Role of antioxidants in rhinovirus-infected airway epithelial cells

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MSc

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Statement of originality

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1α,25(OH)$_2$D$_3$: 1α-25-dihydroxycholecalciferol or 1α-25-dihydroxyvitaminD$_3$
2A^{pro}: viral protease 2A
3CD^{pro}: viral protease CD
3C^{pro}: viral protease 3C
A549: human alveolar epithelial cells line
AD: adenovirus
AP-1: activator protein-1
Br$^-$: bromide
BECs: bronchial/airway epithelial cells
Calu-3: human airway epithelial cells line
CARDS: caspase activation and recruiting domain
CAT: catalase
Cl$^-$: cloride
COPD: Chronic obstructive pulmanory disease
COX-2: cyclooxygenase-2
CuZnSOD: copper-zinc superoxide dismutase
CYP: cytochrome P$_{450}$
DMSO: dimethyl sulfoxide
dsRNA: double-stranded ribonucleic acid
ELISA: enzyme-link immunosorbent assay
ELAM-1: endothelial leukocyte adhesion molecule-1
EPO: eosinophil peroxidase
FCS/DMEM: foetal calf serum/Dulbecco's modified eagle medium
FCS/MEM: foetal calf serum/minimum essential medium
FEV: Forced expiratory volume in 1 second
FVC: Forced vital capacity
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
γ-GT: gamma-glutamyl transpeptidase
GM-CSF: granulocyte-monocyte-colony stimulating factor
GSH: reduced glutathione
GSHPx: glutathione peroxidase
GSSG: oxidized glutathione
H_2O_2: hydrogen peroxide
Hep-2: human respiratory (larynx) epithelial cells line
HOBr: hypobromous acid
HOCl: hypochlorous acid
ICAM-1: intercellular adhesion molecule-1
IFN: interferon
IFN-k: interferon-kappa
IFN-β: interferon-beta
IFN-γ: interferon-gamma
IFN-λ1: interferon-lambda 1 (interleukin-28A)
IFN-λ2: interferon-lambda 2 (interleukin-28B)
IFN- λ3: interferon-lambda 3 (interleukin-29)
IFN-ω: interferon omega
IKK: IK-B kinase complex
IL-13: interleukin-13
IL-1β: inteleukin-1 beta

IL-4: interleukin-4
IL-5: interleukin-5
IL-6: interleukin-6
IL-8: interleukin-8
IL-9: interleukin-9
iNOS: inducible nitric oxide synthase
IP-10: interferon-gamma-induced protein-10
IRES: internal ribosome entry site
IRF-3: interferon regulatory factor-3
IRF-7: interferon regulatory factor-7
LPS: lipopolysaccharide
MAVS: mitochondrial antiviral signaling protein
MDA-5: melanoma differentiation associated gene-5
MDCK: madin-darby canine kidney epithelial cells line
m-ICAM-1: membranous-intercellular adhesion molecule-1
MMP-9: matrix metalloproteinase-9
MnSOD: manganese superoxide dismutase
MPO: myloperoxidase
MRC-5: human lung fibrolast cells line
mRNA: messenger ribonucleic acid
NAD^+: nicotinamide adenine dinucleotide
NADPH: reduced form of nicotinamide adenine dinucleotide phosphate
NF-κB: nuclear factor-kappa B
O_2^-: superoxide radical
P1: viral genome encoded structural protein 1
P2: viral genome encode non-structural protein 2
P3: viral genome encode non-structural protein 3
PH: plecstrin homology
PI3-k: phosphotidyl inositol-3-kinase
PRR: pathogen recognition receptor
PtdIns(3)P: phosphotidyl inositol-3-phosphate
PtdIns(3,4,5)P_3: phosphotidyl inositol (3,4,5) triphosphate
PtdIns(4,5)P_2: phosphotidyl inositol (4,5) biphosphate
RANTES: regulated upon activation normal T-cell expressed and secreted
RD-ICAM-1: rhabdomyosarcoma expressing intercellular adhesion molecule-1
RIG-I: retinoic acid inducible gene
RNA: ribonucleic acid
ROS: reactive oxygen species
RSV: respiratory syntical virus
RT-PCR: real time-polymerase chain reaction
RV: Human rhinovirus
RV43: human rhinovirus-43 (rhinovirus species A and major group)
RV1B: human rhinovirus-1B (rhinovirus species A and minor group)
RV-A: human rhinovirus species A
RV-B: human rhinovirus species B
RV-C: human rhinovirus species C
RV-D: human rhinovirus species D
SEM: standard error of mean
ssRNA: single-stranded ribonucleic acid
TBARS: thiobarbituric reactive substances

Th: T helper cells (subgroup of lymphocytes)
THF: tetrahydrofuoran
TICAM: Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule-1
TLR: toll like receptor
TNF-α: tumor necrosis factor-alpha
TRIF: TIR domain-containing adapter inducing interferon-beta
URTI: upper respiratory tract infection
UTR: untranslated region
VCAM-1: intervascular adhesion molecule-1
VP1: viral capsid protein 1
VP2: viral capsid protein 2
VP3: viral capsid protein 3
VP4: viral capsid protein 4
VPg: viral small protein
vRNA: viral ribonucleic acid
XO: xanthine oxidase
Zn: Ion zinc
$ZnSO_4$: Zinc sulphate

Publication arising from this thesis

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2. FH Addnan, PAB Wark, LG Wood, ML Garg: Antiviral activity of antioxidants against rhinovirus infection. Conference Abstracts Nutrition Society of Australia and Nutrition Society of New Zealand 2011, Australasian Medical Journal 2011, 4, 12, 739-788.

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Abstract

Human rhinovirus are associated with the majority of exacerbations of asthma and chronic obstructive pulmonary disease. The epithelial cells of the airway are the primary target for invading rhinovirus and the alterations on the airway epithelium by the virus are believed to be central in enhancing the airway inflammation that leads to asthma exacerbations. The development of a conventional vaccine is not practical to fight against rhinovirus, due to the fact that there are more than 100 serotypes. Natural agents capable of interfering with viral replication warrant exploration, because as yet, no licensed effective antiviral is currently available. Hence, this thesis is conducted to provide a promising candidate against rhinovirus infection.

We utilized natural potent antioxidant compounds including resveratrol, lycopene, zinc and vitamin D at physiologically relevant concentrations, to prevent inflammatory response of airway epithelial cells induced by rhinovirus. In this thesis, we studied the anti-inflammatory effect of resveratrol, lycopene, zinc and vitamin D against the major group of human rhinovirus, (consist of 90% rhinovirus serotypes) which is using intercellular adhesion molecule-1 on host cells to gain infection. We found that enriched Calu-3 cells with those antioxidant compounds prior to rhinovirus infection, significantly prevent the virus from replicating efficiently. However, the antioxidants failed to significantly decrease the inflammatory response of Calu-3 cells induced by rhinovirus. Rhinovirus infection cause significant secretion of interleukin-6, interleukin-8 and interferon-gamma-induced protein-10 into the cultured media, hence confirming the model used for investigating the effect of antioxidant compounds against the virus.

Thorough mechanism studies to unfold antioxidants' mode of action against rhinovirus replication were conducted. The study revealed that phosphotidyl inositol-3-kinase is required during RV internalisation, and enriched Calu-3 cells with resveratrol, lycopene and vitamin D decreased the activation level of phosphotidyl inositol-3-kinase, hence explained the significant decrease of viral titers observed earlier in the rhinovirus infection study. Verification studies were done using wortmannin which is a specific inhibitor of phosphotidyl inositol-3-kinase and visualizing AlexaFluor 555-labelled rhinovirus entry into Calu-3 cells by confocal microscopy. Antioxidant compounds were found not to have any significant effect in the course of viral translation and viral replication steps.

Resveratrol, lycopene, vitamin D and zinc, were demonstrated to have beneficial roles in limiting rhinovirus replication in Calu-3 cells. Preventing or ameliorating rhinovirus replication will hopefully bring significant impact towards managing asthmatic patients who are at high risk of suffering rhinovirus-induced asthma exacerbations.

CHAPTER 1: BACKGROUND

1.1 Introduction

Asthma is a chronic inflammatory disorder of the airways in which many cells and elements play a role. A number of features characterize asthma including airway hyper-responsiveness, reversible airflow obstruction, and airway inflammation [1, 2]. Recent evidence shows that airway inflammation is an integral component in the pathogenesis of asthma and a driving force in airway hyper-responsiveness and the propensity to airflow obstruction. Virus infections, in particular human rhinovirus (RV), are associated with the majority of exacerbations of asthma and chronic obstructive pulmonary disease (COPD) [3, 4]. Johnston et al.[3] found that about 80-85% of asthma exacerbations in school age children are associated with upper respiratory viral infections and picornaviruses (mostly RV) accounted for two thirds of these viral infections. An earlier study conducted by Nicholson et al. [4] found that respiratory virus infections commonly cause, or are associated with, exacerbations of asthma in adults. In addition, a recent review article by Dulek and Peebles [5] on viruses and asthma reported that RV has a clear association with acute asthma exacerbation and RV infection during early life is associated with the development/inception of asthma in later life.

The development of a conventional vaccine is not practical to fight against human respiratory viruses causing the ‘common cold’, particularly RV (accounting about 50% number of cases) [6], due to the fact that there are more than 100 serotypes. Natural agents capable of interfering with viral replication warrant exploration, because as yet, no licensed effective antiviral is currently available for the treatment of the common cold [7]. The epithelial cells of the airway are the primary target for invading RV. The airway epithelium acts as physicochemical barrier to the external environment (from

harmful irritants and noxious substances) and is well documented as being fundamental to asthma pathogenesis [8]. The alterations on the airway epithelium by RV are believed to be central in enhancing the airway inflammation that leads to asthma exacerbations. RV targets the epithelial cells in which they replicate [9] and initiate innate immune responses [10, 11].The epithelial cells produce various pro-inflammatory mediators which result in significantly increased airway inflammation, including increased interleukin-6 (IL-6) [9, 12], interleukin-8 (IL-8) [9, 13-15], interferon-gamma induced protein-10 (IP-10) [16], and intercellular adhesion molecule-1 (ICAM-1) [17]. RV infection also leads to the intracellular production of the highly reactive superoxide anion and results in increased oxidative stress [18]. Although the human airway epithelium is equipped with very high concentration of GSH [19, 20], this concentration is probably inadequate to scavenge the damaging effect of free radicals produced due to RV infection. One consequence of this increased oxidative stress is activation of redox-sensitive transcription factors, such as nuclear factor-kappaB (NF-κB) and activator protein (AP-1), which are critical to transcription of pro-inflammatory genes (IL-8, IL-6 and TNF-α) [21-23]. Membrane bound-ICAM-1 (m-ICAM-1), the receptor for 90% of RV, is an adhesion protein that has a central role in inflammatory cell recruitment following RV infection [24]. The expression of m-ICAM-1 by respiratory epithelial cells is via a mechanism that is NF-κB dependent [18]. Research has revealed that virus infections frequently result in the generation of oxidative stress in the infected cells. Thus the life cycle of several viruses are influenced by host-cell redox state [25-29].

Based on the numerous studies demonstrating the central role that the airway epithelium plays in regard to RV infection, it may therefore be beneficial to equip these cells with antioxidant compounds. Antioxidants may alleviate the oxidative stress-

mediated pro-inflammatory response generated by viruses. In this regard, antioxidant compounds such as vitamin D [30], zinc (Zn) [31], lycopene [32] and resveratrol [33] may be beneficial in protecting against RV infection. There has been an increase in scientific evidence dealing with the protective role of polyphenols during oxidative stress. The beneficial effects of polyphenols relate to their anti-inflammatory and antioxidant properties. Retrospective and prospective epidemiological studies have found protective effects of high consumption of fruits and vegetables (rich in polyphenols) in the diet against chronic diseases [34-37]. Those effects have been linked and credited to the antioxidant vitamins and polyphenol compounds they contain. Resveratrol (3,4,5-trihydroxystilbene) is a naturally occurring polyphenol presents in many plants, nuts and fruits and is abundant in grapes. It's prime function is to protect the plant from injury, UV irradiation, and fungal attack. Resveratrol has been proposed to exhibit antioxidative [38, 39], antiproliferative [40], and anti-inflammatory properties [41]. The anti-inflammatory effects of resveratrol have been associated with inhibition of the transcription factor NF-κB and AP-1 [42], inhibition of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-8 release, and COX-2 expression [43].

This study examines the effect of resveratrol, and other antioxidant nutrients, including lycopene, vitamin D and zinc, on the inflammatory response to RV infection. RV is the major cause of exacerbation in asthma and COPD [4, 44, 45]. Guan et al. [46] has studied the cytopathogenic inhibitory effect of resveratrol using different types of cells (i.e., MDCK, A549, Hep-2 cell and MRC-5) exposed separately to respiratory viruses, specifically the influenza virus type AFM1 strain, rhinovirus type R_{14}, RS virus and AD virus type 7. They found that resveratrol significantly inhibited the cytopathogenic effect of AD virus type 7 at the concentration of 120 ug/ml, however,

the mechanism behind this effect needs to be clarified. This project will extend these observations, by examining resveratrol and other antioxidant compounds, in relation to RV infection, in an airway epithelial cell line. This is particularly relevant to asthma and COPD, as epithelial cells are the initial site of virus infection. This thesis will employ *in vitro* models of airway epithelial cell infections. Calu-3 cells will be used as an *in vitro* model of airway epithelial cell infections as they provide an efficient preclinical model to study human respiratory viruses [47-50]. Calu-3 cells are a well differentiated and characterized cell line, that is believed to be derived from human bronchial submucosal glands serous cells [49], demonstrating similar characteristics of the primary normal human bronchiol epithelial cells *ex vivo* [51]. Calu-3 cells also has been used as a respiratory model in drug delivery studies [52, 53]. The investigations will focus on the inflammatory response of the airways to RV infection, the roles played by the antioxidants on the RV life cycle, and the mechanisms that lead to that response.

1.2. Etiological factors in the development of asthma

The main factor involved in the pathophysiology of asthma is inflammation and evidence suggests that airway inflammation is a driving force in airway hyper-responsiveness, resulting in airflow obstruction. Airway cells that are involved in the inflammatory cascade include lymphocytes, mast cells, eosinophils, neutrophils, macrophages and also epithelial cells. The recruitment of inflammatory cells to the site of injury is due to inflammatory mediators, regarded as chemokines, that are highly expressed by epithelial cells [54].

It is well recognized that allergen, occupational agents, infections, pollutans and drugs are asthma potent triggering factors that are involved in the inception or

worsening of the disease. Allergens has been well demonstrated to induced airway inflammation and airway hyperresponsiveness [55]. These conditions induced by allergens can be explained via role-play by mast cells. Mast cells is well regarded as a type of cells that plays an important role in coordinating the early event of innate and adaptive immunity due to allergens contact [56]. Allergens has been shown to activate the maturation of CD4+ T helper cells [57], which then resulted in production of immunoglobulin (Ig) E. Mast cells reside at airway epithelium, then bind to IgE (mast cells wall membranes has Fc receptor), and subsequently activating mast cells. Activated mast cells resulted in secretion of wide range of mediators and these include histamine, tryptase, oxygenated of arachidonic acid derived leukotriene (LTC_4, $LTCD_4$, $LTCE_4$), prostaglandin (PGD_2) and HETEs, and cytokines (IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, GM-CSF, TNF-α). The mediators secreted by mast cells play an important roles in attracting basophils, eosinophils, neutrophils and macrophages, hence provide a robust explanation that allergen is strongly involve in inducing airway inflammation and airway hyperresponsiveness [58].

Environment related workplace contain various agents (over 300 agents) which have been identified in development of asthma (or known as occupational asthma). In general, these agents are classified either as high molecular weight agents (consist of animal-derived material, plant-derived material, enzymes) or low molecular weight agents (including spray paints, wood dust, biocides) or irritant agents (including chlorine, acetic acid, isocyanates) [59]. These agents induced asthma through either immunological mechanisms (IgE-dependent or IgE-independent) or through non-immunological mechanisms [59].

Pollutants such as sulphur dioxide (SO_2), nitrogen dioxide (NO_2), ozone and particulate matter air pollutants have been identified as an agent implicated in the inception or exacerbations of asthma [60, 61]. These pollutans have been shown to induce airway inflammation [62], airway hyperresponsiveness in a number of studies (ozone) [63-65]. Exposure to nitrogen dioxide has been shown to cause wheezing bronchitis [66].

Nonsteroidal anti-inflammatory drugs (NSAIDs), in particular aspirin has been shown to induce asthma [67, 68]. However, the exact mechanism of how aspirin-induced asthma is not well understood, but it is believed that this drug induced asthma through abnormality of arachidonic acid metabolism which produces biologically active lipid mediators. This drug induced bronchoconstriction via blocking cyclooxygenase enzyme 1 and 2 of arachidonic acid catabolism, which then lead to oxygenation of arachidonic acid only via lipoxygenase enzyme. Hence, result in the production of leukotrienes which causes bronchoconstriction to the airway walls.

Evidence suggests that the development and regulation of inflammation is associated with both innate and adaptive immune responses [69]. Human airways produce T helper (Th) 2 lymphocytes due to exposure to common environmental allergens in order to protect the airway from damage. Th2 cells produce a family of cytokines (such as IL-4, IL-5, IL-6, IL-9, and IL-13) that are involved in allergic inflammation. The balance between Th1 and Th2 cytokine is an important determinant in allergic asthma and numerous studies have reported a shift towards Th2 cytokine [8, 70-72]. Th1 cells play an important role for defending the airways from events like infection via activated defense mechanism by producing IL-2 and interferon-γ (IFN-γ).

1.3 Diet and asthma

Dietary change is one of several causal factors implicated in development of asthma. In the past two decades the body of evidence suggesting a relationship between diet and asthma has increased substantially. The emerging research on diet and asthma are originally based on the hypothesis developed by Seaton et al.[73]. Seaton et al.[73] proposed that the increase in asthma prevalence during the 18th and 19th centuries (during industrial revolution period) was not due to exposure to exogenous factors (such as indoor or outdoor pollutants, cigarette smoke and allergen). Rather, they identified evidence to support the hypothesis that host resistance has declined due to the alterations in dietary pattern that are associated with the western lifesyle [73]. Inflammation is considered as the central factor in asthma pathogenesis, and dietary factors associated with a western dietary pattern have been shown to modulate inflammation [74]. Most studies have focused on dietary antioxidants i.e., vitamin C, vitamin A, vitamin E, selenium, zinc and polyphenols and also omega 3 fatty acids. Vitamin C, β-carotene, magnesium and selenium have been shown to provide protection against asthma in some studies [75-79], which may be due to the inhibition of lipid peroxidation (prevents or limits an inflammatory response) and restoration of the oxidant-antioxidant imbalance in the airways. There is substantial evidence that antioxidant defences are reduced in asthma [80-83]. Based on these observations, the intervention or management of asthma via increasing the consumption of dietary antioxidants has merit. Unfortunately, to date, studies using antioxidant supplementation in asthma are inconclusive (Table 1.3.1). For instance, Gao and colleagues [84] conducted a meta-analysis of 10 observational studies using asthma or wheeze and lung function as an outcome. They found that, a higher dietary intake of antioxidants vitamins C, E and β-carotene was not associated with a lower risk of

having asthma [84]. Yet other studies have demonstrated that supplements of vitamins E and C supplements are beneficial in asthmatic adults [85]. In summary, further evidence from randomised-controlled trials are needed to understand the specific role of antioxidant supplementation in patients with asthma [86].

Table 1.3.1. The relationship between antioxidant nutrients (including lycopene) and asthma.

