosteroids.

Time course experiments were first performed to identify appropriate exposure times to inflammatory stimuli so that suitable stimulation window was present. As these molecular events usually involve phosphorylation or translocation processes that are known to occur rapidly, timings of up to 1 h are typically sufficient for the western blotting experiments (Jang et al., 2007; Newton et al., 1998).

Although it is ideal that cells are derived from groups of patients which are well-matched in demographics, this was not feasible in this study due to the limited availability of patient samples. This limitation did not appear to be important since there was no difference between the western blot data from different patient groups.

NF- κ B regulates the transcription of a wide array of inflammatory genes including IL-6 and CXCL8. Isorhapontigenin and pinostilbene showed marked inhibition of NF- κ B transcription activity but had little effects on the upstream I κ B α and p50/p65 nuclear translocation. This implies that their NF- κ B inhibitory activities likely involved effects downstream of NF- κ B nuclear translocation such as its interaction with various

proteins and transcriptional co-activators including HDACs and p300 (Hoesel and Schmid, 2013; Zhong et al., 2002). These data appear to contradict findings from previous studies which showed that resveratrol exerted its effects through the I κ B α or p65 nuclear translocation pathways (Adams et al., 2005; Adhami et al., 2003; Holmes-McNary and Baldwin, 2000; Liu et al., 2010; Ren et al., 2013). However, the similar lack of effect of dexamethasone on these pathways observed in the study herein corroborates with a previous report using the same cell line and stimulus (Newton et al., 1998), supporting the notion that the mechanisms are specific to the cell type, stimuli and experimental conditions used. One interesting observation in these experiments is that the highest concentration of stilbene compounds and dexamethasone resulted in slight increase in I κ B α levels. This has also been observed in a similar study on dexamethasone in A549 cells previously (Jang et al., 2007). This slight increase in I κ B α was insufficient in reducing the nuclear translocation of the NF- κ B subunits. It is unlikely that the stilbene analogues and dexamethasone share the same mechanism despite showing similar weak effects at the I κ B α and p50/p65 levels. This is because when their effects on NF- κ B dependent transcription were examined, dexamethasone only showed a maximal inhibition of approximately 60% while pinostilbene and isorhapontigenin could almost completely block transcription.

For the AP-1 transcription factor pathway, the inhibition of c-Jun protein levels appeared to be highest for isorhapontigenin, which was congruent with data in Chapter 7.3.2 that identified isorhapontigenin as

the most potent stilbene analogue in cytokine inhibition. Furthermore, isorhapontigenin also exhibited highest suppression of the induction of c-Jun mRNA levels amongst the compounds tested, indicating that the reduced protein levels of c-Jun could be largely due to reduced mRNA levels. For the other stilbene analogues and dexamethasone, regulation at the mRNA level was less marked and the changes in protein levels could also be contributed by post-translational modifications of c-Jun such as inhibition of its phosphorylation (Caelles et al., 1997; Musti et al., 1997). In contrast to c-Jun levels, the protein levels of the other major AP-1 subunit, c-Fos, were increased by the stilbene compounds. Despite this, the transcriptional activity of AP-1 is likely to be reduced as c-Fos requires heterodimerisation with c-Jun to form transcriptionally active dimers (Halazonetis et al., 1988). The resveratrol-mediated reduction of c-Jun protein levels in A549 cells observed in the present study could explain the abrogation of AP-1 transcriptional activity by resveratrol in another study (Donnelly et al., 2004).