Nutrients	Outcomes	Authors
Vitamins A, C and E	A total of 40 studies were included in a systematic review and meta analyses to determine whether vitamins A, C, and E are associated with asthma. The study found that low dietary intakes of vitamins A and C are associated with significant increased odds of asthma and wheeze, whilst vitamin E does not appear to be related to asthma status.	Allen et al. [87]
Vitamin C, α-tocopherol, lycopene and β-carotene	The plasma levels of vitamin C, α-tocopherol, lycopene and β-carotene were significantly lower in children with asthma compared with healthy controls.	Sackesen et al. [88]
Vitamin C	Plasma levels of vitamin C was found significantly lower in symptomatic than in asymptomatic cases of asthma.	Patel et al. [78]
Vitamins C, E and β-carotene	A meta-analysis study was undertaken to examine the association between intake of vitamin C, E and β-carotene and the risk of asthma. The study found that, a higher dietary intake of vitamins C, E and β-carotene was not associated with a lower risk of having asthma.	Gao et al. [84]
Vitamins E, C	A double-blind crossover study was designed to evaluate the effects of intake of vitamins C and E on ozone-induced bronchial hyperresponsiveness in asthmatic adults. Supplementation with vitamins C and E was shown protective against low level of ozone exposure followed by sulphur dioxide challenges.	Trenga et al. [85]

1.4 Inflammation in asthma

As described previously, asthma is an inflammatory disease of the airways, involving infiltration of the epithelium with activated mast cells, macrophages, eosinophils as well as neutrophils. Epithelial cell defence against inflammatory triggers (such as human RV infection) involves activating the host defence and tissue-repair responses. Inflammation is described as an adaptive response that is triggered by noxious stimuli and conditions, leading the affected host tissue to take an immediate action to restore homeostasis. Asthma is regarded as a chronic inflammatory disease of the airways due to the involvement of a complex interaction between many inflammatory and structural cells. Neutrophils and eosinophils infiltrate the airways, mast cells degranulate, membranes thicken, epithelial cells lose integrity, and mucus occludes the bronchial lumen. In addition, bronchial smooth muscle undergoes hyperplasia and hypertrophy. All these features are consequences of inflammation. Bronchial specimens from asthmatic patients show marked inflammation and inflammatory processes occurring within the airways [89-91].

1.4.1 Mediators of Inflammation in Asthma

In asthma, based on their origin, mediators of inflammation can be classified into general and structural cells. General cells include mast cells, macrophages, eosinophils, T lymphocytes, dendritic cells, basophils, neutrophils and platelets. On the other hand, airway epithelial cells, smooth muscle cells, endothelial cells and fibroblasts are classified as structural cells. The secretion of inflammatory molecules will have effects such as bronco-constriction, mucus secretion, attraction and activation of inflammatory cells and structural changes in the airways. The structural changes in the airways include fibrosis, developed as a consequence of collagen deposition, thickening of

airway smooth muscle due to hyperplasia and hypertrophy, development of angiogenesis and mucus-secreting cells. This following section focuses on mediators of inflammation that are relevant to the bronchial epithelial cells, and considers their role in the development of asthma as an inflammatory disease.

1.4.2 Cytokines

Cytokines are signalling proteins produced by many different cell types. Cytokines provide a mechanism for interaction between cells. They act on target cells to cause a wide array of cellular functions including activation, proliferation, chemotaxis, immuno-modulation, release of other cytokines or mediators, growth, cell differentiation and apoptosis. Cytokines with chemotactic properties are better known as chemokines and are actively involved in attracting leukocytes into tissues. Chemokines play a significant role in the pathogenesis of asthma as demonstrated by presence of several cell types in asthmatic airways.

1.4.3 Pro-inflammatory cytokines

Cytokines classified as pro-inflammatory include: interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and granulocyte-macrophage colony stimulating factor (GM-CSF). The primary function of TNF-α is to stimulate airway epithelial cells to produce cytokines including secreted protein RANTES, interleukin-8 (IL-8), and GM-CSF [92-94]. TNF-α has been reported to have synergistic effects with interleukin-4 (IL-4) and interferon-γ (IFN-γ), to increase the expression of vascular adhesion molecule-1 (VCAM-1) and lead to increased adhesion of inflammatory molecules such as leukocytes to the site of inflammation [95]. IL-6 is secreted by bronchial epithelial cells in association with viral infection [96, 97]. Bronchial epithelial

cells are a main source of GM-CSF, that function primarily to enhance the survival rate of eosinophils in the lumen of asthmatic airways [98]. In addition to producing pro-inflammatory cytokines, bronchial epithelial cells also produce chemokines during the inflammatory process. For example, RANTES is a potent chemoattractant for eosinophils, basophils and T-lymphocytes [99] and is expressed by bronchial epithelial cells after stimulation with interleukin-1β (IL-1β), TNF-α and IFN-γ [92]. Similarly, interleukin-8 (IL-8) has a significant role as a chemotactic agent and activator of neutrophils. Epithelial cells produce IL-8 after treatment with IL-1β or TNF-α [100] or after infection with respiratory virus [101].

1.4.4 Transcription Factors

The dominant transcription factors that are involved in the inflammatory process in asthma are NF-κB and AP-1. In general, transcription factors are DNA-binding proteins that play an important role in regulating the expression of inflammatory genes and expression of enzymes involved in the synthesis of inflammatory mediators. In asthma, the expression of inflammatory mediators is regulated at a transcriptional level [102, 103]. Therefore, down-regulation of these transcription factors may be useful in preventing asthma. Chronic inflammation in asthma has been associated with the activation of NF-κB. Once activated, NF-κB induces genes for mediators of inflammation including pro-inflammatory cytokines, synthesis of enzymes involved in secretion of inflammatory molecules and recruitment of eosinophils by chemokines and adhesion molecules.

NF-κB resides in the cytoplasm of the unstimulated cells. In unstimulated cells it is binded to its inhibitory proteins (IκB). Pathogenic stimuli causes the release of IκB

from the NF-κB and subsequently NF-κB translocates to the nucleus, binds to promoter region on DNA, and thereby induces the synthesis of various pro-inflammatory cytokines including IL-6, IL-8, IP-10 [12, 16, 104, 105]. Oxidative stress [106-108], dsRNA [105], cytokines including TNF-α [104, 109], and IL-1α [110] were demonstrated to induce activation of NF-κB. NF-κB is a family of seven structurally related transcription factors namely p65 (RelA), p50, RelB, C-Rel, p52, p105 and p100 [111]. The activated form of NF-κB is heterodimer which usually consists of two subunits, p65 and p50, and this form is widely studied in relation to RV infection on human epithelial cells [14, 105, 109, 110].

In unstimulated cells, this heterodimer subunits p65/p50 (NF-κB) bind to its inhibitory proteins (IκB). The inhibitory protein, IκB is phosphorylated by IκB kinase, resulting in cleavage of the IκB from the NF-κB complex and destruction of IκB in the proteosome [112]. IκB kinase (IKK) complex consist of three subunits, IKKα, IKKβ, and IKKγ. IKKα and IKKβ are catalytic subunits of IKK. Both catalytic subunits exhibit similar substrate specificities, targeting two specific serines on regulatory domain of IκB proteins [113]. However, IKKβ is more potent than IKKα in phosphorylation of IκB proteins [114]. In addition, IKK was also shown to phosphorylate p65 on p65/p50 heterodimer, for activation of NF-κB (nuclear function) [109]. Hence, it is very clear that activation of IκB kinase provides a central regulatory point in controlling the activation of NF-κB.

A number of evidence have shown that enhanced NF-κB pathway in asthmatics tissues, including peripheral blood mononuclear cells (PBMC), bronchial biopsies and sputum cells, when compared to non-asthmatics [115], hence, have provided strong

basis on the importance of NF-κB pathway in asthma. However, targeting the NF-κB pathway in providing efficacy against the disease is yet to be established [115].

1.5 Oxidative stress in asthma

Oxidative stress has been shown to be important in the pathophysiology of asthma [116]. Oxidative stress is a consequence of host antioxidant defences being overwhelmed by an excessive quantity of free radicals, including reactive oxygen and nitrogen species. Among the most prominent manifestations of oxidative stress are events such as remodelling of blood vessels, elevated mucus secretion, apoptosis and cell proliferation. Increased levels of reactive oxygen species (ROS) have been further implicated in initiating inflammatory responses in the lungs through the activation and translocation of transcription factors, signal transduction and gene expression of pro-inflammatory mediators [117-119].

The inflammatory cells recruited to the bronchial sites have been reported to produce increased amounts of reactive oxygen species, which can lead to oxidative stress [120, 121]. Activated eosinophils, neutrophils, monocytes and macrophages can generate superoxide (O_2^-) via the membrane associated NADPH-dependent complex. Endogenous antioxidant enzymes, in particular superoxide dismutase, reacts with the superoxide radical to form hydrogen peroxide (H_2O_2), which subsequently reacts with potent cytotoxic radicals [hypohalous acids (HOCL or HOBr)]. This occurs through its interaction with a halide (Cl^- and Br^-) in a reaction catalysed by myeloperoxidase (MPO) in the case of neutrophil or monocyte-mediated inflammation, or eosinophil peroxidase (EPO) in the case of eosinophil-mediated inflammation [122, 123]. In addition, Wu et al. [124] reported that the end product of nitric oxide ($^{\cdot}NO$) metabolism,

nitrite, is used by MPO and EPO together with H_2O_2 to promote formation of reactive nitrogen intermediates. Eosinophils have higher capacity, compared to neutrophils, to exert oxidative injury because they can generate higher amounts of O_2^- and H_2O_2, and higher concentrations of EPO [125, 126].

Asthmatics have increased oxidative stress in the airways compared to healthy controls and this increases with disease severity [127]. Research has also found that there is a strong association between asthma progression and the excessive production of oxidants in blood leucocytes, lipid peroxidation and reactive products in the urine, and plasma in asthmatic patients [120, 121, 128, 129]. These findings have highlighted the potential role for antioxidants as an intervention strategy in relation to the pathogenesis of asthma.

1.6 Viruses and asthma

Virus infection is another important player that can induce the development of asthma. Several studies have demonstrated that virus infection is the most common cause of exacerbations in asthma [130-135]. Exacerbations of asthma involve a progressively worsening shortness of breath, cough, wheezing and chest tightness or any combination of these symptoms as measured by a decrease in expiratory airflow [136]. The health burden of asthma exacerbations is massive. For instance, 37830 hospitalisation were recorded in Australia just in the period of 2010 to 2011 [137]. In the United State of America (USA) alone, asthma exacerbations accounted for 14.7 million outpatient visits, 1.8 million visits to emergency room, 497000 hospitalisation and 4055 deaths, in the year 2004 [138]. In a study by Kuga and colleagues [139], a strong relationship was found between human RV infection and exacerbations of

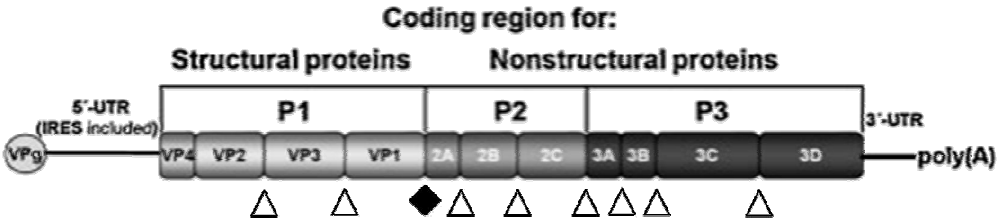
bronchial asthma. The authors reported that human RV contributes to approximately 94% of picarnovirus infection-induced exacerbations in asthmatic patients, detected using real time (RT)-PCR.

The airway epithelium is the first line of defence against RV infection and activates an immune response to the cytotoxic effects caused by RV invasion. Subauste and colleagues [9] successfully developed a model system of human RV infection using a human respiratory epithelial cell line. This model has been successfully used by Wark et al in a number of studies [97, 140, 141], providing a useful tool for examining the early events/mechanisms associated with the pathogenesis of RV infections leading to exacerbations in asthmatic patients. RV infection has been shown to induce increased production of IL-8, IL-6 and GM-CSF from epithelial cells. In addition, the secretion of the chemokine IL-8 correlates with RV replication in the first 24 hr of infection. The stimulation of IL-8 by infection with human RV is mediated through the production of oxidative species [142] and the subsequent activation of the redox signalling molecule, NF-κB. Papi and colleagues [18] also demonstrated that RV infection of human respiratory epithelial cell lines induces a rapid increase in intracellular oxidative species (in particular superoxide anion). The increased level of oxidative stress by respiratory virus infection affected the balance between reduced glutathione (GSH) and oxidized glutathione (GSSG) within the cells [26]. Following RV infection, human epithelial cells have been shown to express intracellular adhesion molecule-1 (ICAM-1), which is the receptor for the majority (90 %) of RV [18, 143] and also upregulates the level of the LDL-receptor family [144] which is the receptor for minor group RV [145]. Thus, ICAM-1 and the LDL-receptor family may also play a crucial role in promoting airway inflammation during virus induced exacerbations in asthma.

1.6.1 Human Rhinoviruses (RV)

RV is a non-enveloped, icosahedral virus of about 30 nm in diameter (icosahedral protein shell), consisting of uncapped single stranded (+) RNA genome of approximately 7200 -7400 bases [146, 147]. The icosahedral protein shell is composed of 60 separate identical units called protomers, with each protomer consisting of 4 proteins VP1, VP2, VP3 and VP4. The VP1, VP2 and VP3 are located on the surface of the capsid, whereas VP4 is located inside the virion [146]. The viral RNA genome is flanked by the 5' and 3' untranslated region (UTR) (Figure 1.6.1.1). A small protein known as VPg is covalently attach to the 5' UTR containing internal ribosome entry site (IRES), while the 3' UTR has a polyadenylated tail like cellular messenger RNA [147]. The viral genome encodes a single large polyprotein of approximately 250 kD using cellular ribosomes. A single large virus polyprotein is matured by two virus-encoded proteases, 2A ($2A^{pro}$) and 3C ($3C^{pro}$) resulting in 11 functional proteins (4 structural and 7 non-structural) (Figure 1.6.1.1). At first, $2A^{pro}$ and $3C^{pro}$ release themselves from the polyprotein by self cleavage. Then, $2A^{pro}$ cleavage between P1 and P2, after that $3CD^{pro}$ is released from the P3 by self cleavage, then $3C^{pro}$ and $3CD^{pro}$ cleavage proteins of the P1 (capsid regions), P2 and P3 (non-structural proteins) region (Figure 1.6.1.1). The non-structural protein 3D functions as an RNA-dependent RNA polymerase [148], 3B possibly functions as a primer for RNA synthesis [149] and the other non-structural proteins (2B, 2C, 3A) are associated with RNA replication (Figure 1.6.1.1). RV belongs to the genera of *enterovirus,*classified in the family of *picornaviradae*. RV formerly belongs to the genera *rhinovirus*, however due to high similarities in terms of genome sequence and organization (Figure 1.6.1.1) from *enterovirus*, both genera are combined together (www.picornastudygroup.com/taxa/species/species.htm). In general, the family of *picornaviridae* have 8 genera namely *enterovirus*, *hepatovirus*, *cardiovirus*,

kobuvirus, teschovirus, erbovirus, aphthovirus and *parechovirus*. Recently, RV has been broken down into 4 species namely RV-A, RV-B, RV-C and RV-D (www.picornastudygroup.com/taxa/species/species.htm). Based on the antigenicity, the RV species have been further broken down into serotypes. To date, about 100 RV serotypes have been discovered and according to currently approved taxonomy, 75 and 25 serotypes belong to RV-A and RV-B species, respectively. Based on the way RV serotypes attach to the host cells, the serotypes are grouped into either major group (90% of RV attach via ICAM-1 surface receptor) or minor group (10% of RV attach via a family of LDL receptor). This thesis employed RV from both serotype subgroups which are RV-43 (major group) and RV-1B (minor group), in order to broaden the scope and implications of the study. In addition, both serotypes are RV-A species.



The coding regions are divided for structural proteins [P1 (VP4, VP2, VP3, VP1)]) and non-structural proteins [P2 (2A, 2B, 2C), P3 (3A, 3B, 3C, 3D)]. The genome codes for a large polyprotein are matured by virally encoded proteases
(◆$2A^{pro}$ cleavage site; △$3C^{pro}$ cleavage sites) [147, 150].

Figure 1.6.1.1. Enterovirus genome organization

1.7 RV, oxidative stress and cellular dysfunction

The ability of RV to induce oxidative stress can be demonstrated by alterations in a variety of cellular functions. For example, xanthine oxidase (XO) activity is altered by RV infection. XO is an enzyme system which is found exclusively in the cytosol and

expressed and secreted by numerous surface epithelia, [151]. The primary function of XO is on purine catabolism, as it catalyzes oxidation of hypoxanthine to xanthine and subsequently xanthine to uric acid. Uric acid and its oxidation product allantoin are known potent antioxidants, acting as free radical scavengers. In addition, XO has been shown to have bactericidal properties, being activated in response to bacterial infection *in vivo* [152]

During RV infection, the production of the superoxide radical ($O_2^{\cdot-}$) intracellularly by XO enzyme system has been well described [29]. Furthermore, Kaul et. al. [153] demonstrated that following RV infection on BEC, the antioxidant copper zinc superoxide dismutase (CuZnSOD) was significantly upregulated.

Another aspect that could link oxidative stress and anti-viral responses could involve the 'cellular power house' organelle known as mitochondria. Oxidative stress induces mitochondrial dysfunction and subsequently impairs responses to the RV infection. Currently (unpublished data from our group) has found that impairment of mitochondrial antiviral signaling (MAVS) by oxidative stress impair the RV-infected cell ability to induce proteins responsible for antiviral action (interferon-α and interferon-γ), thus facilitate the RV infectivity. A study conducted by Seth et al.[154] has shown that silencing MAVS expression through RNA interference abolishes the activation of NF-κB and IRF-3, hence abrogated the production of antiviral protein (IFN-β). Mitochondrial modifications may also lead to unwanted effects such as induction of apoptosis. It has been demonstrated that apoptosis is triggered by cells infected with RV, contributing to the destabilization of the infected cell, thus facilitating viral progeny release [155]. RV infection has been shown to induce apoptosis on HeLa

cells [156]. Deszcz et al. [155] have shown that following RV-14 infection, apoptotic events were observed in airway epithelial cells via the activation of caspase-9 and release of cytochrome-c from the mitochondria to cytosol. Based on the findings, it is concluded that RV infection triggered airway epithelial cells-induce apoptosis via mitochondrial dysfunction [157]. However, the role of oxidative stress in this process is yet to be elucidated.

Emerging accumulated evidence has shown that viral infection can act as an intracellular oxidative stressor [25, 26, 29, 158]. Among the first line of defence within the cell is glutathione. Reactive oxygen species (ROS) such as the superoxide anion (O_2^-), are counteracted by the superoxide dismutase enzyme and hydrogen peroxide (H_2O_2) is produced. Subsequently, H_2O_2 is detoxified by either CAT or GSHPx to yield water, oxygen, and oxidised glutathione (GSSG). The increase in oxidized glutathione triggers signal transduction, involving Nf-κB and the release of pro-inflammatory cytokines such as IL-6 and IL-8.

1.8 RV and innate immunity

Multiple pathways are involved in the innate immune response of bronchial epithelial cells (BECs) to invading RV. The primary objective of these pathways is to activate NF-κB and interferon response factor-3 (IRF-3) to secrete cytokines (inflammatory) and anti-viral proteins (interferon) [159]. The infection of BECs by RV is recognized by proteins called pattern recognition receptors (PRRs), which can be broken down into toll-like receptors (TLRs) and RIG-like receptors (RLR), including retinoic acid inducible protein-I (RIG-I) and melanoma differentiation associated gene-5 (MDA-5)[159]. Based on the current literature, the innate immune pathways involve the

recognition of virus by TLRs (localized on the membranes of the host cells and on the vesicles membranes in the cytoplasm) and RLR (localized in the cytoplasm).

TLRs are activated by recognition of specific molecular patterns found in microbial pathogens [160] and trigger inflammatory and anti-viral responses [161]. On the other hand, RLRs are activated by recognition of viral RNA [162, 163]. RIG-I has been shown to be activated by single-stranded RNA (ssRNA) containing a terminal 5-triphosphate [164, 165] and by linear double-stranded RNA (dsRNA) no longer than 23 nucleotides [166, 167]; whilst MDA-5 has been shown to be activated by long strands of dsRNA [166, 167].