A clear distinction between the molecular mechanism of stilbene analogues and dexamethasone was evident in the activation of MAPK pathway, Dexamethasone inactivated all three MAPK proteins likely through binding to the glucocorticoid receptors and inducing the dual-specificity MAPK phosphatase 1 (DUSP1) (Jang et al., 2007; Shah et al., 2014). On the other hand, the stilbene analogues did not suppress the activation of MAPK proteins by IL-1 β . While isorhapontigenin showed marginal effects on the phosphorylation of MAPK proteins,

resveratrol and to a larger extent, pinostilbene, showed concentration-dependent increase in phosphorylation of p38, JNK and ERK. This finding appears to contradict observation in the present study that the stilbene analogues inhibited the release of cytokines from A549 cells in Chapter 7. However, regulation of cytokine release often involves more than one pathways and the increased activation of MAPK proteins could have been counteracted by other effects. The relatively small effect of isorhapontigenin on the MAPK proteins could explain its higher potency in the inhibition of cytokines and its retention of CXCL8 release inhibition in primary cells.

Among the stilbene analogues, isorhapontigenin showed the highest inhibition of Akt phosphorylation and this correlated well with its position as the most potent stilbene analogue for inhibition of the release of inflammatory cytokines. The involvement of PI3K/Akt pathway in the release of IL-6 in A549 cells stimulated with IL-1 β has already been established while it is also involved in release of CXCL8 from A549 cells stimulated with thrombin (Eda et al., 2011; Lin et al., 2011). One crucial observation in this study is that in contrast to its strong inhibition on MAPK proteins, dexamethasone only showed weak inhibitory effects on Akt activation in both A549 and primary cells. This Akt-independent effect of dexamethasone has been reported in A549 cells and other cell types such as MCF-7 breast cancer cells (Jang et al., 2007; Machuca et al., 2006). Since the stilbene analogues rapidly inhibited activation of both phosphorylation sites in a manner similar to LY294002, a PI3K inhibitor, they were likely to engage direct and quick

regulatory mechanisms on the PI3K/Akt pathway. The activation of Akt phosphatases such as PP2A and PHLPP is an unlikely mechanism since these enzymes show higher specificity for one phosphorylation site only and would not cause the observed uniform reduction in both phosphorylation sites of Akt (Andjelkovic et al., 1996; Brognard et al., 2007; Stambolic et al., 1998). The direct kinases of Akt, PDK-1 and mTORC2, are also unlikely targets due to the same reason described above. It is likely that the stilbene analogues targeted the regulation of PIP₃ levels which could be demonstrated by immunostaining of PIP₃ levels. However, this experiment could not be carried out successfully as the negative isotype control antibody used in the experiment showed high non-specific staining after many attempts. In support of this deduction, there are enzymatic studies which have shown that resveratrol and its analogue, piceatannol are direct inhibitors of PI3K (Choi et al., 2010; Frojdo et al., 2007). However, currently the lack of an appropriate cellular PI3K assay impedes validation of this target in cells.

Downstream of the PI3k/Akt pathway, FoXO3A is a major transcription factor that becomes phosphorylated, leading to cytoplasmic retention, ubiquitination and eventually degradation (Huang and Tindall, 2011). Reduced nuclear levels of FoXO3A in COPD airway epithelial cells has been shown to promote inflammation and oxidative stress by increasing the release of CXCL8 and reducing levels of antioxidants such as manganese superoxide dismutase (MnSOD) (Ganesan et al., 2013; Kops et al., 2002). The MAPKs and PI3K/Akt pathways are also

associated with the transcription factors described earlier. It appears that NF- κ B activity is dependent on both the MAPK and PI3K/Akt pathways as both dexamethasone and stilbene analogues could reduce its activity. These links have also been shown in other studies using the same cell lines (Donnelly et al., 2004; Jang et al., 2007; Jung et al., 2003; Lin et al., 2011; Newton et al., 1998). On the other hand, although inactivation of MAPK proteins by dexamethasone led to reduced c-Jun protein levels, the repression of c-Jun levels by stilbene analogues was not dependent on their inhibition of Akt phosphorylation. This observation was consistent with the MAPK-dependent and Akt-independent regulation of cJun mRNA and phosphorylation in A549 cells reported in another study (Lee et al., 2010b).