It has been shown that, induction of the innate immune response is mediated by TLR 3 and MDA-5 during RV invasion of BEC [168]. The authors found that, knocking down either TLR-3 (through adaptor TRIF/TICAM) or MDA-5 caused significantly decreased expression of RV1B-induced mRNA expression of IFN-β, IFN-λ1, IFN-λ2/3, interferon regulatory factor (IRF)-7 and IP-10/CXCL-10. Paradoxically, knock down of MDA-5 significantly increased in RV1B-induced mRNA expression of IL-8 [168]. In addition, they found decreased IRF-3 function downstream of MDA-5 and TLR-3. The silencing of IRF3 significantly reduced RV1B-induced expression of IFN-β, IFN-λ1, IFN-λ2/3, interferon regulatory factor (IRF)-7 and IP-10/CXCL-10. Based on the findings by Wang et al. [139], the activation of IRF-3 is crucial not only in the induction of the type I IFN but also of type III IFN. The induction of interferon-β (IFN-β) is not only useful in limiting viral replication, but also has anti-inflammatory effects [169].

The presence of dsRNA is sensed by TLR-3, RIG-I and MDA-5. Once the host cells detect the dsRNA via TLR-3, RIG-I and MDA-5, the proteins then bind to their unique adaptor proteins known as TIR-domain-containing adaptor-inducing interferon-β (TRIF) for TLR-3 and interferon-β promoter stimulator (IPS)-1 (also called VISA) for RIG-I and MDA-5. The engagement of TRIF initiates specific signalling leading to activation of transcription factors IRF-3 and NF-κB, and the consequent induction of type I interferon and inflammatory cytokines. Both RIG-I and MDA-5 contain homologous cytoplasmic helicases that contain two-amino terminal caspase activation and recruitment domains (CARDS) and a carboxy-terminal DExD/H-Box RNA helicase domain. Both RIG-I and MDA-5 sense dsRNA via the helicase domain and signal through CARDS domain to its adaptor as mentioned earlier.

Interferons play an important anti-viral function. In general, interferons can be classified into three classes which are type I, type II and type III. Type I interferons (IFN) can be broken down into IFN-α, IFN-β, IFNK, IFN-ω and IFN-v.The most widely studied are IFN-α and IFN-β, due to their important role in innate immune responses to viral infection. IFN-γ belongs to the type II class of interferons; whilst IFN-λ1 (IL-28A) and IFN-λ2/3 (IL-28B/IL-29) belong to the type III class of interferons. Contoli et al. [170] have demonstrated that type III IFNs are secreted by BECs following RV infection. It has been shown that, asthmatic subjects have deficient innate immune responses to virus infection, as demonstrated by a reduced induction of type I IFNs (IFN-β) [141] and type III IFNs (IFN-λ1 and IFN-λ2/3) [170]. Therefore, interferons play very important roles in eliminating the infectivity of RV and also in halting pro-inflammatory anti-viral responses.

1.9 Replication of RV

In general, the replication of RV in the cell will go through several steps, including receptor binding (will determine the amount of virus that can bind and enter the cells), viral entry, uncoating of the capsid, viral RNA penetration into the cytoplasm and finally replication (forming the dsRNA). An explanation on how antioxidants may possibly interfere with RV replication and infectivity is best divided into two parts, which are firstly, virus internalization and secondly, the recognition of viral capsid and dsRNA by pathogen recognition receptors (PRRs) mediating downstream signalling toward releasing antiviral proteins. Virus internalization is a term describing the virus entering the cell followed by virus attachment and is achieved by a process known as endocytosis. Evidence suggests that upon production of RV dsRNA, PRRs express the RNA helicase MDA-5 to mediate the immunomodulatory responses. Recently, the immunomodulatory mediated pathway has been known to be controlled and coordinated by proteins found in the cell mitochondria. RV enters the cells via endocytosis and then accumulates in the intracellular compartment (endosome). The binding of RV to the ICAM-1 receptor triggers uncoating of the viral RNA genome from the viral capsid. The release of the RNA genome is followed by the synthesis of RV protein [translation of viral genome protein (VPg)] with the involvement of cellular (host) ribosomes. The RNA genome then serves as a template for RNA replication in association with cellular membranes (membrane vesicle). In the membrane vesicle, positive-strand RNA is copied by viral RNA polymerase to form negative-strand RNA which then is used to produce positive-strand RNA (forming viral double-stranded RNA). The positive RNA strands are packaged into new viral capsid (viral assembly) in the cytosol, then the completed virions are released from the host cells, completing the cycle. It is suggested that the completed virions are released from host cell via cell lysis [171], however the

mechanism of this step is largely unknown. The steps of viral replication are shown in Figure 1.9.1. The inhibition of virus attachment (binding), capsid uncoating, protein and RNA synthesis are aspects of this pathway that could be modified by antioxidants, in order to interfere with viral replication.

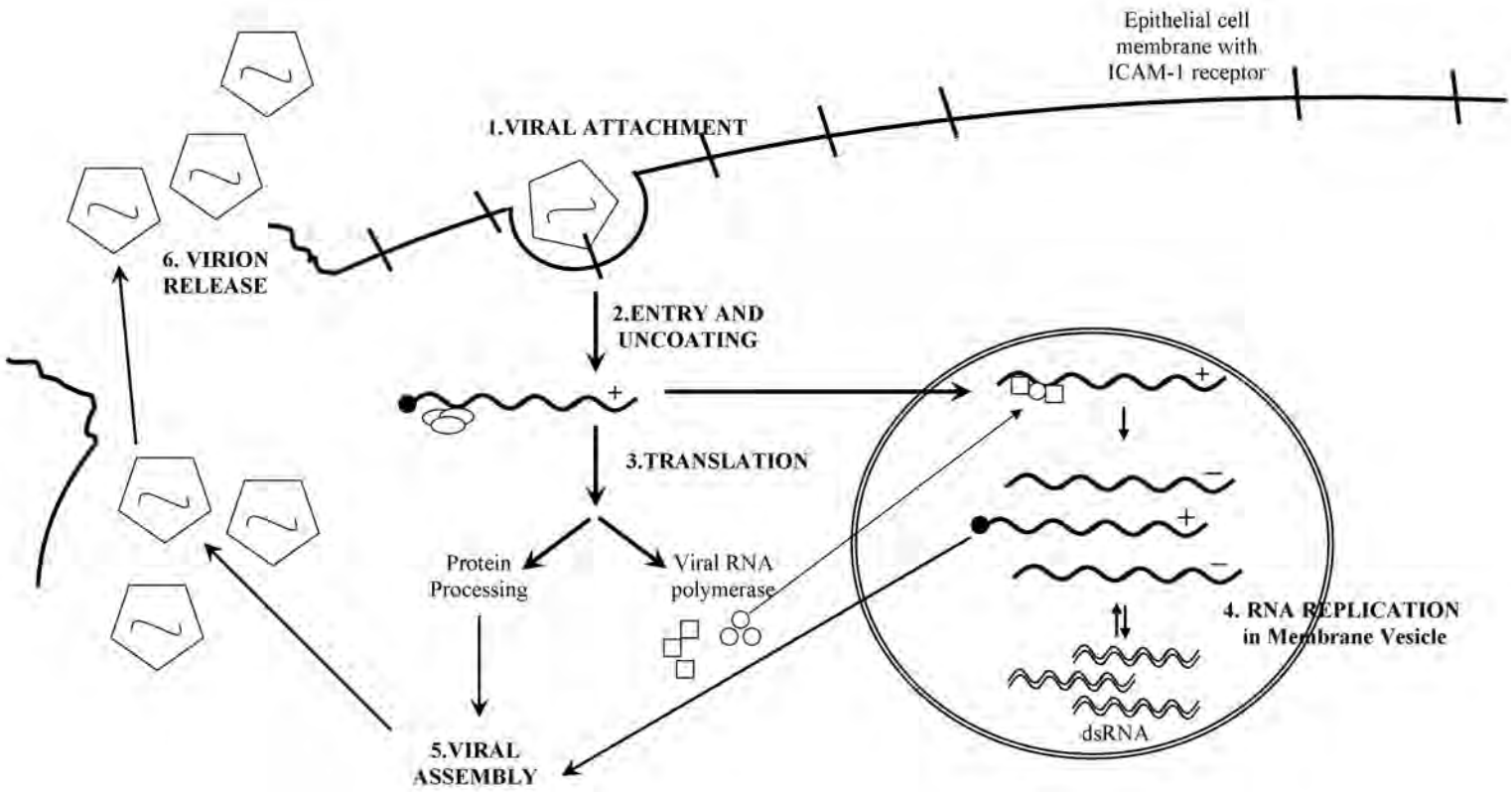


Figure 1.9.1. Overview of the rhinovirus (major group) replication

Based on the current knowledge of viral replication, important steps have been identified for the development of anti-viral compounds. These steps have been targeted by numerous natural products and synthetic compounds in order to determine their anti-viral property [110, 172-180]. The steps including prevention of virus attachment/uncoating, virus endocytosis (the delivery of viral RNA genome to the cytosol), protease inhibitors (prevent maturation of viral polyprotein), inhibition of viral RNA synthesis and finally the release of viral progeny.

RV endocytosis requires participation of many cellular proteins. Upon attachment of RV to ICAM-1 receptors, a signalling cascade is triggered, which is not only responsible for RV internalization and replication, but also responsible for initiating and amplifying the pro-inflammatory responses. The ability of RV intermediate replication products, induce production of antiviral proteins (interferons) has been described in the earlier section. Based on the present literature, those proteins involved in the RV internalization are ezrin (a cytoskeletal protein that is abundantly expressed in epithelial cells that possesses an ITAM-like motif), tyrosine kinase p60 SRC, Syk tyrosine kinase, PI3-kinase (heterodimer of p85α regulatory subunit and p110β catalytic subunit) and serine/threonine kinase AKT [181-183] (Figure 1.9.2). Upon engagement of RV with ICAM-1, ezrin rapidly binds to the cytoplasmic tail of ICAM-1. Two tyrosine residues on ezrin are rapidly phosphorylated by SRC tyrosine kinase, leading to recruitment of spleen tyrosine kinase (Syk), then the activation of Syk. This induces direct binding of the p85α regulatory subunit of PI3-k, therefore activating the PI3-k pathway involving Akt.

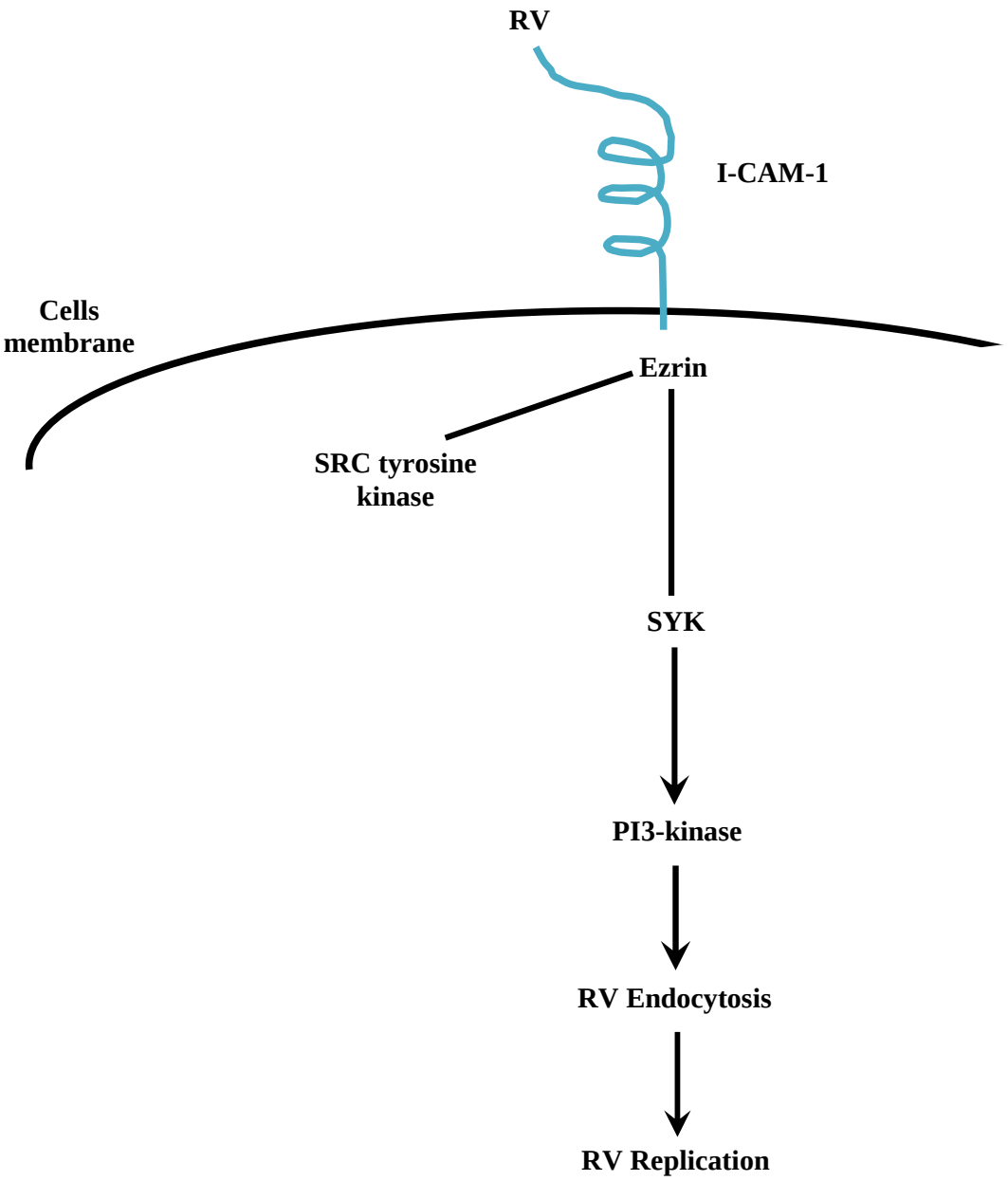


Figure 1.9.2. General overview on RV endocytosis pathway

1.9.1 RV and PI3-kinase

Phosphatidylinositol 3-kinases (PI3-kinases) are a family of cellular enzymes with various roles including cell growth, proliferation, differentiation, survival and

intracellular trafficking, which in turn is required for infectivity of RV in BEC [181, 182, 184]. In general, PI3-kinases have 3 classes, including class I, II, and III, based upon domain structure, type of lipid substrate they use, and mode of regulation [185]. However, only class I of PI3-kinases have been the focus of research and therefore have been recognized as a potential therapeutic point of interest. PI3-kinase is a heterodimeric molecule consisting of catalytic and regulatory subunit proteins. Class I PI3-kinase catalytic subunits can be broken down into class IA (isoforms α, β, δ) and class IB (γ isoform). The catalytic subunits of class IA PI3-kinase form heterodimers with one of five Src-homology 2 (SH2) domain-containing regulatory subunits namely p85α, p85β, p55γ, p55α and p50α. Among the downstream effectors of PI3-kinase, protein serine/threonine kinase Akt (also called protein kinase B) regulates the activation of NF-κB and IL-8 expression in relation to RV infection in BEC [184]. The phosphorylation of Akt activates the IκB kinase (IKK) which then degrades the IκBα, an inhibitor protein of NF-κB [184, 186]. The upstream activator of PI3-kinase (class IA isoform) has been shown to be tyrosine kinase p60Src [181]. PI3-kinases phosphorylate the 3 position hydroxyl group of the inositol ring of phosphatidylinositol which is among the phospholipid present in the inner leaflet of lipid bilayer (converting the phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2) to phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3]. Once the phosphate group binds to position D3 of inositol ring, it will attract any signalling protein which has plecstrin homology (PH) domain on it structure. AKT and phosphoinositide-dependent kinase 1 (PDK-1) are two important proteins that are activated [187]. Binding to the membrane (PtdIns(3,4,5)P_3) brings these proteins close to each other and facilitates the phosphorylation of Akt by PDK-1[188]. The major group of RV activates the translocation of Src from the perinuclear region of the cell to the peripheral sites of the HRV and I-CAM-1 complex.

It has been shown that the p85 regulatory subunit of PI3-kinase acts as a substrate for binding with Src [189]. Class IA PI3-kinase contains heterodimeric subunits which consist of the p85 regulatory subunit and p110 catalytic subunit (either p110α or p110β or p110δ). Catalytic subunit p110 α and p110β are ubiquitously expressed, while p110δ is predominantly expressed in leukocytes [190]. In BECs infected with major group RV, it was found that the p110β catalytic subunit of PI3-kinase is expressed in relation to viral attachment with ICAM-1[181]. In RV-infected BECs, two major roles of PI3-kinase (Akt) have been described, which are to amplify IL-8 release and to participate in viral endocytosis. It has been shown too, even though unable to replicate, UV-inactivated RV is able to induced IL-8 expression due to the involvement of PI3-kinase during early events of viral internalization (attachment to the cell surface receptor).

1.10 Antioxidants and PI3-kinase

Interfering with PI3-kinase activity, either by blocking or preventing, provides a potentially interesting therapeutic approach in respiratory diseases associated with RV infection. Leslie et al. [191] demonstrated that both exogenous and endogenous oxidants play a crucial role in deactivating tumor suppressor phosphates and tensin homologue deleted on chromosome ten (PTEN), which is the main regulator of PI3-kinase signalling. Hence, antioxidants may have the potential to modulate the main regulator of PI3-kinase signalling, via neutralizing the free radicals to preserve the protein PTEN. Many PI3-kinase inhibitor molecules have been developed to improve the understanding of the role of this enzyme as a key regulators of many signalling cascades. To date, it has been reported that resveratrol is able to inhibit the catalytic subunits p110α and p110β of class IA PI3-kinase induced by insulin in human primary myotubes and muscle derived cell lines [192]. It has also been shown that, resveratrol

(administered at 100 μM for 24 hrs) reduced the phosphorylation of Akt (by down regulating PI3-kinase) in human U251 glioma cells [193]. Due to its action as a PI3-k inhibitor, resveratrol may be useful in preventing RV internalisation. Contrary to resveratrol, zinc has been shown to increase activation of the PI3-kinase/Akt signalling pathway [194, 195], which is believed due to effect of zinc on ubiquitin-associated proteolytic of PTEN in human airway epithelial cells [195].

1.11 Antioxidants and respiratory disease

1.11.1 Resveratrol

1.11.1.1 Description, sources and measurement

Resveratrol has been studied extensively to demonstrate that this molecule was effective in inhibiting critical pathways for all three major stages (i.e., initiation, promotion, and progression) in cancer [40]. Resveratrol (3,4',5-trihydroxystilbene) is a naturally occurring phytoalexin present in at least 72 plants including grapes (*Vitis* spp.), berries (*Vaccinium* spp.) and peanuts (*Arachis* spp), with relatively high concentrations in grapes. This molecule is synthesized in response to fungal, viral, bacterial attacks and damage from exposure to ultraviolet radiation (UV), conferring disease resistance in plants [196, 197]. Resveratrol exists in two isomeric forms, that is *cis*- and *trans*- , with isomerisation of *trans* to *cis* facilitated by UV exposure. Its stilbene structure is related to the synthetic estrogen diethylstilbestrol. Two phenol rings are linked by a styrene double bond to generate 3,4',5-trihydroxystilbene (Figure 1.11.1.1.1). A number of pharmacological effects observed in experimental systems have used the t*rans* form of resveratrol rather than the *cis* form [198]. The *cis* form has been shown 9 times weaker than *trans* form in inhibiting lipid peroxidation *in vitro*, probably because of its non-planar conformation structure [199].