ROS has been implicated in the activation of PI3K/Akt pathway (Ito et al., 2007). Although IL-1 β did not increase ROS levels in the present study, the stilbene analogues, in particular isorhapontigenin, were effective in reducing the levels of ROS. This could be the reason underlying the ability of stilbene analogues to reduce Akt phosphorylation below baseline. One of the factors accounting for the higher anti-oxidant effect of isorhapontigenin compared to resveratrol is the additional meta-methoxy group in its structure which has been associated with increased anti-oxidant potential (Hasiah et al., 2011).

Sirtuins, in particular SIRT1 and SIRT6, are increasingly recognised as novel therapeutic targets to combat the inflammation and oxidative stress in COPD (Chun, 2015). The present study showed that in

addition to resveratrol, a well-established activator of SIRT1 (Hubbard et al., 2013), isorhapontigenin was also able to activate SIRT1 but to a lesser extent. Activation of SIRT1 could be one of the mechanisms leading to the anti-inflammatory effects of these compounds but is unlikely to be the major mechanism since isorhapontigenin, the most potent anti-inflammatory stilbene analogue identified in this study, was not the most potent activator of SIRT1. The lack of significant changes in the levels of SIRT1 and SIRT6 as well as in immunoprecipitated SIRT1 proteins from A549 cells treated with stilbene analogues suggest that the Akt-inhibitory effects of the stilbene analogues were not associated with changes in the sirtuin enzymes. The direct activation of SIRT1 enzymes could be an additional, independent anti-inflammatory mechanism of stilbene analogues in cells but this would require further verification.

One of the reasons underlying the use of combination therapies in COPD is the ability to improve the anti-inflammatory effects of corticosteroids (Barnes, 2002; Cosio et al., 2009a). It was rational to combine isorhapontigenin with dexamethasone since both compounds have been shown to mediate their effects through different mechanisms. Using isorhapontigenin at lower doses that are achievable by oral dosing will simplify its use clinically since oral administration is the most convenient route. The addition of isorhapontigenin resulted in a trend of further inhibition of IL-6 and CXCL8 and enhanced efficacy of dexamethasone in the inhibition of IL-6 but the experimental variability and small sample size meant that

statistical significance was not achieved. However, this pilot experiment demonstrates the feasibility of combining both compounds to achieve superior anti-inflammatory effects that were additive. In peripheral blood mononuclear cells (PBMCs), the PI3K/Akt pathway modulated by isorhapontigenin is an attractive therapeutic target in COPD as its inhibition leads to improvement in corticosteroid responsiveness (Rossios et al., 2012; To et al., 2010).