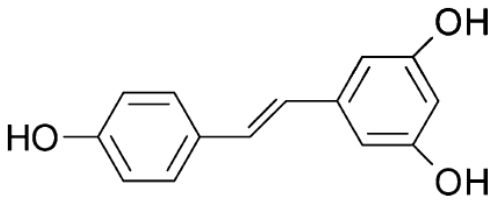
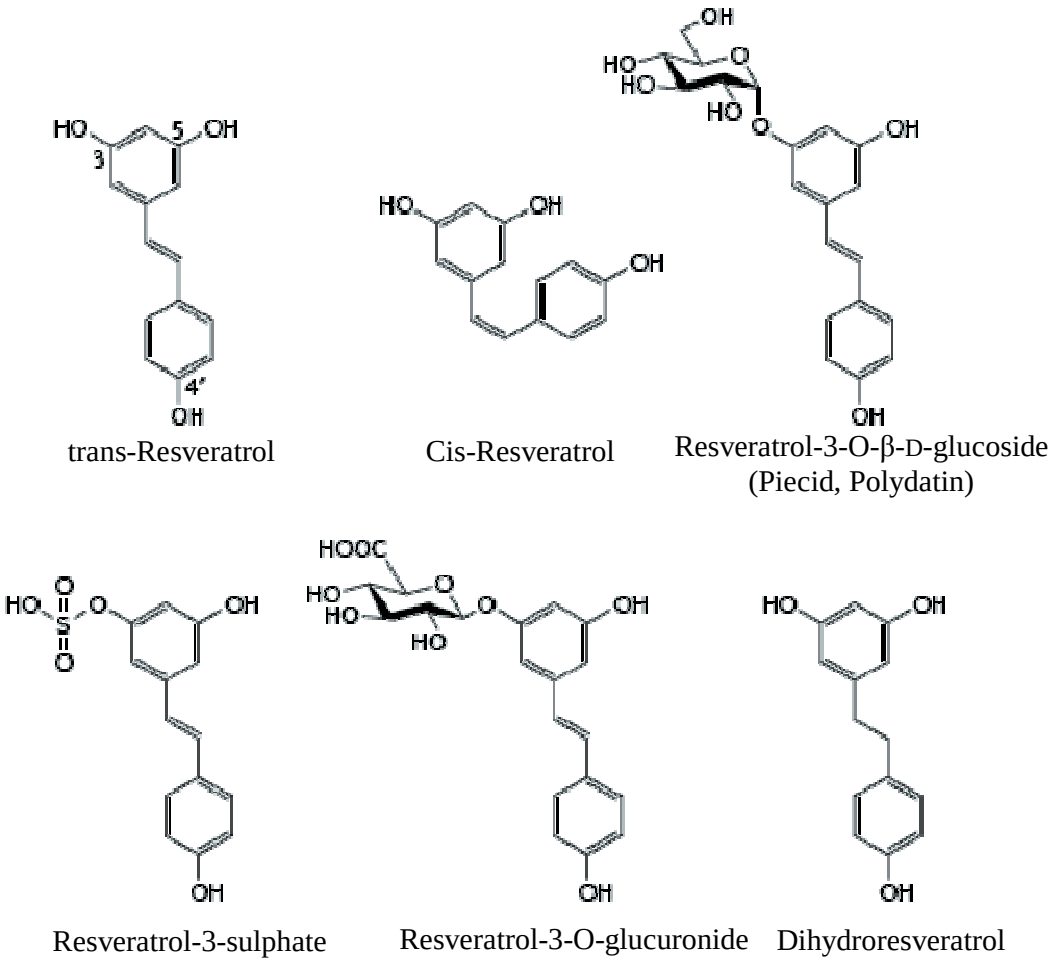


Figure 1.11.1.1.1. Chemical structure of trans-resveratrol

The stability of resveratrol under different conditions was described by Wang et al. [200]. They found that resveratrol was stable for up to 5 days at 4 °C in the dark, but was not stable at room temperature without protection from light [200]. Resveratrol has been found to be stable to moderate heating, but will react when exposed to low or high pH [201]. Another study presented by Bhat and collegues [202], studied the effects of storage conditions and exposure to lab illumination on resveratrol stability. They found that resveratrol, when dissolved in DMSO, is stable (about 90% of trans-form of resveratrol is preserved) at the end of 7 days, when continuously exposed to fluorescent light 18 hours/day as quantified by reverse phase-high performance liquid chromatography (RP-HPLC).



Piecid is found in grapes and other natural sources of resveratrol. Resveratrol-3-sulphate, resveratrol-3-O-glucuronide and dihydroresveratrol are metabolites of resveratrol. The positions of hydroxyl groups are indicated on the parent molecule.

Figure 1.11.1.1.2. Related structures of resveratrol molecule (top 3) and metabolites of resveratrol (bottom 3)

1.11.1.2 Resveratrol and respiratory disease

The pathogenesis of respiratory diseases such as asthma and COPD, involves the excessive accumulation of free radicals, leading to oxidative stress. Therefore, alleviating oxidative stress with an antioxidant supplement may be effective therapy in respiratory disease. Recently, Lee et al. [203] has demonstrated that resveratrol inhibits

mucin gene expression, its protein synthesis and secretion from airway epithelial cells *in vitro*. In addition, TNF-α or phorbol 12-myristate 13-acetate induced NF-κB activation were inhibited by resveratrol [203]. A number of studies have reported the effects of resveratrol on airway function. Lee et al. [204] has demonstrated that resveratrol significantly inhibits increases in T-helper-2-type cytokines (IL-4 and IL-5 levels) in both plasma and bronchoalveolar lavage fluid; suppressed airway hyperresponsiveness, eosinophilia and mucus hypersecretion; in the ovalbumin-induced allergic mouse model of asthma. In another *in vivo* study employing a rodent model of LPS induced airway inflammation, resveratrol was shown to reduces lung tissue neutrophilia and pro-inflammatory mediators (TNF-α, IL-1β, and MPO) in the lung tissues [205]. In an *in vitro* study using A549 cells line (lung epithelial cells), resveratrol has been demonstrated to significantly inhibits airway inflammatory mediators (IL-8 and GM-CSF) secretion from the cells [206]. Resveratrol also has been shown to protect the cells against oxidative stress induced by cigarette smoke extract on A549 cells and human primary airway epithelial cells [207].

1.11.1.3 Antioxidant effects – *in vitro* and *in vivo* evidence

The antioxidant properties of resveratrol are known due to the availability of a hydroxyl group (-OH) and the presence of conjugated double bonds in the molecule, which have the effect on stabilizing the antioxidant-derived radical. Gulcin [33] found that resveratrol possessed strong antioxidant and radical scavenging activities which findings were based on various *in vitro* antioxidant assays including DPPH•, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$, $O_2^{\bullet -}$ and H_2O_2 scavenging, total antioxidant activity, reducing ability, and Fe^{2+} chelating activities. Resveratrol has been shown to exhibit antioxidant properties by preventing the oxidation of low density lipoprotein (LDL), preventing lipid

peroxidation, and reducing several oxidative stress markers. It is well established that the oxidation of LDL molecules is strongly associated with the progression of heart related diseases and resveratrol has been reported to prevent LDL oxidation *in vitro* [208, 209]. In a study employing human subjects, resveratrol was detected within LDL molecules following consumption of red wine and subsequently prevented the molecules from oxidation injury [210]. Resveratrol has also been shown to reduce markers of oxidative stress including 8-hydroxydeoxyguanosine and glycated albumin, in urine and serum respectively, in stroke-prone spontaneously hypertensive rats [211]. In addition, a recent study by Mokni et al [212] reported that resveratrol, when administered intraperitoneally to rats, decreased brain malondialdehyde levels in a dose dependent manner, whereby, the brain antioxidant defence system (i.e. SOD, CAT and glutathione peroxidase activities) increased in a dose dependent fashion. Resveratrol also exerted a cardioprotective effect by preventing rat hearts from ischaemia reperfusion injury [213, 214]. Lipoxygenase, is a dioxygenase with peroxidase activity, involved in the synthesis of mediators in inflammatory, atherosclerotic, and carcinogenic processes. Resveratrol has also shown to inhibit the dioxygenase activity of lipoxygenase [215]. Resveratrol also effectively scavenges superoxide anion radicals [216]. Most relevant to respiratory disease,is a recent study by Kode et al. [217], demonstrating that resveratrol attenuates cigarette smoke-mediated oxidative stress, by quenching reactive oxygen species and by upregulating the synthesis of glutathione via activation of an Nrf2-dependent mechanism in human lung epithelial cells. Thus a variety of antioxidant properties of resveratrol have been demonstrated, which may prove beneficial in respiratory disease.

1.11.1.4 Anti-inflammatory effects- *in vitro* and *in vivo* evidence

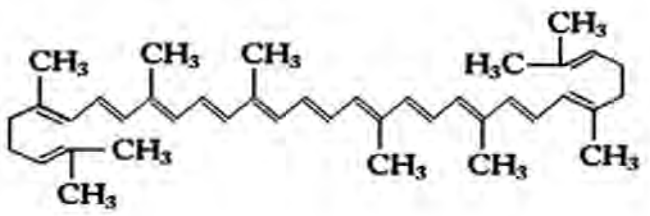
Recent *in vivo* and *in vitro* studies show a potential anti-inflammatory role for resveratrol. Resveratrol has the capability to down regulate the activity of cyclooxygenase 2 (COX-2) and lipoxygenase. The cyclooxygenase enzymes are important in the production of pro-inflammatory molecules by both the cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) pathways. Resveratrol has been shown to inhibit both 5-LOX and COX activities in arachidonic metabolism in rat peritoneal leukocytes, resulting in a reduced accumulation of the lipoxygenase product 5S-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and COX product heptadecaatrienoic acid (HHT) and thromboxane B_2 [218]. In another study, Birrel et al. [219] demonstrated that resveratrol can inhibit inflammatory cytokine expression in response to lipopolysaccharide (LPS) challenge in rat lungs. Another study conducted by Lee et al [220], using a mouse model of allergic asthma, showed that resveratrol markedly reduced airway hyper-responsiveness, significantly inhibited total inflammatory cell counts and eosinophil counts in broncheoalveolar lavage fluid (BALF), reduced the levels of inflammatory cytokines, IL-4, and IL-5 in serum and BALF and substantially inhibited both eosinophilia in lung tissue and mucus hypersecretion by airway goblet cells. Other researchers have demonstrated that resveratrol inhibits NF-κB and AP-1 activation in both monocytic U937 cells and alveolar epithelial A549 cells [42, 43]. It has been shown that resveratrol suppresses the activity of NF-κB by inhibiting the phosphorylation and subsequent degradation of its inhibitory protein, IκBα and by blocking the binding of phosphorylated p65 of NF-κB onto the promoter region of the DNA sequence Resveratrol has been shown to block PMA-induced COX-2. It has been established that this gene and inducible nitric oxide synthase (iNOS) gene are regulated by NF-κB activation [221]. Resveratrol hampers the expression of COX-2 and iNOS by

inhibiting NF-κB activation. These molecules are known to be regulated by NF-κB via matrix metalloproteinase-9 (MMP-9), intercellular adhesion molecule-1 (ICAM-1), endothelial leukocyte adhesion molecule-1 (ELAM-1), and vascular cell adhesion molecule-1 (VCAM-1). Ferrero et al. [222] have demonstrated that resveratrol inhibited the cell-surface adhesion molecule expression and it is believed to be associated with the inhibiting NF-κB. In a recent study, Yu et al. [223] have demonstrated resveratrol inhibited TNF-α mediated MMP-9 expression by downregulating NF-κB activity. In yet another study, Zhu et al. [224] reported that resveratrol demonstrated an anti-inflammatory effect on TNF-α-induced MCP-1 expression in adipocytes mediated by directly repressing NF-κB binding activity, as well as indirectly inhibiting NF-κB dependent transcription activity. Recently, Papi et al. [29] has proposed a possible mechanism by which resveratrol may protect against infection of epithelial cells with RV. Following viral infection, xanthine oxidase is activated and as a consequence, the superoxide radical is produced which can be detoxified by GSH, (catalysed by glutathione peroxidase). The reaction converts GSH to GSSG. As the redox balance has shifted toward the pro-oxidant state, the signalling molecule NF-κB is activated, which regulates the expression of damaging inflammatory cytokines. NF-κB also regulates expression of the receptor for human RV, that is responsible for entry of the virus into the cell. The expression of inflammatory cytokines by epithelial cells might thus be prevented by resveratrol via i) scavenging of reactive oxygen species produced by viral infection, ii) inducing and preserving GSH, iii) preventing signalling (oxidative stress) that can phosphorylate IκB, thus suppressing NF-κB from translocation into the nucleus. This proposed mechanism, by which resveratrol and potentially lycopene, vitamin D and zinc may prevent or down-regulate the expression of inflammatory cytokines, is described in Figure 1.13.1.

1.11.2 Lycopene

1.11.2.1 Description, sources and measurement

Lycopene is a fat soluble, red coloured plant pigment molecule (Figure 1.11.2.1.1), one of the carotenoid compounds which doesn't possesses pro-vitamin A activity, which is due to the absence of a β-ionone ring structure. Lycopene is a natural pigment widely present in plants and microorganism. Its functions as an accessory-light harvesting pigment and protects the chlorophyll pigment from toxic effects caused by light and oxygen during photosynthetic reactions [225]. Furthermore, an increasing body of evidence has shown that lycopene possesses health benefits to humans. Lycopene has been implicated to protect against disorders or diseases including prostate cancer, atherosclerosis, inflammation, sun protection, macular degeneration, infertility and high blood pressure associated with pregnancy [226]. Lycopene health benefits are hypothesised to be primarily due to its strong antioxidative activity. In humans, most intake of lycopene is from consumption of fresh tomato or tomato-based processed products [227]. Other food types that are rich in lycopene include pink grapefruit, guava, watermelon and papaya [227]. Lycopene has been found to be the predominant carotenoid present in human blood (0.76-0.86 μmol/L) [228] and human tissues (liver, kidney and lung) [229]. Carotenoids are strongly bound to macromolecules in the physical food matrix and this affects its absorption. Because carotenoids are highly lipid soluble compounds, co-consumption of dietary fat is required for its absorption. Studies have shown heating tomato products prior to ingestion improves lycopene bioavailability [230]. The absorption of carotenoids (including lycopene) into parietal cells of the gastrointestinal tract has been demonstrated to occur via both passive and facilitated diffusion (using scavenger receptor class B type 1 (SR-B1) transport protein) [231-233].



Alternate conjugated double bonds on its structure is the key for quenching and scavenging radicals

Figure 1.11.2.1.1. Molecular structure of lycopene

1.11.2.2 Lycopene and respiratory diseases

Characterized as a potent antioxidant carotenoid [226], lycopene is hypothesised to be beneficial for disorders related to respiratory system associated with oxidative stress. It has been shown that children with severe asthma experienced significant burden oxidative stress as had been measured with level of reduced GSH, increased malondialdehyde, 8-isoprostanes, and hydrogen peroxide concentration in their bronchoalveolar lavage [234]. In agreement with those findings, adult asthmatics given low antioxidant diet were shown to have reduction in lung function and increased risk of asthma exacerbations compared to subjects with high antioxidant diet [235]. A case-control study carried out by Riccioni et al. [236] has found serum lycopene levels were significantly lower in asthmatic subjects than in healthy control subjects. In regard to that, Wood et al. [237] has carried out a randomized, cross-over study design trial to investigate whether lycopene supplementation to asthmatic adults can improve the disease condition. They found that the administration of low antioxidant diet to the asthmatic adults resulted in decreased plasma carotenoids levels, percentage of forced expiratory volume in 1 second (FEV_1) and percentage of forced vital capacity (FVC); and increased sputum neutrophils [237]. Interestingly, treatment with tomato (which rich in lycopene) resulted in decreased airway neutrophil influx, and reduced sputum

neutrophil elastase activity [237]. In a study employing a mouse model of allergic asthma, lycopene supplementation was shown to significantly suppresses Th2 responses and lung eosinophilia [238]. Daily lycopene supplementation (30mg) for 7 days to patients with exercise-induced asthma resulted in 55% of the subjects protected against exercise-induced asthma [239]. It was found that significant increases of serum lycopene levels was noted in the lycopene-supplemented subjects compared to the subjects in the placebo group [239]. The beneficial effect of lycopene observed on exercise-induced asthma may be due to lycopene ***in vivo*** antioxidative effect, as oxidative stress is implicated in the exercise-induced asthma. In another double-blind, randomized, crossover design study, it was shown that a daily dose of lycopene (30 mg) to young athletes (with exercise-related difficulty in breathing) for 7 days resulted in no improvement on their lung function (shown by no difference in FEV_1 levels) [240].

1.11.2.3 Antioxidant effects-*in vitro* and *in vivo* evidence

The antioxidant properties of lycopene stem from the fact that carotenoids have a strong ability to quench $^1O_2$ [241, 242]. In fact, lycopene has been shown to be the most potent singlet oxygen quencher among the carotenoids, being more effective than astaxanthin, canthaxanthin, a-, β- and γ-carotene, bixin, zeaxanthin and lutein [241].$^1O_2$ has been demonstrated to be produced both intracellularly and extracellularly. Notable sources of $^1O_2$ include respiratory burst of neutrophils and eosinophils [243-245] and neutrophil phagocytosis [246, 247]. $^1O_2$ is capable of causing lipid peroxidation [248, 249] and damaging biomolecules including proteins [250] and DNA [251]. Being a potent $^1O_2$ quencher, lycopene may be relevant to any disorder that is mediated by its production. However, to date, even though $^1O_2$ is recognized as one of the oxidants in biological systems, it is difficult to determine its importance in disease

pathogenesis due to the lack of studies investigating its biological effect. Rao and Agarwal [252] conducted a randomized, crossover design study to investigate whether lycopene acts as an antioxidant *in vivo*. The study was conducted on 19 healthy human subjects receiving dietary lycopene provided by tomato juice, spaghetti sauce, and tomato oleoresin for a period of one week each. Blood samples were collected at the end of each treatment to determine serum lycopene, serum thiobarbituric acid-reactive substances (lipid oxidation), protein thiols (protein oxidation) and 8-oxodeoxyguanosine contents of lymphocyte DNA (DNA oxidation). Serum lycopene levels increased, thiobarbituric acid-reactive substances decreased significantly, while a trend towards lowered protein and DNA oxidation was observed. These results indicate that lycopene from these sources is readily absorbed by the human body and may act as an effective *in vivo* antioxidant [252]. In an *in vitro* assay, Shi et al. [253] demonstrated that lycopene is more efficient in protecting liposomes from oxidation (as assessed by TBARS concentration) than -, β-carotene and lutein. In another study, the synergistic antioxidant action of lycopene with rutin (a phenolic compound) was noted in an *in vitro* assay investigating the effect of lycopene on LDL-oxidation *ex vivo* [254]. The extent of LDL-oxidation was determined by formation of conjugated dienes (membrane oxidation) and loss of tryptophan fluorescence (marker of apoB-100 LDL-oxidation). [250]. The LDL enrichment with lycopene failed to prevent oxidation of LDL. However, the combination of rutin and lycopene markedly reduced LDL and protein oxidation [254]. It has been shown previously that carotenoids localize in the hydrophobic core of LDL particles [255], thus the antioxidant action is confined inside the LDL. Phenolics such as rutin are bound at the outer surface [256]. Thus, the different sites of action may explain the synergistic effects that were observed.

Cigarette smoke is of particular importance in respiratory diseases, and it is one of the damaging environmental factors associated with asthma and COPD. Böhm et al. [257] demonstrated the protective effect of lycopene on *ex vivo* lymphocytes exposed to $^1O_2$ and nitrogen dioxide (NO_2), which are ROS derived from cigarette smoke. Lymphocytes were derived from healthy volunteers whom had been supplemented with pre-warmed tomato juices for 2 weeks. The administration of tomato juice resulted in increased levels of lycopene in the serum (0.15-1.5 μM). The cells were then treated with $^1O_2$ and NO_2 and cells membrane destruction and cell death were monitored [257]. Lycopene protected against $^1O_2$ and NO_2 damage [257], which is in agreement with other studies [258] [259, 260]. CSE has been shown to induce oxidative stress (ROS) intracellularly via NADPH oxidase (membrane associated enzyme), and in another study, lycopene was shown to protect the cells (THP-1) from oxidative stress, assessed via measuring ROS and NADPH oxidase-4 levels [261]. Hence, from the accumulated evidence, it is appears that lycopene exhibits potent antioxidant *in vitro* and this is well translated *in vivo*.