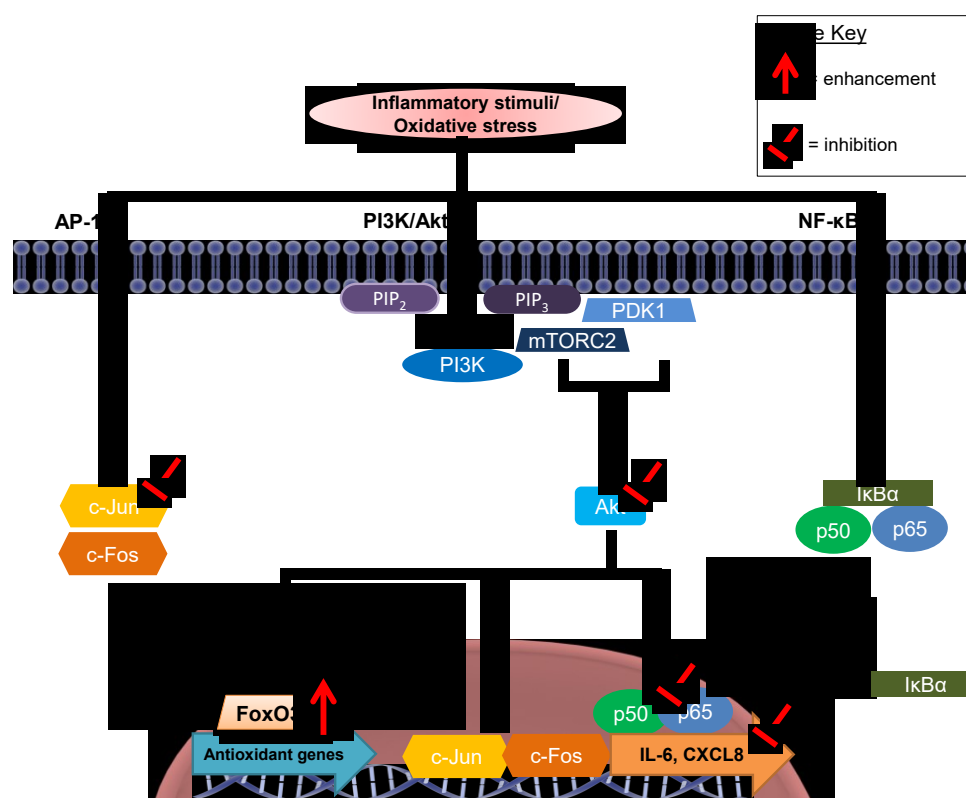


Figure 8.27 Summary of effects of stilbene analogues on inflammatory pathways demonstrated in the present study.

8.5 Conclusion

This chapter examined the anti-inflammatory mechanism of stilbene analogues. These compounds demonstrated inhibitory effects on the corticosteroid-insensitive PI3K/Akt pathway and showed more efficacious repression of NF- κ B activation compared to dexamethasone. They were also able to suppress the increase of c-Jun stimulated by IL-1 β . These compounds were also effective antioxidants and activation of SIRT1 could contribute to their anti-inflammatory effects. Their main mechanism of PI3K/Akt pathway inhibition was also observed in primary human airway epithelial cells. Isorhapontigenin was clearly the most potent stilbene analogue and when combined with dexamethasone, it showed a tendency to further suppress the release of inflammatory cytokines. Isorhapontigenin is a promising candidate for development as novel anti-inflammatory agent for COPD.

CHAPTER 9
GENERAL DISCUSSION AND FUTURE
PERSPECTIVES

9.1 General Discussion

The burgeoning healthcare cost associated with COPD has fueled research into its underlying mechanisms in recent years which have fundamentally advanced our knowledge of the important therapeutic targets. It is now understood that one of the key drivers of this complex disease is chronic inflammation that leads to the relentless decline in lung function and it is poorly reversible by corticosteroids. As such, novel anti-inflammatory pharmacological agents are urgently needed.

The pharmacology of resveratrol has been extensively characterised and its therapeutic effects in airway inflammation are well-documented (Baur and Sinclair, 2006; Birrell et al., 2005; Culpitt et al., 2003a; Donnelly et al., 2004; Kode et al., 2008). Although relatively less prominent, other stilbene analogues have shown potential as superior pharmacological agents. This forms the basis of the hypothesis in this thesis which is that analogues of resveratrol have superior *in vivo* pharmacokinetics and *in vitro* anti-inflammatory effects compared to resveratrol and they exert their effects through a pathway distinct from corticosteroid.

The first part of the hypothesis on pharmacokinetics was partially correct as the investigations presented in this thesis revealed that DHS and isorhapontigenin had better pharmacokinetic profiles than resveratrol whilst the pharmacokinetics of pinosylvin were worse. Moreover, pterostilbene was found to be more widely distributed compared to resveratrol. The synthetic stilbene compound, DFS, in