1.11.2.4 Anti-inflammatory effects-*in vitro* and *in vivo* evidence

A growing number of studies have demonstrated that lycopene is also a potent anti-inflammatory agent. Lee et al [262] conducted a study to investigate whether lycopene suppresses inflammatory events in an ovalbumin (OVA)-induced murine asthma model. The authors found that administration of lycopene significantly decreased the infiltration of inflammatory cells (neutrophils, eosinophils, lymphocytes and macrophage) into the airway lumen. The administration of lycopene also significantly decreased airway hyperresponsiveness to methacholine. Additionally, lycopene was shown to significantly decrease the expression of inflammatory mediators

including matrix metalloproteinase-9 (MMP-9) and eosinophil peroxidase (EPO) [262]. In an *in vitro* study using RAW 264.7 cells (macrophages cell line), pre-treatment of the cells with lycopene significantly decreased LPS-induced nitric oxide (NO) levels, mRNA expression of inducible nitric oxide synthase (iNOS) and IL-6 (protein and mRNA expression) [263]. The anti-inflammatory action of lycopene against LPS-induced inflammation in macrophages was shown to be mediated by the effect of lycopene on inhibition of the ERK, p38MAP kinase (MAPK) and Nf-κB pathways [263]. Recently, another similar study conducted by Simone et al. [261] also demonstrated the anti-inflammatory action of lycopene against cigarette smoke (CSE)-induced inflammation in human THP-1 cells (macrophages cell lines). In the study, lycopene was shown to significantly decrease CSE-induced IL-8 at mRNA and protein levels. The inhibition of IL-8 production was explained by the inhibition of the NF-κB pathway through NF-κB DNA binding, p65 nuclear translocation and phosphorylation of IKKα and IkBα. In addition, lycopene also inhibited CSE-induced phophorylation of the redox-sensitive ERK1/2, JNK and p38MAPK pathways [261].

1.11.3 Zinc

1.11.3.1 Description, sources and measurement

Zinc (Zn) is a trace mineral that is needed by the human body for healthy functioning. The recommended daily allowance of Zn is between 7-11 mg per day in various countries (USA, Germany, European Union, Ireland, Norway, United Kingdom and WHO) [264]. Most intracellular Zn is bound to metallothionein [265]. In general, Zn is required due to its various biochemical functions as a cofactor during the biosynthesis of heme, as a cofactor for enzymes responsible for DNA replication, gene transcription, RNA and protein synthesis, as a regulator of gene expression and cell

signalling, cell replication and normal growth [265, 266]. In addition, Zn has been under investigation over the past two decades as an antioxidant. Many food sources contain Zn, however the most bioavailable forms are found in animal flesh and red meat [267]. Whole grains, leafy and root vegetables contain good amounts of Zn, however, the presence of phytate and fibre suppresses the absorption of Zn (indigestible zinc) [267].A systematic review on the assessment of Zn status in the human body has recommended that in healthy individuals, plasma, urinary and hair Zn are reliable biomarkers to reflect human zinc status [268].

1.11.3.2 Zinc and respiratory diseases

The possible beneficial effects of Zn on respiratory diseases may be due from its properties as an anti-inflammatory [269-272] and antioxidant [31, 272] agent. Many original articles on Zn and respiratory diseases associated with viral infection have been published following a seminal report which found that Zn inhibits RV replication *in vitro* [273]. The mechanisms of the antiviral action of Zn are suggested to be due to the direct inhibition of RV replication [178] and the induction of antiviral protein [274]. Jason et al. [275] has written a review article regarding the efficacy of Zn on the common cold, or RV. The authors concluded that Zn, when administered intranasal to the subjects, reduces the duration of common colds [275]. Recently, an excellent systematic review and meta analyses which include 62 epidemiological studies (consisting of cohort, case-control and cross-sectional studies) was published to provide an insight on the role of nutrients, including Zn, in the prevention of asthma [276]. The authors found that (from a total of 8 epidemiological studies which investigated the roles of Zn in prevention of asthma), the efficacy of Zn in asthma prevention is very weak (the authors considered the methodology used in the studies were weak). Hence,

prompt a well designed randomized controlled trial study to be conducted in order to draw a conclusive evidence in regard to the role of Zn on asthma prevention.

1.11.3.3 Antioxidant effects - *in vitro* and *in vivo* evidence

In 1990, Bray et al. [277] published a review paper on the antioxidant activity of Zn in the biological system. It has been shown that Zn antioxidative activity is related to its protective effect on the sulfhydryl group of proteins , as well as the prevention of hydroxyl radical ($OH^{\cdot}$) production via the Haber-Weiss and Fenton reaction [277]. Zago and Oteiza [278] studied the ability of Zn against membrane lipid oxidation in liposomes. The authors found that Zn prevents iron (Fe^{2+})-induced lipid peroxidation by preventing iron binding to the membrane. Zn also functions as scavenger of hydroxyl radical ($HO^{\cdot}$). $HO^{\cdot}$ is considered the most powerful oxidizing species in biological systems, thus preventing the formation or scavenging $HO^{\cdot}$ is highly important and will have significant effects on minimizing oxidative stress. In this aspect, metallothionein protein, which has been shown to be induced by Zn [279], acts as an excellent scavenger of $HO^{\cdot}$ [280].

The antioxidant effects of Zn have been successfully translated in *in vitro* and *in vivo* systems. A number of articles have been published stating that a deficiency of Zn leads to increased oxidative stress *in vitro* [281-283] and *in vivo* [284-286]. Supplementation of primary rat endothelial cells with Zn prevented hydrogen peroxide-induced cell death via increasing cellular reduced glutathione (GSH) levels [287]. The cellular uptake of Zn by airway epithelial cells has resulted in significant reductions in lipid peroxidation [288]. In a study of healthy human subjects who received daily oral Zn supplementation (45 mg) for 8 weeks, subjects were found to have increased plasma

Zn levels and significantly decreased markers of oxidative stress at the end of the study period, including plasma lipid peroxidation (malondialdehyde) and DNA oxidation (8-hydroxy-2-deoxyguanosine) levels [272]. To date, the evidence on the antioxidative effects of Zn can be regarded as compelling, hence the use of Zn as a treatment or adjuvant against many chronic diseases involving oxidative stress merits further scientific investigation.

1.11.3.4 Anti-inflammatory effects - *in vitro* and *in vivo* evidence

Zn has been shown to have anti-inflammatory effects in some settings. In a study conducted by Prasad et al. [272], *ex vivo* peripheral mononuclear cells were obtained from normal healthy subjects after giving daily oral Zn supplementation for 8 weeks. The authors found that the level of mRNA for TNF-α and IL-1β in LPS-induced mononuclear cells was reduced in Zn-supplemented subjects compared to the placebo group. In addition, the authors also observed that in Zn supplemented subjects, the activation of Nf-κB was reduced, due to involvement of Zn in up-regulating expression of protein A20 and the binding of A20 transcription factor to DNA [272]. Recently, an experiment using HL-60, human umbilical vein endothelial cells and SW480 cells lines revealed the mechanism underlying Zn anti-inflammatory properties was due to the induction of protein A20 [269]. The increased incidence of infections seen in the elderly were associated with Zn deficiency and decreased in immune function. It has been reported that immunodeficiency occurs in the elderly and is associated with Zn deficiency, hence the elderly are prone to microorganism infections. Cakman et al. [289] demonstrated that *ex vivo* monocytes treated with virus infection from healthy elderly subjects were producing less IFN-α compared to those from healthy young subjects. The level of IFN-α in the monocytes from elderly subjects was restored after

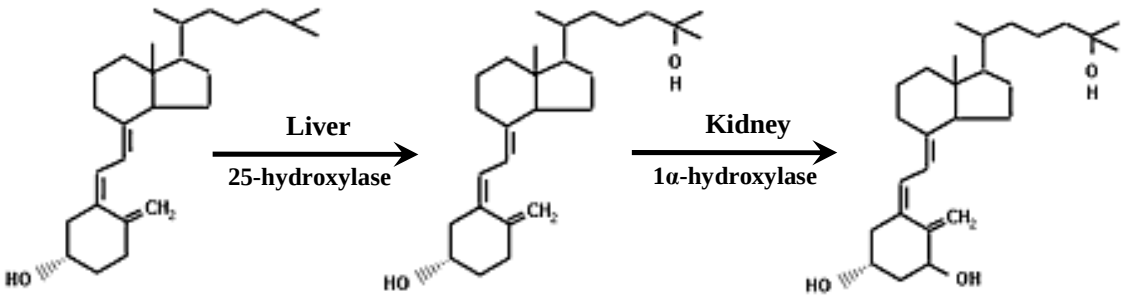
supplementation with Zn [289]. Another similar study conducted by Prasad et al. [290] also found that zinc supplementation over a 12 month period to elderly subjects resulted in significant decreases in the number of infections and significant decreases in TNF-α. Additionally, elderly subjects had significantly decreased plasma Zn levels compared to younger subjects. Zn deficiency among the elderly resulted in higher production of inflammatory cytokines (IL-1β and TNF-α) and endothelial cell adhesion molecules [290]. Studies done on the effect of Zn on inflammation have yielded promising evidence of Zn as an anti-inflammatory agent in various conditions. Therefore, we hypothesize that supplementation of Zn to airway epithelial cells will reduce virus-induced inflammation.

1.11.4 Vitamin D

1.11.4.1 Description, sources and measurement

Vitamins are required from dietary sources by humans in small quantities (micrograms or milligrams per day), for the maintenance of normal health and metabolic integrity. Vitamins are classified based on their biological and chemical activity, and in general, are grouped either as fat-soluble (vitamin A, D, E, K) or water-soluble (vitamin B, C). Specifically, vitamin D is referring to a molecule called 1-α-25-dihydroxyvitamin D_3, 1-α-25-dihydroxycholecalciferol or calcitriol. Vitamin D from plants, yeast and fungi is referred to vitamin D_2 or ergocalciferol, while vitamin D of animal origin (including human) is referred to vitamin D_3 or cholecalciferol. To avoid confusion regarding terminology, vitamin D generally refers to 1-α-25-dihydroxycholecalciferol unless stated. It has been reported that dietary sources contributing to vitamin D status include fatty fish (regarded as an excellent source) and meats (including meat products) [291].

7-dehydrocholesterol (provitamin D_3) is used to synthesize cholecalciferol (previtamin D_3) *de novo*. 7-dehydrocholesterol is intermediate molecule during *de novo* cholesterol biosynthesis. Upon exposure to the sun UV-B radiation (290-315 nm wavelength), 7-dehydrocholesterol is converted to cholecalciferol (previtamin D_3). Recently, the origin of 7-dehydrocholesterol present in the skin are under debate, however it is believed to be synthesized after oxidation of cholesterol (via 7-dehydrocholesterol reductase), or regulated via cholesterol biosynthesis negative feedback regulation [292]. To be biologically functional (1-α-25-dihydroxycholecalciferol), cholecalciferol needs both 25- and 1-α-hydroxylation at the carbon 25 and 1, respectively. 25-hydroxylation occurs in the liver due to mitochondrial enzymes 25-hydroxylase [cytochrome P_{450} (CYP) CYP27A1] producing 25-hydroxycholecalciferol (also called calcifediol or calcidiol). The latter hydroxylation occurs due to 25-hydroxycholecalciferol-1α-hydroxylase (CYP27B1) in the kidney, producing 1-α-25-dihydroxycholecalciferol (also called calcitriol) (Figure 1.11.4.1.1). The conversion to 1-α-25-dihydroxycholecalciferol not only occurs in the kidney (where the enzyme is expressed at the highest concentration), but also in numerous tissues such as the skin, placenta, bone cells, prostate and also cells from the immune system [293]. As scientific knowledge grows exponentially, vitamin D is not only recognized as a nutrient required for bone health [294] but is also demonstrated to have non-classical roles via vitamin D receptor (VDR) activation. These include many physiologic activities such as key regulation of a very large number of human genes [293, 294]. The level of circulating 25-hydroxycholecalciferol is scientifically recognized as clinical biomarker for monitoring the adequacy of vitamin D status [295-297], and can be measured by either radioimmunoassay [298-300] or HPLC [291, 301, 302].



7-dehydrocholesterol **25-hydroxycholecalciferol (calcidiol)** **1α-25-dihydroxycholecalciferol (calcitriol)**

Figure 1.11.4.1.1. The conversion of provitamin D_3 to vitamin D_3 through 2-steps hydroxylation

1.11.4.2 Vitamin D and respiratory disease

Considering the highest prevalence of asthma is in Westernized, modernized countries [303], vitamin D deficiency has been proposed in the inception and development of asthma [304]. Litonjua [304] has recently reviewed the roles of vitamin D associated with asthma, ranging from examining VDR polymorphisms, vitamin D induction of bactericidal peptide, and involvement of vitamin D in immune system (development and exacerbations of asthma). Human airway surface fluid, a mucin-rich fluid covering the respiratory epithelium, contains antimicrobial components, and of particular interest is a bactericidal peptide known as cathelicidins [305]. It has been shown that patients with cystic fibrosis have a deficiency in circulating levels of 25-hydroxycholecalciferol [306], which may reduce their ability to induce this antimicrobial peptide [307, 308]. Of particular important, Yim et al. [309] has shown that, vitamin D is able to induce cathelicidin in normal and cystic fibrosis of BEC. Ginde et al. [310] conducted a secondary analysis of the Third National Health and

Nutrition Examination Survey (a probability survey of the US population conducted between 1988 and 1994), and reported that circulating levels of 25-hydroxycholecalciferol were inversely associated with episodes of upper respiratory tract infection (URTI). The finding suggests that vitamin D may play an important role in protecting the URT. In support of this study, Ginde et al. [310] reported that vitamin D significantly decreased the effect of RSV-induced NF-κB driven genes in airway epithelial cells [311]. In an earlier study, it was shown that airway epithelial cells constitutively convert previtamin D_3 to vitamin D_3 during respiratory virus infection, suggesting the involvement of vitamin D on host defence [312]. Significantly, another study has demonstrated that human macrophages induced expression of CYP27B1 (1α-hydroxylase) and VDR when treated with *Mycobacterium tuberculosis* [307], explaining the induction of cathelicidin and bacterial killing in response to previtamin D_3. However, to date the involvement of vitamin D in exacerbations of asthma associated with RV infection (the most common infection pathogenesis of common cold [6]) has not been examined and merits investigation.

1.11.4.3 Antioxidant effects-*in vitro* and *in vivo* evidence

To the best of our knowledge, the first study reporting the antioxidant property of vitamin D was conducted in 1993 [30]. Wiseman [30] demonstrated that all the vitamin D metabolites (from provitamin D to vitamin D) tested are membrane antioxidants, as they inhibit iron-dependent liposomal lipid peroxidation. In a small intervention study, the deficiency of 25-hydroxycholecalciferol is associated with a reduction in endothelial function and increased lipid peroxidation (TBARS) [313]. Vitamin D has also been reported to having a protective effect on DNA molecules as quantified by 8-hydroxy-2-deoxyguanosine (8-OH-dG) [314, 315]. Fedirko et al. [315] has found that normal

colorectal mucosa patients have high levels of the DNA damage product (8-OH-dG) and by supplementing with vitamin D, the marker was significantly reduced after a 6 month intervention period. Among the major proteins involved in detoxification of ROS in the cells is GSH. Vitamin D has been shown to upregulate the level of GSH in a brain astrocytes model [316, 317]. Astrocytes are the cells present in the brain and they play major roles in the central nervous system detoxification pathway. In an experiment done by Garcion et al. [316], the detoxification process was initiated when the astrocytes were treated with endotoxin (LPS). Vitamin D was found to enhance detoxification by potentiating the expression of γ-glutamyl transpeptidase (γ-GT), a membrane-bound enzyme required for the synthesis of intracellular GSH. In addition, antioxidant enzymes such as SOD and CAT (involved in detoxification of ROS) are suggested to be influenced by vitamin D. Recently, vitamin D has been reported to significantly increase the level of erythrocyte SOD and CAT in a clinical trial in patients with atopic dermatitis [318]. In another study, Bao et al. [319] demonstrated that vitamin D prevents non-malignant prostate cells from oxidative stress-induced cell death by elimination of ROS-induced cellular injuries, via increasing glucose-6-phosphate dehydrogenase (G6PD) activity and GSH level. However, efficacy of vitamin D on regulation of either GSH or antioxidant enzymes levels has not yet been investigated in a model of RV-induced oxidative stress in airway epithelial cells.

1.11.4.4 Anti-inflammatory effects- *in vitro* and *in vivo* evidence

Hansdottir et al. [311] has demonstrated that vitamin D inhibits respiratory syncytial virus action in airway epithelial cells by inducing IκBα protein production, an NF-κB inhibitor [311]. *Ex vivo* studies carried out by Szeto et al. [320] and Sun et al. [321] found that fibroblast cells lacking VDR exhibit increased NF-κB activity due to

the reduction in IκBα levels and the lack of VDR-p65 interaction. Sun et al. [321] also found that IκBα degradation induced by TNF-α was inhibited by vitamin D, and induction of IL-6 due to TNF-α or IL-β treatments has much more robust in cells lacking VDR compared to normal cells. The effect of vitamin D on regulation of NF-κB activation is not only seen in airway epithelial cells, but also has been reported in lymphocytes, dendritic cells and keratinocytes. Vitamin D suppresses the increase in p50 and c-Rel of NF-κB proteins in activated lymphocytes [322], vitamin D directly suppresses Rel B of NF-κB protein transcription [323], vitamin D inhibits the NF-κB signalling pathway in dendritic cells [324]and vitamin D regulates NF-κB binding activity (IL-8 binding sequence) via an increased expression of IκBα in cultured normal human keratinocytes [325]. In a study conducted by Cantorna et al. [326], vitamin D was shown to slow the progression of arthritis in a murine model. In this study, supplementation with vitamin D dramatically decreased arthritic syndrome (inflamed, swollen ankles and paw) induced by either collagen injection or *B. burgdorferi* infection.

1.12 Conclusions

Asthma is a chronic inflammatory airway disease, and a large body of evidence has shown that oxidative stress is involved in the induction of inflammation. Asthma exacerbations occurs due to chronic inflammation of the airways and RV infection is the major environmental driving factor associated with this unwanted event. Research has revealed that upon infection with RV, cells experience oxidant burden. This activates redox sensitive signalling pathways and initiating and amplifying cytokine and chemokines responses, leading to acute and chronic inflammation. Though the role of RV in the pathogenesis and exacerbation of asthma is well documented, currently no

vaccine or antiviral compound/drug is readily available to 'deactivate' the virus. Several nutrients with antioxidant actions are investigated in this thesis, as a potential anti-RV therapies. Positive results in these experiments would highlight the potential for improved treatment/management of respiratory viruses simply by increasing the consumption of foods containing particular dietary antioxidants. The data would also direct future *ex vivo* and *in vivo* studies that would be needed to complement the *in vitro* findings and provide definitive information. To conclude, many centuries ago Hippocrates (460-370 BC) said, "let your food be your medicine and medicine be your food".

1.13 Hypothesis

It is hypothesised that enrichment of BEC with antioxidants (resveratrol, Zn, vitamin D and lycopene) and their intracellular utilization will reduce the host cell response to virus infection (inflammatory and viral replication), which is associated with the pathogenesis and exacerbations of asthma. Therefore the general objectives of the current study are as follows:

1. To investigate methods of enriching BEC with antioxidants and determine if this can occur without significant toxicity.
2. To examine whether enrichment of BEC with antioxidants will have anti-inflammatory and antiviral effects on virus-infected cells.
3. To elucidate the mechanism by which antioxidants affect pro-inflammatory mediator release by BECs infected with RV.
4. To investigate the mechanisms by which antioxidants affect RV replication.