addition to showing improved plasma pharmacokinetics compared to resveratrol, was also substantially metabolised into another active compound. Interesting relationships between structure and pharmacokinetics were revealed from comparing the plasma pharmacokinetic profiles of the compounds in Chapter 4. A study on the clearance of pairs of compounds which differ by the presence of hydroxyl group showed that only half of the compound pairs exhibited higher clearance for the hydroxylated analogue although it is known that hydroxyl groups have increased susceptibility to Phase II metabolism (Miao and Obach, 2010). The data in this thesis were in line with such observations and further extended by identifying that the presence of hydroxyl groups in meta-position increases susceptibility to clearance. However, methylation proved to be an effective solution to increased clearance as isorhapontigenin, which has an additional meta-hydroxyl group that is methylated, displayed similar clearance to resveratrol. Importantly, this additional methoxy group also improved the oral bioavailability of isorhapontigenin compared to resveratrol. These observations can help in the design of molecules with improved pharmacokinetic properties by reducing susceptibility to clearance and increasing oral bioavailability. The pharmacokinetic profiles acquired from the studies presented herein can also be widely applied in different therapeutic areas to help make an informed decision on the ideal route of administration.

The second part of the hypothesis regarding the *in vitro* anti-inflammatory effects of stilbene analogues was also partially correct

since the compounds displayed different extents of cytokine inhibition in the various airway inflammation models with isorhapontigenin consistently showing a trend of higher potency compared to resveratrol. Structural activity relationships that emerged from these studies such as the importance of the 4'-OH moiety in the inhibition of IL-6 release in A549 cells will aid in the design of molecules to achieve superior anti-inflammatory effects. The present study also showed the utility of transformed cells as a screening platform but confirmation in primary cells will be crucial as there are fundamental differences in the characteristics between these cell types.

Based on the pharmacological properties of the various stilbene analogues shown in this thesis, isorhapontigenin has emerged as a better drug candidate compared to resveratrol. The significantly better oral pharmacokinetics of isorhapontigenin is crucial since it implies that substantial levels of isorhapontigenin can be achieved in the systemic circulation following administration through the most convenient and well-accepted route. Furthermore, isorhapontigenin is a more potent anti-inflammatory agent which can achieve the desired therapeutic effects at lower compound concentration. The data from this thesis, using the pharmacokinetics of isorhapontigenin in rats and cytokine inhibition in primary airway epithelial cells as a gauge, at least 30% inhibition of stimulated IL-6 release should be attained through a single oral dose of isorhapontigenin. However, *in vivo* experiments will be required to confirm this.

The third part of the hypothesis was correct as the stilbene analogues clearly inhibited the PI3K/Akt pathway which was relatively insensitive to corticosteroids. Furthermore, these compounds did not inhibit the MAPK pathways which were potently repressed by corticosteroids, therefore clearly demonstrating a different anti-inflammatory profile compared to corticosteroids. The PI3K/Akt pathway is an attractive inflammatory target that has seen accumulating evidence of its importance in COPD (Barnes, 2013b; Ito et al., 2007; Marwick et al., 2010). Other than being associated with the release of inflammatory cytokines as shown in the present studies, the PI3K/Akt pathway is also involved in the release of MMP9 that leads to the degradation of extracellular matrix components in COPD (Lee et al., 2010b). Furthermore, oxidative stress, which amplifies the inflammatory processes, and the PI3K/Akt pathway are intertwined. Oxidative stress has been shown to be involved in activating and is also increased by the PI3K/Akt pathway (Los et al., 2009; Qin and Chock, 2003). As demonstrated herein, the stilbene analogues rapidly reduced oxidative stress levels and inhibited Akt resulting in the increased nuclear translocation of FoxO3A, an important transcription factor of anti-oxidant genes.

One of the most important discoveries in recent years regarding the role of PI3K/Akt pathway in COPD is its involvement in corticosteroid resistance. In macrophages and monocytes, inhibition of the PI3K- δ isoform was found to be a viable strategy to increase the sensitivity of corticosteroids (Marwick et al., 2010; To et al., 2010). PI3K was

implicated in the reduction in HDAC2 activity which is essential for the anti-inflammatory effects of corticosteroids. However, combinations of stilbene analogues and dexamethasone proved to be additive and not synergistic but more work is required to understand any interactions.