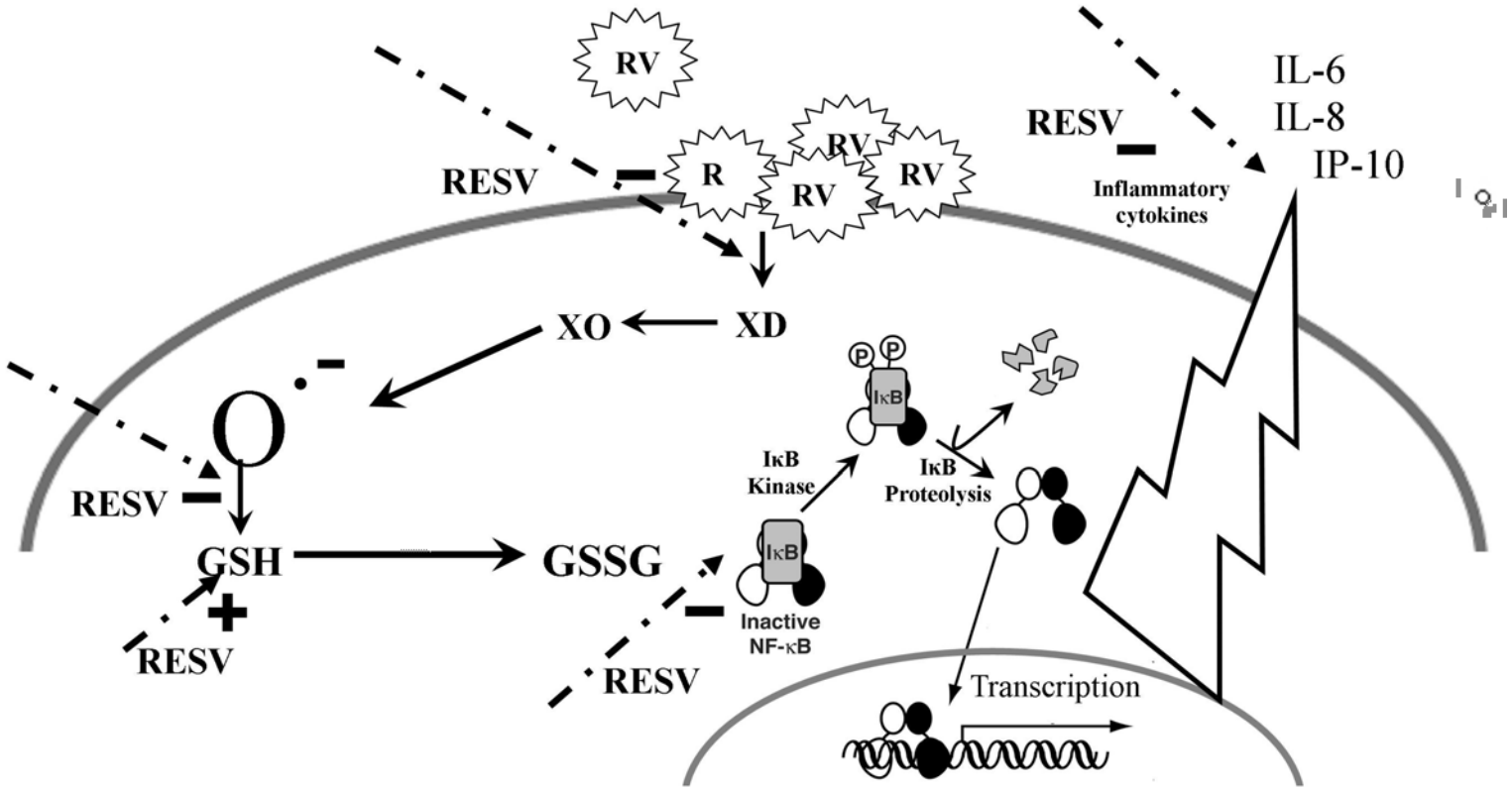


Figure 1.13.1. Hypothesised mechanisms of antioxidant (for example RESV) action on the pro-inflammatory response to viruses [26, 29, 217, 327]. RESV, resveratrol; RV, rhinoviruses; XD, xanthine dehydrogenase; XO, xanthine oxidase; $O_2^{\cdot -}$, superoxide; GSH, reduced glutathione; GSSG, oxidized glutathione, NF-κB, signalling molecule nuclear factor-kappaB, - preventing; + up-regulating.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Introduction

This thesis investigated the role of antioxidant compounds (resveratrol, zinc, vitamin D and lycopene) against rhinovirus (RV)-infected airway epithelial cells in *in vitro* model. The bronchial epithelial cells (Calu-3) was pre-treated with the antioxidants prior to RV infection. The delivery of antioxidants (for example resveratrol) into the cells was assessed by RV-HPLC. Cells viability was assessed via Trypan blue staining and Annexin V and PE staining followed by flow cytometry. The inflammatory cytokines were determined by flow cytometry and ELISA; and viral titres ($TCID_{50}$/ml) was done to determine the amount of infectious virus present in the conditioned medium. The mechanism study was done to investigate how antioxidant capable in preventing RV infection via focusing on the virus itself (viral attachment, internalisation, translation, and replication). Flow cytometry was performed to investigate viral attachment; western blotting, RV labelling and confocal microscopy were carried out to determine RV internalisation step; western blotting and RT-qPCR was done to determine the virus RNA translation and RNA replication steps, respectively. Finally, study on PTEN mRNA silencing using siRNA was ensued to determine whether antioxidant has a direct effect on PI3-kinase mediating RV internalisation.

2.2 Cell cultures experiments

2.2.1 In vitro epithelial cell culture

Human airway epithelial cells (Calu-3, from ATCC, USA) were cultured in minimum essential medium containing 10% foetal calf serum (10%FCS/MEM) containing 2% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine, and 2.2g/l $NaHCO_3$ (all were purchased from Invitrogen Corporation, Carlsbad, USA) at 37ºC in the presence of 5% CO_2. The cells were supplemented with 1% FCS/MEM during enrichment of antioxidants (resveratrol, zinc, vitamin. D, and lycopene) and during virus infection experiments to minimize the rate of cell differentiation.

2.2.2 Preparing virus stock and $TCID_{50}$/ml assay

In this study, RV serotypes RV-43 and RV-1B were used to infect the Calu-3 cells. Both of the viruses were cultured in RD-ICAM-1 cell line, this is a transformed cell line enriched with ICAM-1 receptor expression. These cells were cultured in complete Dulbecco's Modified Eagle Medium containing 5% foetal calf serum (5%FCS/DMEM) containing 2.2g/l $NaHCO_3$, 100U/ml penicillin, 100µg/ml streptomycin, 1mM sodium pyruvate, 20mM HEPES and 2mM L-glutamine (all were purchased from Invitrogen Corporation, Carlsbad, USA) at 37ºC in the presence of 5% CO_2. Confluent RD-ICAM-1 (about 80-90 % confluent) in T175 culture flask (NUNC, Roskilde, Denmark) was infected with 1 ml of either RV-43 or RV-1B stock and incubate at 33ºC in the presence of 5% CO_2. The temperature at 33ºC provides an optimum temperature (as established in our lab and others) for the virus to replicate more efficiently. Approximately 24-30

hrs following infection, significant cytopathological effects (CPE) in the RD-ICAM-1 cells was observed by light microscopy and the cell culture supernatant collected and centrifuged for 10 min at 2000rpm. Clarified supernatant was then aliquoted and stored at -80ºC. An aliquot of the supernatant was used to determine the virus titres using $TCID_{50}$ assay as described below.

$TCID_{50}$ experiments were performed using confluent RD-ICAM-1 cells seeded in 96-well tissue culture plates (NUNC, Roskilde, Denmark). After overnight seeding the cells or the cells reach 60-80% confluence, the cells were then infected with media alone or media containing virus at varying dilutions as described below. To determine the $TCID_{50}$ from virus stock, serial ten-fold dilutions (ranging from 10^{-1} to 10^{-12}) were prepared and seven individuals wells were infected with each dilution, while twelve individual control wells were prepared with media alone. For samples, serial ten-fold dilutions of the samples were prepared and four individual wells were infected with each dilution. For titration of samples 7 dilutions were prepared and 1 control well was prepared with media alone. The plates were reviewed daily and read, after 5 days the plates were finally read and the $TCID_{50}$ calculation made. Infected wells were scored based on the CPE observed. A positive result was given to a well with more than 50% CPE demonstrated by light microscopy.

Viral titers of the samples determined by cell titration assay using RD-ICAM-1 cells was calculated and expressed as tissue culture infectious dose at 50% in log value ($TCID_{50}$ $\log_{10}$) [328] and viral titres were calculated based on the Kaber formula for the tissue culture infective dose 50% ($TCID_{50}$) [329] as below:

$\text{Log}(TCID_{50}) = a - D(\Sigma p - 0.5)$

Where a = dilution index for last dilution where all wells are infected

D = log of dilution factor (i.e., for a dilution factor of 10: log 10 = 1)

Σp = sum of p between x and y

Where p = proportion of well infected

x = greatest dilution at which all are infected

y = lowest dilution at which none are infected

The Karber formula provides an estimate as to the dilution factor that is required to cause infection in 50% of test wells.

2.2.3 Rhinovirus-43 fluorescent labelling

2.2.3.1 Virus purification

Virus stock (as described in section 2.2.2) (110ml) was transferred into 4 tubes of 38.5 ml UltraClear tubes (open top) (Beckman Coulter). The tubes were place in a Beckman SW32Ti rotor and spun at 10 000g at 4ºC for 30 minutes. Clarified virus supernatant were transferred into 6X Amicon Ultra-15 100K centrifugal filter devices (Millipore) (18 ml virus into each unit), and spun at 4000g (swing bucket) at 4ºC for 20 minutes to concentrate the virus (do not concentrate until dryness). DPBS was added to 15ml again to the centrifugal filter devices and re-spun until the solution was as clear as the DPBS, and this step was repeated 3 times. The concentrated virus was then transferred into 10 ml centrifuge tubes. The concentrated virus was then slowly overlaied on an 8ml solution of 30% sucrose, 1M NaCl, 20mM NaHEPES pH 7.5 cushion in 12.5 ml UltraClear tubes (open top) (Beckman Coulter). The tubes were place in a Beckman SW41Ti rotor and were spun (Beckman Coulter Optima L-100 XP Ultracentrifuge) at 41000 rpm at 15ºC for 6 hours. The supernatant was removed and a very small, faint pellet was recovered. The pellet (purified virus) was resuspended in 1 ml of DPBS and transferred into tubes.

2.2.3.2 Fluorescent labelling of virus

One vial of AlexaFluor 555 carboxysuccinimidyl ester (Molecular Probes™) was dissolved in 10µl of DMSO. The solution was added to 1ml of purified virus in 4 ml tube. Then, the tube was mixed with 100µl of 0.1M $NaHCO_3$ and incubated at 4ºC for 1 hour. The volume in the solution was brought to 4ml with DPBS and then transferred to a 15ml conical centrifuge tube and then added with 308µl of 5M NaCL, 20µl of 20% BSA and 0.6ml of 50% PEG-8000 (Sigma). The tube was tumble mix with MACS MIX (mx001, Miltenyl Biotech) at 4ºC for overnight before spun at 2000g (swing bucket) at 4ºC for 30 minutes. The fluid (supernatant) was carefully removed over the visibly colour pellet, and the pellet was resuspended in 200 µl of 0.1% BSA/DPBS. The labelled virus was kept at 4ºC and was used within 1 week. The labelled virus was titre as described in section 2.2.2.

2.2.4 Confocal microscopy

2.2.4.1 Collagen-coated coverslip

Sterile glass coverslips (13 diameters, Menzel-Glasses Coverslips) were sprayed with 70% ethanol and placed on 24-well plate in cell culture hood. Then, 200µl of diluted collagen (1:500 placenta collagen with DPBS solution) were added on the coverslips and incubated for 1 hour in the hood. Solution containing collagen was drained from the plate, and the plate containing collagen-coated coverslips was used in the section 2.2.4.2.

2.2.4.2 Cell preparation for confocal microscopy

Calu-3 cells at a density of 6 x 10^4 cells per well were cultured on collagen-coated coverslips. After 2 days the cells were treated with the antioxidants (the concentrations of resveratrol, vitamin D and lycopene are given in the individual chapters) for 2,18 and 24 hours prior to labelled-RV infection, at a multiplicity of infection (MOI) of 0.5. MOI was calculated using formula as describe below:

$$\text{Volume (ul) of virus stock} = \frac{1000 \times (\text{MOI} \times \text{number of cells})}{\text{TCID}_{50}\ \text{Rhinovirus-43}}$$

After 1 hour of infection, the cells were washed with DPBS, and were fixed in 1% paraformaldehyde/DPBS for 20 minutes at 4ºC. The cells were then washed three times with ice cold DPBS. The coverslips were carefully removed (cells facing above) and placed onto a microscope glass slide. A drop of ProLong® Gold Antifade Reagent with DAPI (Molecular Probes) was then added to the coverslips, and another coverslip (rectangular shape) was carefully placed on top of this. Images were obtained using a Olympus IX81 Fluoview FV1000 Confocal Laser Scanning Biological Microscope and the FV10-ASW Version 2.0 image software.

2.2.5 Preparing medium containing resveratrol-DMSO

Resveratrol (Sigma-Aldrich, Australia) was prepared in the medium containing DMSO (Sigma-Aldrich, Australia). The stock solution of resveratrol (250 mM) was prepared by dissolving 0.05705 g or resveratrol powder in 1 ml of DMSO, and stored at -80ºC. Then a working stock of resveratrol (100 mM) was prepared by diluting 1:2.5 ratio of

resveratrol stock with DMSO. The appropriate volume of the resveratrol working stock was added to the 1% FCS/MEM to achieve 5, 10, 25, 50, 100 μM resveratrol containing medium. The final concentration of DMSO in resveratrol containing medium was always less than 0.1 %.

2.2.6 Preparing medium containing lycopene

Lycopene (Sigma-Aldrich, Australia) was prepared in medium containing THF (Sigma-Aldrich, Australia). The stock solution of lycopene (2000 μg/ml) was prepared in THF by dissolving 0.001 g of lycopene powder in 0.5 ml of THF, and stored at -80ºC. The appropriate volume of the lycopene stock was added to the 1% FCS/MEM to achieve 2.5, 5 and 10 μg/ml lycopene containing medium. The final concentration of THF in lycopene containing medium was 0.5 %.

2.2.7 Preparing medium containing zinc salt (zinc sulphate)

Zinc sulphate heptahydrate (Sigma-Aldrich, Australia) was prepared in medium containing sterile ultra pure water (Millipore Inc, USA). The stock solution of zinc salt (100 mM) was prepared in ultra pure water by dissolving 0.115 g of zinc salt in 4 ml of milliQ water, and stored at 4ºC. The appropriate volume of the zinc salt stock was added to the 1 % FCS/MEM to achieve 10, 25 and 50 μM zinc salt containing medium.

2.2.8 Preparing medium containing 1α,25-dihydroxyvitaminD3

1α,25-dihydroxyvitaminD3 (vitamin D) (Sigma-Aldrich, Australia) was prepared in medium containing absolute ethanol. The stock solution of vitamin D was prepared in ethanol by dissolving 10 μg of vitamin D powder in 24 μl of ethanol, and stored at -20ºC. The appropriate volume of the vitamin D stock was added to the 1 % FCS/MEM to achieve 0.01, 0.1 and 1 μM vitamin D containing medium. The final concentration of ethanol in vitamin D containing medium was 0.1 %.

2.2.9 Supplementation of Calu-3 with resveratrol, lycopene, zinc and vitamin D

Calu-3 cells were incubated with 1 % FCS/MEM containing final concentrations of resveratrol, lycopene, zinc salt and vitamin D as described in the previous sections.

2.2.10 Primers

Genes expression including PTEN, GAPDH and ribosomal 18S in Calu-3 cells were quantitated by RT-qPCR using specific primers as tabulated in Table 2.2.10.1. RNA for rhinovirus was also quantitated by RT-qPCR using primers as shown in Table 2.2.10.2.

Table 2.2.10.1. Commercial assays used and list of primer-probes for RT-qPCR

Primers RT-qPCR (gene)	Probes (5' fluorescent dye and 3' quencher	Assay/sequence (5'-3')
PTEN	FAM - MGB	TaqMan® gene expression assay Hs02621230_s1 (Applied Biosystems)
GAPDH	FAM - MGB	GGGCGCCTGGTCACCAGGGCTGCTT
18S	VIC - MGB	Proprietary

Table 2.2.10.2. List of primer-probes and sequences for RV in RT-qPCR

Primers for RV	Sequence (5'-3')
RV forward primer (RVFWD)	GCACTTCTGTTTCCCC
RV reverse primer (RVRVS)	GGCAGCCACGCAGGCT
RV probe 1	FAM-AGCCTCATCTGCCAGGTCTA-TAMRA
RV probe 2	FAM-AGCCTCATCGACCAAACTA-TAMRA

2.2.11 Common buffers for protein analysis

PBS: 120mM NaCl, 2.7mM KCl, 10mM phosphate salts pH 8.0

TBS: 120mM NaCl, 10mM Tris.HCl pH 8.0

TBS-T: 120mM NaCl, 10mM Tris.Cl pH 8.0, 0.05% (v/v) Tween 20

Towbin transfer buffer: 192mM Glycine, 25mM Tris.HCl pH8.3, 20% Methanol

Radioimmunoprecipitation assay (RIPA) buffer: 50mM Tris.HCl pH 7.4, 150mM NaCl, 2mM EDTA, 1% Triton X-100, 2% sodium deoxycholate, 0.1% SDS

2.3 Biochemical analysis

2.3.1 Resveratrol analysis

Concentrations of extracted resveratrol from cell cultured media and cultured cell pellets were quantified by reverse phase- high performance liquid chromatography (RV-HPLC). The resveratrol content were quantified according to the method described by Zhu et al.[330] All chemicals reagent used were of HPLC grade and were filtered through nylon, 0.2µm filter, (Alltech, Australia) prior to use.

2.3.1.1 Resveratrol extraction

Enzymatic hydrolysis of conjugates was carried out according to the method developed by Soleas et al.[331] A working solution was freshly made each day comprising 80 mg of powder (β-glucuronidase; which was purchased from Sigma-Aldrich, Australia), it was dissolved in 1 ml of 0.58 M acetic acid. Samples (250 µl) was pipetted into a clean culture tube, then added with 50 µl of working enzyme solution representing 1 mg of original enzyme. The mixture was vortexed, incubated in water bath at 37ºC for 2 hr, then extracted with 250 µl of methanol according to the method described by Boocock et al.[332] with slight modification. The samples were then be spun at 13 000 g in a microcentrifuge at 4ºC for 15 min to discard denatured proteins. The supernatant was decanted off carefully using a glass Pasteur pipette, into a glass high recovery 10 ml tube and dried down at room temperature in the dark under ultra pure nitrogen gas (Linde, Australia) using nitrogen evaporator (Organomation Associates, Inc, USA). Once dry, the samples were reconstituted in 200 µl of methanol, vortexed and

transferred to a high recovery glass insert in an amber glass HPLC vial and 40 µl was injected into HPLC system.

2.3.1.2 HPLC-Resveratrol

The HPLC system consists of an Agilent quaternary pump, autosampler with a refrigeration unit, UV–vis detector and in-line vacuum degasser. The detection was carried out at 310 nm. Chromatographic separation were accomplished by injecting the sample onto a Agilent ZORBAX Eclipse XDB-C18 (5 µM, 150 x 4.6 mm I.D.), using a column oven set at 35 ◦C with a flow rate of 1 mL/min. An isocratic elution was employed consisting of acetonitrile: 25 mM sodium phosphate monobasic (30:70 v/v). Stock solutions of 100 µg/ml trans-resveratrol was prepared in methanol. A calibration standard curve was prepared ranging from 156 ng/ml to 20000 ng/ml of trans-resveratrol and was used to determine raw concentration of resveratrol in samples. A final, corrected concentration was obtained by incorporating the dilution factor into the calculations.

2.3.2 Analysis of inflammation biomarkers

Pro-inflammatory cytokines (chemokines), including IL-6 and IP-10 in cells cultured media were analyzed using BD CBA Soluble Protein Flex Set System according to manufacturer's instruction. The assay will be run simultaneously by using BD™ Cytometric Bead Array (CBA) Human IL-6 Flex Set and BD™ Cytometric Bead Array (CBA) Human IP-10 Flex Set, all purchased from BD Biosciences, San Jose, USA. Sample (100 µl) was diluted with 900 µl assay diluent and standard was prepared in

assay diluent. Mixed capture beads (for IL-6 and IP-10) was prepared by diluting 2 µl of capture beads (1 µl of IL-6 + 1 µl of IP-10) with 48 µl of capture beads diluent. A PE detection reagents (for IL-6 and IP-10) was prepared by diluting 2 µl of detection reagent (1 µl of IL-6 + 1 µl of IP-10) with 48 µl of detection reagent diluent, and was kept in dark. Freshly prepared diluted mixed capture beads (50 µl) was added to flow cytometry tube. Then, 50 µl of samples or standards was added to the tube, mixed gently and was incubated at room temperature in dark for 1 hour. Then, 50 µl of freshly prepared diluted PE detection reagents was added to the tube, mixed gently and was incubated at room temperature in dark for 2 hours. After incubation, 1 ml of wash buffer was added to the tube and was spun at 200g for 5 minutes. The supernatant was discarded and 300 µl of wash buffer was added to the tube and was gently mixed to resuspend the beads in the tube. Then, 300 µl from the tube was transferred to a 96-well flat bottom culture plate. The plate was then analyzed by flow cytometer (BD FACSCanto^{TM} II Flowcytometer, BD Biosciences, San Jose, CA, USA) and analysed on FACSDiva software.

2.3.2.1 Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of IL-8 was measured by ELISA (RND System, Minneapolis, USA) according to the manufacturer's instruction. Microplate 96 well flat bottom (NUNC) was pre-coated with 100µl of capture antibody in PBS overnight at room temperature. The plate was washed with 400µl of wash buffer (0.05% Tween 20 / PBSpH 7.2) three times, and was inverted and blotted to remove remaining wash buffer. Blocking buffer (1% BSA /5% sucrose/0.05% NaN_3/ PBS) (300µl) was added to each well to block unspecific sites for one hour at room temperature. After three washes with the wash

buffer, 100µl of samples or standards in reagent diluent (0.1% BSA/0.05% Tween 20/Tris-buffered saline pH 7.3, 0.2µm filtered) was added to the wells and incubated at room temperature for two hours. Three washes of the plate were repeated and 100µl of secondary detection antibody was added to each well. After two hours incubation at room temperature, the washing step was repeated and 100µl of streptavidin-HRP was added to each well and incubated at room temperature in the dark for 20 minutes. Three washes were performed again and 100µl of substrate solution was added to the wells and incubated at room temperature in the dark for 20 minutes. The stop solution (1M H_2SO_4) (50µl) was added to complete the reaction. The plate was then read by the microplate reader (BMG Labtech) at 450nm with a wavelength correction at 540nm, and the concentration of the target protein were calculated by the software FLUOstar OPTIMA from the standard curve prepared with each plate.

2.3.3 Analysis of cellular apoptosis, necrosis and viability

Cellular apoptosis, necrosis and viability was analyzed using PE Annexin V Apoptosis Detection Kit I purchased from BD Bioscences, San Jose, USA, according to manufacturer's instruction. The kit contains two fluroforms which are PE Annexin V and vital dye 7-amino-actinomycin (7-AAD). The cells pellets (1×10^6) were collected and washed with PBS once and with 1X staining buffer. After centrifuging at 1,500 rpm for 5 minutes, the cells were then re- suspended in 1X staining buffer with annexin V-PE stain and the vital dye 7-amino-actinomycin (7-AAD) for 15 minutes at room temperature in dark. The cells were then analyzed by the flow cytometer (BD FACSCanto™ II Flowcytometer, BD Biosciences, San Jose, CA, USA) and analysed on FACSDiva software.

2.3.4 Analysis of intacellular adhesion molecule-1 (ICAM-1) surface receptor expression

The cells pellets (1 x 10^6) were collected and suspended in 1%FCS/0.01% sodium azide/ice cold DPBS. The cells suspensions were then transferred to flow cytometry tubes. The cells' suspensions were then mixed gently with 20µl of mouse monoclonal [1H4] to ICAM-1 (FITC) (Abcam). For the isotype control, 20µl of goat polyclonal to mouse IgG-H&L (FITC) (Abcam) was added to the cells suspension in equivalent tubes. The samples were then incubated for 30 minutes at 4ºC. The cells were washed 3X and centrifuged at 1200 rpm for 5 minutes and the cells resuspended in 500µl of 1% FCS/0.01% sodium azide/ice cold DPBS. The cells were then analyzed by flow cytometer (BD FACSCanto^{TM} II Flowcytometer, BD Biosciences, San Jose, CA, USA) and analysed on FACSDiva software.

2.3.5 Total protein concentration quantification

The concentration of proteins in whole cell lysates were determined using BCA Protein Assay Kit (Thermo Scientific). Samples and BCA standards were vortexed and centrifuged, and 10µl from which was loaded in wells of 96 well plate. Solution A and solution B was mixed and 200µl of which was added into each well. The plate was placed on a plate shaker and mixed at 300 rpm for 30 seconds, and incubated at 37°C for 30 minutes. The plate was then read at absorbance of 560nm in a microplate reader (BMG Labtech) and the concentration of the samples was calculated by the FLUOstar OPTIMA software.

2.3.6 SDS-PAGE electrophoresis and western blotting

Protein samples were mixed with an equal volume of 2x loading buffer (125mM Tris.Cl pH 6.8, 4.1% (w/v) SDS, 0.001% (w/v) Bromophenol Blue, 20% (v/v) glycerol, and 300mM β-mercaptaethanol for reducing gels. The samples were boiled at 95℃ for 10 minutes and loaded into the wells of the NuPAGE 4 – 12% Bis-Tris gel (Invitrogen). The gel was electrophoresed in 1X MOPS SDS running buffer using XCell SureLock Electrophoresis cell (Invitrogen) at 200V for one hour. After electrophoresis the gels was equilibrated in towbin transfer buffer for 30 seconds and placed on to a pre-soaked whatman filter paper (Whatman). A pre-soaked PVDF membrane (Hybond) was then placed on top of the gel and followed by another piece of filter paper. The Gel- membrane was then sandwiched between pre-soaked sponges and placed in the XCell II Blot Module (Invitrogen). The transfer was performed at 30V for one hour. The membrane was blocked with 5% (w/v) BSA in TBS-T for one hour at RT, after which the membrane was washed three times for 10 minutes each in TBS-T. The membrane was probed for the following targets at the appropriate dilution with the respective antibody listed in Table 2.2.6.1 as shown below. After 1 hour incubation at room temperature on an orbital shaker, the membrane was washed three times for 10 minutes each in TBS-T. The membrane was then incubated with appropriate secondary antibody in TBS-T for 1 hour at room temperature on an orbital shaker, and washed three times for 10 minutes each in TBS-T. The membrane was then developed by chemiluminescence using SuperSignal West Femto substrate (Thermo Scientific) and visualized using Fujifilm LAS-3000 Intelligent Dark Box with analysis software

Fujifilm Image Reader LAS-3000. The densitometry on the blot was performed using the software Image J (http://rsbweb.nih.gov/ij/index.html).

Table 2.3.6.1. List of antibodies and dilution used to determine protein of interest

Target	Primary antibody	Dilution	Secondary antibody	Dilution
pAKT	Rabbit polyclonal anti-pAKT (Ser473) (Cell Signaling)	1: 3000	Goat polyclonal to Rabbit IgG:HRP (Abcam)	1: 4000
eIF4GI	Rabbit polyclonal anti-eIF4GI (Cell Signaling)	1: 3000	Goat polyclonal to Rabbit IgG:HRP (Abcam)	1: 4000
GAPDH	Goat polyclonal anti-GAPDH (Abcam)	1: 4000	Anti-goat IgG:HRP (RnD System)	1: 6000

2.3.7 RNA analysis

2.3.7.1 RNA harvesting and extraction

Three hundred fifty µl of RLT buffer containing 300mM β-mercaptaethanol was added to the cell monolayer and incubated at room temperature for 20 minutes. The RNAs were extracted from the RLT samples using RNeasy Mini Kit (Qiagen). Equal volume of 70% ethanol was added to the samples and mixed by pipetting. The mixture was transferred into an RNeasy mini spin column and centrifuged for 15 sec at 10,000 rpm. Flow-through was discarded and 700µl of RNeasy Buffer RW1 was added to the column and centrifuged 15 sec at 10,000 rpm. The spin column was transferred to a new collection tube and 500µl of RNeasy Buffer RPE and centrifuged for two minutes at 14,000 rpm. Flow-through was discarded and re-centrifuged for one minute at 14,000 rpm to remove all trace of ethanol. The spin column was transferred to a 1.5ml

eppendorf tube and 50µl of RNase- free water was added and centrifuged for 1m minutes at 10,000 rpm to elute the RNA. The concentration and quality of eluted RNA was determined by NanoDrop 1000 (Thermo Scientific).

2.3.7.2 Reverse transcription of RNA to cDNA

Reverse transcription of RNA to cDNA was done by performing reverse transcriptase-PCR using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Extracted RNA (200ng) was mixed with appropriate volume of nuclease-free water to make 10µl of final volume. Then, 10µl solution containing extracted RNA was mixed with a 10 µl of 2X master-mix containing 2µl of 10X RT buffer, 0.8µl of 25X dNTP Mix (100mM), 2µl 10X random primers, 1µl of MultiScribe™ reverse Transcriptase, and 3.2µl of nuclease-free water. The samples were incubated at 25°C for ten minutes and followed by 37°C for 120 minutes in thermocycler (Analytikjena Flex cycler), and followed by last incubation for five minutes at 85°C in thermocycler (Analytikjena Flex cycler). Then, the samples (cDNA) are transferred into 1.7ml storage tubes and kept in -20°C freezer for RT-qPCR assay.

2.3.7.3 Real-time quantitative polymerase chain reaction (RT-qPCR)

cDNA products (1µl) from the reverse transcription step were used as template for RT-qPCR using Taqman gene Expression (Applied Biosystems). Reaction was performed in duplicate with 24µl of qPCR reaction mix consist of 9µl nuclease-free water, 12.5µl 2X Master mix, 1.25µl of 20X 18S primer-probe (VIC-MGB), and 1.25µl of the primer-probes targeting the genes of interest (Table 2.2.10.1). Ribosomal RNA (18S)

was used as the reference gene, and each target was performed using Eppendorf RealPlex PCR System. The cycles performed in the RT-qPCR reaction is described below.

Steps	Temperature (ºC)	Time (minutes)	Cycles
1	50	2	1
2	95	10	1
3	95	15 seconds	40
	60	1	

Raw data was presented as the threshold cycle (C_t) at which the fluorescence emission is significantly above baseline, and was then normalized by dividing the mean value of the Ct value of 18S RNA and was expressed as an exponent of 2 (2^{Ct}) by the mean value of 2^{CT} for the target gene. The fold induction over the medium control was then calculated by dividing the normalized target value by the normalized control value.

2.3.7.4 Viral RNA RT-qPCR

cDNA products (1µl) from the reverse transcription step were used as template for RT-qPCR using Taqman PCR. Reaction was performed in duplicate with 24µl of RV PCR reaction mix consist of 12.75µl nuclease-free water, 10µl of Eppendorf 2.5X RealMasterMixProbe ROX and 1.25µl of 20X RV mix. List of the RV primer-probes used is included in Table 2.2.10.2. The component of 20X RV mix is shown below in Table 2.3.7.4.1. Quantitative viral RNA was performed using Eppendorf RealPlex PCR System. The cycles performed in the RT-qPCR reaction is described below.

Steps	Temperature (ºC)	Time (minutes)	Cycles
1	95	2	1
2	95	15 seconds	40
	60	1	

Table 2.3.7.4.1. Preparation of 20X RV mix

Component	Volume (µl)	Final concentration in 25 µl
Nuclease-free water	55	
100 µM RV Forward Primer	22.5	900 nM
100 µM RV Reverse Primer	22.5	900 nM
100 µM RVProbe1	12.5	200 nM
100 µM RVProbe2	12.5	200 nM

The raw data was obtained from the threshold cycle (C_t) at which the fluorescence emission is significantly above baseline. Then, using C_t value of the samples, relative viral RNA copies numbers were obtained by extrapolating from a viral RNA standard curve [viral RNA amount (copies) vs C_t]. The viral RNA standard curve was prepared from serial dilutions of RV RNA in range of neat, 1:5, 1:10, 1:50, 1:100, 1:500, 1:1000, 1:5000, 1:10000.

2.3.7.5 RNA interference

The transfection reagent was prepared by diluting siPORT NeoFX transfection reagent (Ambion) (3µl) with 22 µl of OPTI-MEM1 (Invitrogen) and incubated at room temperature for 10 minutes. Small interfering RNA (siRNA) to PTEN [PTEN Silencer Select Pre-designed siRNA (ID: s326; s325; s327)] (Ambion) was diluted to 60nM with OPTI-MEM1, mixed with the transfection reagent by inversion, and incubated at room temperature for 10 minutes. This transfection complex was mixed with freshly trypsinized Calu-3 cells at a density of 1 x 10^5/ml and added to a tissue culture 24-well plate. The transfected cells were incubated at 37ºC and 5% CO_2 incubation for 24 hours before supplementation with antioxidant compounds and RV infection. GAPDH was silenced with siRNA in a separate well as a positive control. The successful rate of mRNA knockdown was assessed by RT-qPCR (2.3.7.3). The transfection efficiency in cells transfected with either PTEN siRNA or GAPDH siRNA were determined by comparing the percentage of the gene expression of those cells to the cells transfected with negative control siRNA.

CHAPTER 3: ANTIOXIDANT SUPPLEMENTATION OF CULTURED AIRWAY EPITHELIAL CELLS

3.1 Introduction

Resveratrol (3,4',5-trihydroxystilbene) is a naturally occurring phytoalexin present in more than 72 plant species, with relatively high concentrations in red grapes. Resveratrol exists in two isomeric forms, *cis*- and *trans*- [333]. A number of pharmacological effects observed in experimental systems have used the t*rans* form of resveratrol rather than the *cis* form [39, 334-336]. The pathogenesis of respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) involve the excessive accumulation of free radicals, leading to oxidative stress [337]. Therefore, alleviating oxidative stress with an antioxidant supplement may be effective therapy in respiratory disease [337]. Using cultured Calu-3 cells, the aim of the thesis as presented in Chapter 1 was to investigate the role played by antioxidants, namely vitamin D, lycopene, zinc and resveratrol, against rhinovirus-induced inflammation and viral replication in human airway epithelial cells. Prior to challenging the epithelial cells with virus it was important to establish whether these antioxidants affect cell viability. The concentrations required to maximise bioavailability of zinc, lycopene and vitamin D has been previously established [50, 288, 312]. However, this information was not available for resveratrol. Hence, the aim of this chapter was to describe the effect of vitamin D, zinc, resveratrol and lycopene on cell viability. In addition, resveratrol uptake and release by Calu-3 cells, a human airway epithelial cell line is described.

3.2 Materials and methods

Study design:

In this study, confluent Calu-3 cells were incubated for up to 48 hours with different concentration of resveratrol, lycopene, vitamin D and zinc dissolved either in DMSO or THF or ethanol or sterilized water, respectively. Cells were visually inspected before

and after supplementation with antioxidant compounds, and media and cells were harvested to analyse the cellular release of resveratrol and intracellular resveratrol concentrations.

Enriching the airway cells with resveratrol:

As described in Chapter 2, human airway epithelial cells (Calu-3, from ATCC, USA) were cultured in 24 well plates in minimum essential medium containing 10% foetal calf serum (10%FCS/MEM) containing 2% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine, and 2.2g/l $NaHCO_3$ (all were purchased from Invitrogen Corporation, Carlsbad, USA) at 37ºC in the presence of 5% CO_2. When cells were confluent, the medium was replaced by fresh medium containing different concentrations of resveratrol/DMSO in 1% foetal calf serum/minimum essential medium (1%FCS/MEM). The final concentrations of resveratrol in the media were 0, 5, 10, 25, 50 and 100 µM. The medium containing DMSO without resveratrol was used as control. Cells were incubated for 1, 2, 4, 6, 12, 24, 48 and 72 hrs at 37ºC in the presence of 5% CO_2. Cell culture experiment was performed three times.

Enriching the airway cells with zinc:

As described in Chapter 2, human airway epithelial cells (Calu-3, from ATCC, USA) were cultured in 24 well plates in minimum essential medium containing 10% foetal calf serum (10%FCS/MEM) containing 2% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine, and 2.2g/l $NaHCO_3$ (all were purchased from Invitrogen Corporation, Carlsbad, USA) at 37ºC in the presence of 5% CO_2. When cells were confluent, the medium was replaced by fresh medium containing a concentration of zinc/sterilized water in 1% foetal calf serum/minimum essential medium (1%FCS/MEM). The final concentrations of zinc in the media were 25µM.

Cells were incubated for 4 hrs at 37ºC in the presence of 5% CO_2. Cell culture experiment was performed three times.

Enriching the airway cells with vitamin D:

As described in Chapter 2, human airway epithelial cells (Calu-3, from ATCC, USA) were cultured in 24 well plates in minimum essential medium containing 10% foetal calf serum (10%FCS/MEM) containing 2% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine, and 2.2g/l $NaHCO_3$ (all were purchased from Invitrogen Corporation, Carlsbad, USA) at 37ºC in the presence of 5% CO_2. When cells were confluent, the medium was replaced by fresh medium containing a concentration of vitamin D/ethanol in 1% foetal calf serum/minimum essential medium (1%FCS/MEM). The final concentrations of vitamin D in the media were 1µM. Cells were incubated for 18 hrs at 37ºC in the presence of 5% CO_2. Cell culture experiment was performed three times.

Enriching the airway cells with lycopene:

As described in Chapter 2, human airway epithelial cells (Calu-3, from ATCC, USA) were cultured in 24 well plates in minimum essential medium containing 10% foetal calf serum (10%FCS/MEM) containing 2% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine, and 2.2g/l $NaHCO_3$ (all were purchased from Invitrogen Corporation, Carlsbad, USA) at 37ºC in the presence of 5% CO_2. When cells were confluent, the medium was replaced by fresh medium containing a concentration of lycopene/THF in 1% foetal calf serum/minimum essential medium (1%FCS/MEM). The final concentrations of lycopene in the media were 4.65µM. Cells

were incubated for 24 hrs at 37ºC in the presence of 5% CO_2. Cell culture experiment was performed three times.

Visual inspection of Calu-3 cells:

Cellular viability of cultured Calu-3 cells was observed before and after antioxidant compounds supplementation (Olympus microscope, TL4).

Analysing extracellular and intracellular resveratrol:

After enriching the cells with different concentrations of resveratrol (0, 5, 10, 25, 50 and 100 µM) for 1, 2, 4, 6, 12, 24, 48 and 72 hrs, media and cells were harvested into microcentrifuge tubes and the concentration of resveratrol in media and cells were measured by HPLC (method described in Chapter 2, Section 2.3.1.1 and 2.3.1.2). Resveratrol concentrations were expressed as µg/mL.

3.3 Results

Effect of antioxidants on cells viability of human airway epithelial cells

Viable Calu-3 cells cultured in 24 well plates seen under the microscope are shown in Figure 3.3.1.

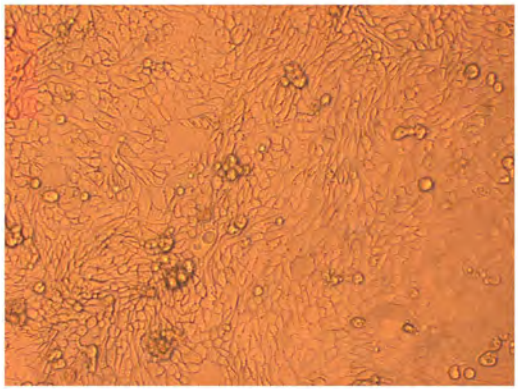


Figure 3.3.1. Calu-3 cells 100% confluent in the bottom of 24-well plate

Calu-3 cells enriched with resveratrol/DMSO, lycopene/THF, zinc/water and vitamin D/ethanol showed no sign of toxicity at all concentrations tested, for a period of

up to 24 hours. The following figures show representative images of Calu-3 cells after incubation with 1%FCS/MEM media containing resveratrol, lycopene, zinc and vitamin D.