9.2 Future perspectives

Studies in this thesis have identified isorhapontigenin as the most potent stilbene analogue among the 11 compounds studied for anti-inflammatory applications in COPD and confirmed that it better pharmacokinetic properties compared to resveratrol. To further develop isorhapontigenin as a novel anti-inflammatory agent for COPD, further investigations would be required.

9.2.1 Anti-inflammatory effects of isorhapontigenin in macrophages

Firstly, it would be interesting to observe if the effects of isorhapontigenin are also replicated in macrophages, which are another important cell type involved in the pathogenesis of COPD. Primary alveolar macrophages are the best cellular model for this purpose. Cells from healthy control subjects and COPD patients should be used to see if the inhibitory effects of isorhapontigenin on the release of cytokines can be retained and whether corticosteroid insensitivity could be overcome in COPD cells.

9.2.2 Molecular mechanism of isorhapontigenin

Mechanistic studies should also be carried out to determine if isorhapontigenin targeted the PI3K/Akt pathway in macrophages. It

would be imperative to confirm that isorhapontigenin can increase the activity of HDAC2 and subsequently enhance the sensitivity of corticosteroid. Greater insight into the specific isoforms of PI3K enzyme targeted by isorhapontigenin can be achieved using enzymatic assays for each isoform. Understanding the specific isoforms inhibited by isorhapontigenin is crucial since PI3K- δ has been linked to corticosteroid sensitivity and PI3K- γ has been associated with release of inflammatory cytokines. Studies in this thesis have shown that the PI3K/Akt pathway was inhibited in cells based on the reduction in Akt activation but further studies would be required to confirm that intracellularly, the inhibition occurred at the PI3K enzyme level or via changes in PTEN or SHIP. This would involve measurement of PIP₃ levels which was unsuccessfully attempted in this project using PIP₃ immunostaining. Alternative methods to do this are using mass spectrometer or radiolabelling (van der Kaay et al., 1997; Wang and Summers, 2003). Ultimately, it would be of keen interest to identify the direct molecular targets of isorhapontigenin. The direct molecular targets of resveratrol have remained controversial and continue to elude us after more than two decades of studies (Britton et al., 2015; Kulkarni and Cantó, 2015). Identifying the targets will provide fundamental knowledge on the biological effects of isorhapontigenin and the various signalling pathways that will be altered. This will help to explain the interesting observation in primary cells where isorhapontigenin and resveratrol showed differential effects on the release of CXCL8.

9.2.3 Efficacy and distribution of isorhapontigenin in *in vivo* models

Isorhapontigenin should be progressed to assays using *in vivo* models of COPD after the cellular experiments. Although it has shown better pharmacokinetic properties compared to resveratrol, this may not necessary translate to better efficacy. To show efficacy in *in vivo* models, the compound would need to reach the therapeutic concentration in the respective tissues. The levels of compounds attainable after administration will depend on its pharmacokinetics while the therapeutic concentration can be inferred from its potency in cellular assays. It would be ideal to study the lung distribution of isorhapontigenin as it is the main drug target organ. A pilot study to investigate this property was not successful as no compound could be detected in the harvested lung tissues probably due to degradation during sample handling. Sample stabilization techniques such as immediate cryopreservation of harvested tissues may be able to overcome this issue. With the appropriate dose computed from pharmacokinetic studies, *in vivo* efficacy studies can be carried out. A suitable preliminary *in vivo* experimental model is aerosolised LPS-challenged rats (Birrell et al., 2005). This is an acute inflammation model that will allow the efficacy of isorhapontigenin on airway inflammation to be studied quickly. This model can be a confirmation of suitable dose as the concentration of isorhapontigenin that is achieved in the body can be correlated to the anti-inflammatory effects observed.

Following this, further studies can be performed using the commonly used mouse model of COPD where inflammation is induced by chronic exposure to cigarette smoke (Guan et al., 2013; Moodie et al., 2004; To et al., 2010). Oral administration of isorhapontigenin is preferred since it is the most convenient route of administration. However, if this route fails to achieve the required therapeutic levels, pulmonary delivery of isorhapontigenin would be necessary. This can be in the form of intratracheal administration of isorhapontigenin solution (Birrell et al., 2005). Various samples such as bronchoalveolar lavage fluid, lung and blood can be collected to assay for inflammatory and oxidative stress markers (Guan et al., 2013; Marwick et al., 2009).

9.2.4 Assessment of safety of isorhapontigenin

Another pertinent aspect in pre-clinical evaluation is the toxicological properties of isorhapontigenin. The well-known stilbene analogue resveratrol is regarded as a safe compound found in numerous commonly consumed food such as grapes and red wine. The excellent safety profile of resveratrol is evident from pre-clinical studies which documented no significant changes in biochemical, histological or hematological variables in models such as rats dosed with 20 mg/kg of resveratrol for 28 days (Juan et al., 2002), rats dosed with up to 1000 mg/kg of resveratrol for 90 days or dogs dosed with up to 1200 mg/kg of resveratrol for 90 days (Johnson et al., 2011). In addition, clinical trials have also largely shown that the adverse effects of resveratrol are limited to mild gastrointestinal symptoms when used at up to 5 g/day for 28 days (Almeida et al., 2009; Boocock et al., 2007; Brown et al.,

2010). Although isorhapontigenin closely resembles resveratrol structurally and can also be found naturally in plants, its safety cannot be confirmed until similar toxicity evaluations on isorhapontigenin are carried out as part of its drug development programme.

9.2.5 Formulation of isorhapontigenin

Before isorhapontigenin is evaluated in clinical trials, design of appropriate formulation for its delivery is essential. If oral administration is used, isorhapontigenin can be delivered in the form of capsule or suspended or solubilised in appropriate vehicle. Higher lung concentrations of isorhapontigenin can be achieved by using pulmonary delivery methods such as aerosols or dry powder inhalers. Cautious assessment of important parameters such as morphological characteristics will be needed to ensure optimal delivery (Trotta et al., 2016).

9.2.6 Applications in other disease

Given the extensive medicinal applications of resveratrol, it is possible that isorhapontigenin may have wide-ranging therapeutic effects too. Furthermore, inflammatory diseases may share common pathological pathways which can be ameliorated by the same compound. Severe asthma is clearly one related area of application as it is also relatively refractory to the anti-inflammatory effects of corticosteroids with certain underlying mechanisms showing similarity to COPD (Barnes, 2013a). The suppressive effects shown by resveratrol on inflammation and airway hyperresponsiveness in pre-clinical asthma models substantiate exploration of effects of isorhapontigenin in similar systems (Lee et al.,

2009; Royce et al., 2011). Colitis, in particular the subset which is severe and resistant to corticosteroids, is another area of potential application for isorhapontigenin. In this application, isorhapontigenin is expected to achieve therapeutic levels easily since it should be easily absorbed into intestinal cells after oral administration similar to resveratrol (Walle et al., 2004).

9.3 Conclusion

This thesis has expanded the understanding of the pharmacology of stilbene analogues, a class of biologically active molecules that have attracted significant interests in recent years. By comparing to resveratrol, a well-known analogue, the studies identified specific structural determinants of pharmacokinetics such as the increased susceptibility of the meta-hydroxy groups to clearance compared to para-hydroxy groups of the molecule. Furthermore, pterostilbene was shown to be an analogue which is extensively distributed and it was ascertained that the main metabolite of DFS was another active compound. These substantiate further drug development of these compounds. Notably, isorhapontigenin emerged as a more potent anti-inflammatory agent than resveratrol with better oral pharmacokinetics. Its corticosteroid-distinct mechanisms further support its development as a novel anti-inflammatory agent for COPD.

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