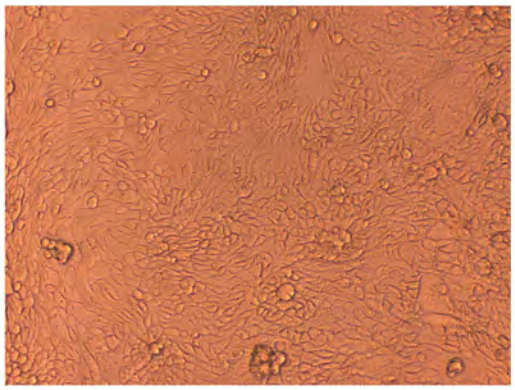


Figure 3.3.2. Calu-3 cells after incubation with resveratrol for 24 hours

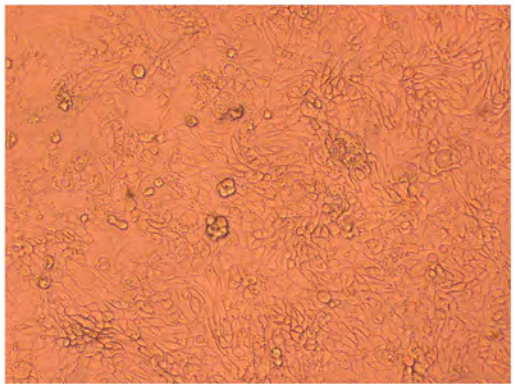


Figure 3.3.3. Calu-3 cells after incubation with 1µM of vitamin D for 24 hours

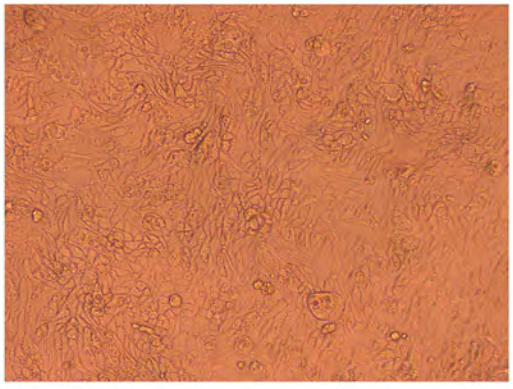


Figure 3.3.4. Calu-3 cells after incubation with 2.5µg/mL of lycopene for 24 hours

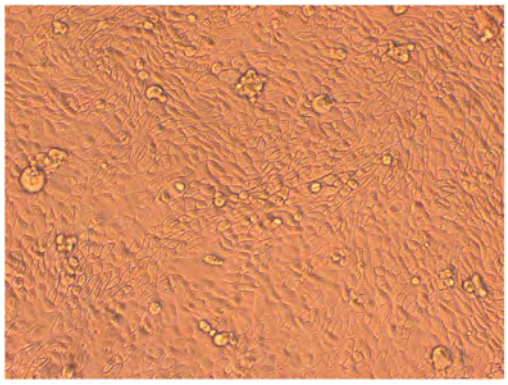
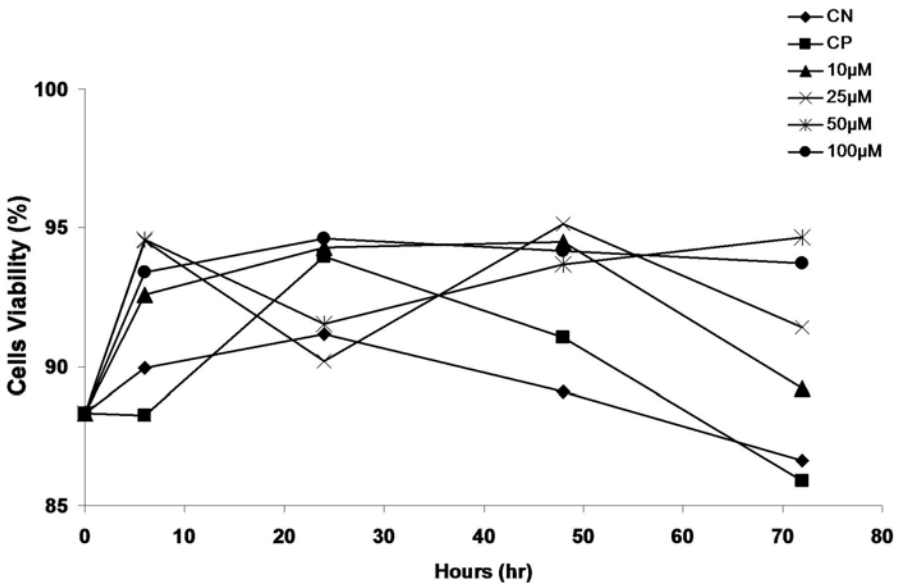


Figure 3.3.5. Calu-3 cells after incubation with 25 µM of zinc for 24 hours

Trypan blue staining was used to quantitatively measure the viability of cells. In this widely used technique, dead cells are expected to be stained with Trypan blue, thus under the microscope the cells will appear blue. Using a haemocytometer, the viable and dead cells were counted. The following figure shows a representative experiment to quantitatively demonstrate an effect of resveratrol on Calu-3 cell viability (Figure 3.3.6). Calu-3 cells were exposed to varying concentrations of resveratrol in media for 72 hours. Throughout the period, cells were still showing good viability based on the Trypan blue staining method (Figure 3.3.6).



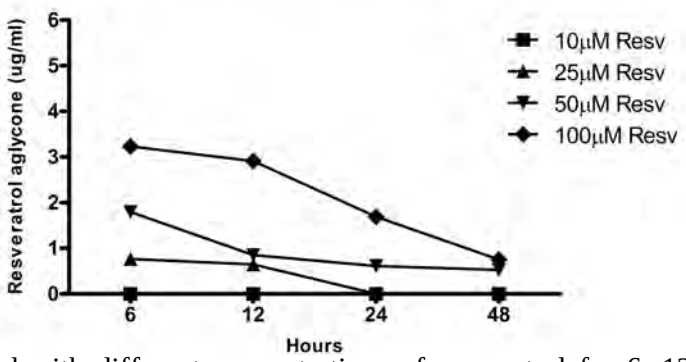
Cells were cultured with different concentration of resveratrol for 6, 24, 48 and 72 hours. Resveratrol was dissolved in the media containing 0.1% DMSO. After each time point, cells were harvested and cell viability was estimated based on Trypan blue dye exclusion. CN, cells were administered with normal media; CP, cells were administered with media containing 0.1% DMSO; 10 μM, 25 μM, 50 μM and 100 μM, cells were administered with different concentrations of resveratrol.

Figure 3.3.6. Cytotoxicity of resveratrol on Calu-3 cells

Resveratrol bioavailability on human airway epithelial cells:

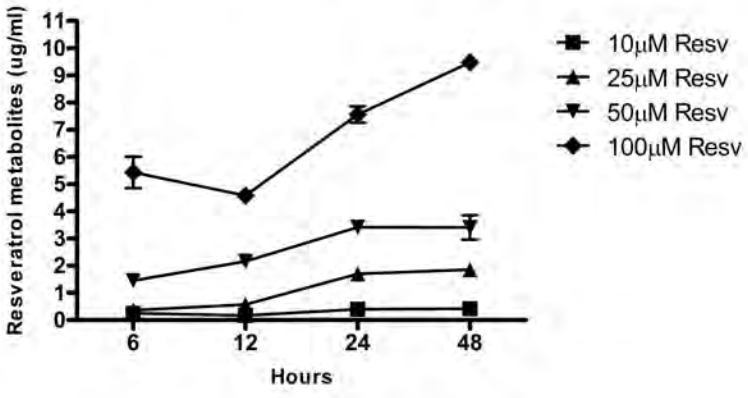
Trans-resveratrol (resveratrol) was administered at 5 different concentrations namely 5, 10, 25, 50 and 100 μM and 1.141, 2.282, 5.705, 11.41, and 22.8 μg/ml are the equivalent amount of resveratrol, respectively given to human airway epithelial cells. The primary objective of the experiments on bioavailability of resveratrol was to determine whether the cells can uptake resveratrol compounds and to determine achievable intracellular resveratrol concentrations. Dimethyl sulfoxide (DMSO) was used to dissolve the resveratrol and to transport the resveratrol inside the cells. Resveratrol metabolites (glucuronide and sulphate) were indirectly measured after hydrolysis with the enzyme β-glucuronidase (also containing sulphatase activity). As shown in Figure 3.3.7, resveratrol aglycone (the free from of resveratrol) was decreased

at the end of study (48 hours). The availability of resveratrol aglycone in the media decreased in a dose-dependent fashion. In contrast, the extracellular level of resveratrol metabolites increased in a dose dependent manner at the end of the study (Figure 3.3.8). Both figures (Figure 3.3.7 and Figure 3.3.8) show that, resveratrol was metabolized by the cells. However, the availability of resveratrol aglycone intracellularly, was not detected.



Cells were cultured with different concentrations of resveratrol for 6, 12, 24 and 48 hours. Resveratrol was dissolved in the media containing 0.1% DMSO. After each time point, media was harvested and concentrations of resveratrol were determined using HPLC.

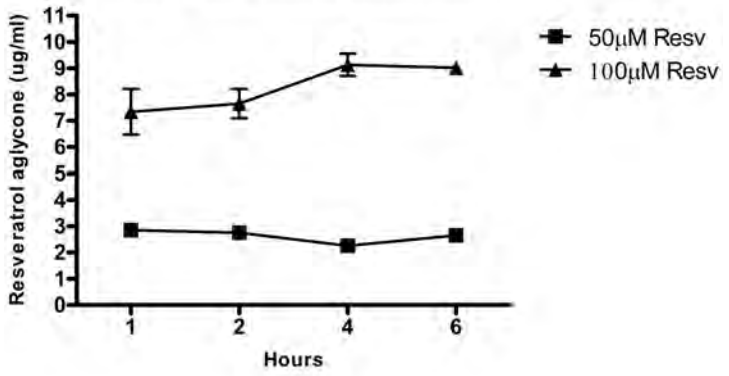
Figure 3.3.7. Concentration of resveratrol (aglycone) in extracellular (media) Calu-3 cells



Cells were cultured with different concentrations of resveratrol for 6, 12, 24 and 48 hours. Resveratrol was dissolved in the media containing 0.1% DMSO. After each time point, media were harvested and concentrations of resveratrol were determined using HPLC.

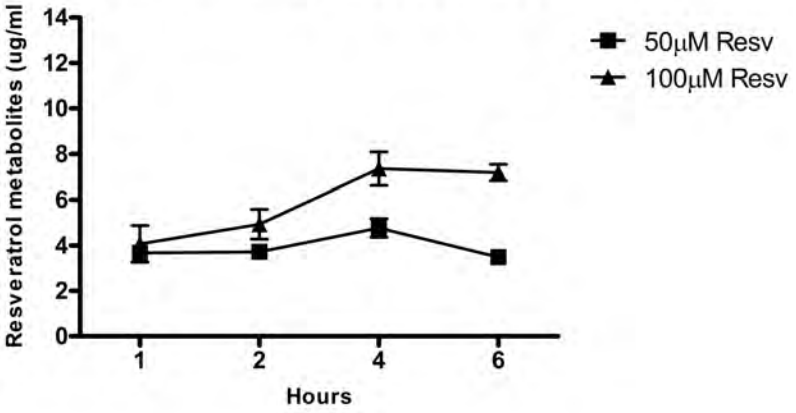
Figure 3.3.8. Resveratrol metabolites in extracellular (media) Calu-3 cells

Bioavailability of resveratrol within the first 6 hours is shown in Figure 3.3.9, 3.3.10 and 3.3.11. In this study, resveratrol bioavailability was examined after incubating the cells with high concentrations of resveratrol, that is 50 and 100 μM. As shown in Figure 3.3.9, the level of resveratrol in the media remained similar at the end of the study (6 hours). However, the release of resveratrol metabolites into the media (extracellular) was increased slightly at the end of the study (Figure 3.3.10) suggesting resveratrol metabolism via conjugation with polar groups. The free form of intracellular resveratrol was not detected in this study; only resveratrol metabolites were detected. The concentration of resveratrol metabolites was found approximately to be 0.4 μg/ml, when the cells were incubated with either 50 μM or 100 μM resveratrol (Figure 3.3.11). From this observation the experiments were extended to determine whether administration of resveratrol at low concentration would result in detectable amounts of resveratrol metabolites. Figure 3.3.12 shows detectable amounts of resveratrol metabolites after cells was administered with 5, 10 and 25 μM resveratrol. In summary, the bioavailability study of resveratrol has found that, detectable intracellular concentrations (0.3 to 0.4 μg/ml or 1.3 to 1.8 μM respectively), can be achieved following incubation with 5 μM to 100 μM resveratrol for 2 hours.



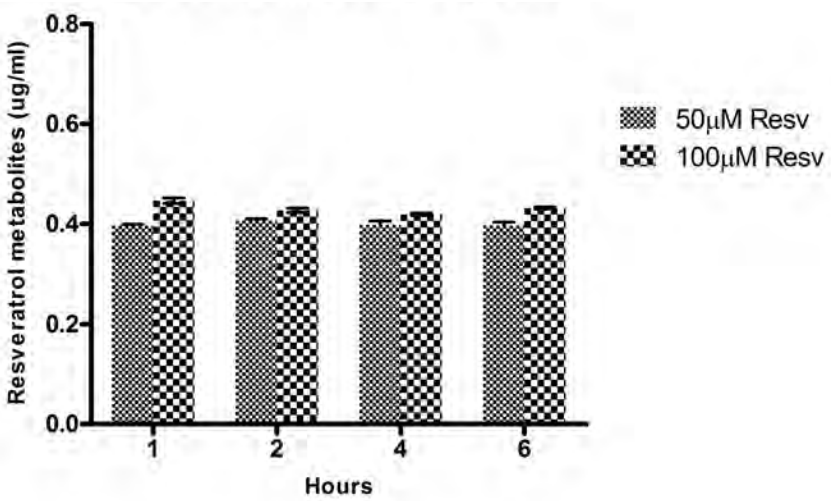
Cells were cultured with 50 μM and 100 μM of resveratrol for 1, 2, 4 and 6 hours. Resveratrol was dissolved in the media containing 0.1% DMSO. After each time points, media were harvested and concentrations of resveratrol were determined using HPLC.

Figure 3.3.9. Resveratrol aglycone (within 6 hours) in extracellular (media) Calu-3 cells



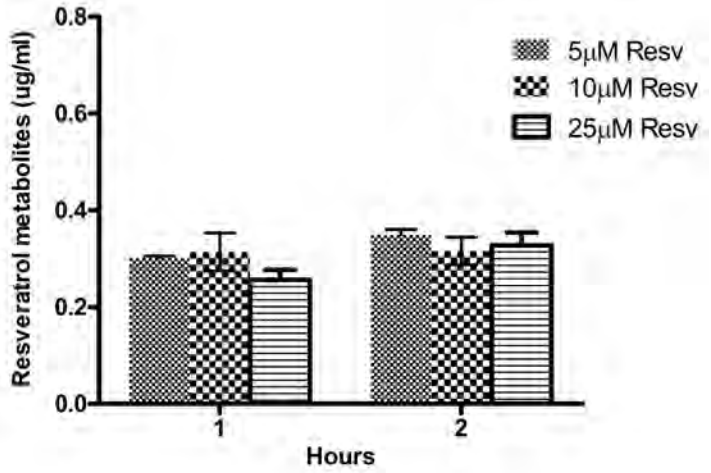
Cells were cultured with 50 μM and 100 μM of resveratrol for 1, 2, 4 and 6 hours. Resveratrol was dissolved in the media containing 0.1% DMSO. After each time points, media were harvested and concentrations of resveratrol were determined using HPLC.

Figure 3.3.10. Resveratrol metabolites (within 6 hours) in extracellular (media) Calu-3 cells



Cells were cultured with 50 μM and 100 μM of resveratrol for 1, 2, 4 and 6 hr. Resveratrol was dissolved in the media containing 0.1% DMSO. After each time points, cells were harvested and concentrations of resveratrol were determined using HPLC.

Figure 3.3.11. Resveratrol metabolites (within 6 hours) intracellular of Calu-3 cells after 50 and 100 μM of resveratrol administration



Cells were cultured with 5, 10 and 25 μM of resveratrol for 1, 2, 4 and 6 hours. Resveratrol was dissolved in the media containing 0.1% DMSO. After each time points, cells were harvested and concentrations of resveratrol were determined using HPLC. Resveratrol was not detected at 4 and 6 hours after resveratrol administration.

Figure 3.3.12. Resveratrol metabolites (within 6 hours) intracellular of Calu-3 cells after 5, 10 and 25 μM of resveratrol administration

3.4 Discussion

Antioxidant was administered once cells have reached confluence level. In the present study, we have shown that resveratrol, lycopene, vitamin D and zinc at concentration tested were not causing toxicity to the Calu-3 cells. In agreement with our study, resveratrol has shown no toxicity effects when administered in cultured A549 cells and human primary epithelial cells [43], MDA-MB-231 and ZR-75-1 breast cancer cells [338].

The present study is the first study to demonstrate on the cellular uptake and release of resveratrol on human airway epithelial cells. This study demonstrates that resveratrol is actively taken up into Calu-3 cells when incubated with varying concentrations in the culture medium. Preliminary studies showed that (data not shown) when resveratrol was dissolved in 0.1% ethanol, the presence of resveratrol (free and metabolites form) intracellularly remained undetected. However, with DMSO, an agent to deliver the drug into the site of action, resveratrol can be successfully delivered into the cells.

It is also noted in the present study, resveratrol aglycone [the form used in the study (trans-resveratrol)] could not be detected intracellularly. This finding explains the low bioavailability of resveratrol as been widely demonstrated by others [339-341]. Resveratrol is rapidly metabolized by the cells via conjugation either through glucuronidation or sulfation for the cells to excrete the compound. Due to resveratrol low bioavailability, it is proposed that the resveratrol metabolites could be responsible to biological actions of resveratrol [339].

As shown in the results section, it was noted that amount of resveratrol aglycone present in the extracellular (media) between in the earlier experiment (Figure 3.3.7) and

in the later experiment (Figure 3.3.9) was different, even though the amount of resveratrol metabolites in the extracellular for both experiments was showing similar amounts. These observed finding was possibly due to the differences in the exactly (practically) number of cells present in the wells during administration of resveratrol. Another reason might be due to the differences in the passage number between earlier experiment and later experiment.

Table 3.4.1. Physiological concentration of antioxidants in human: resveratrol, zinc, lycopene and vitamin D

Compounds	Plasma/Serum	Authors
Lycopene	Approximately 1µM *	Cohn et al. [342]
	0.25 µM*	Böhm and Bitsch [343]
	0.57 µM*	Edward et al. [344]
	0.95 µM*	Hoppe et al. [345]
	0.545 – 0.784 µM $^{\Psi}$	Hadley et al. [346]
	1.2 - 1.3 µM $^{\Psi}$	Zhao et al. [347]
Resveratrol	0.065 – 0.74 µM (glucuronides metabolite)$^{\text{ɤ}}$	Vitaglione et al. [348]
	0.3- 3.9 µM (glucuronides metabolite)$^{\text{þ}}$	Vitaglione et al. [348]
	2 µM (sulfates and glucuronides metabolites)	Walle et al. [340]
Zinc	16.05 µM $^{\Psi}$	Prasad et al. [272]
	15.9 µM $^{\Psi}$	Prasad et al. [290]
Vitamin D	0.047 – 0.12 µM (25-hydroxyvitamin D)	Jakobsen et al. [302]
	0.07 µM $^{\varphi}$ (25-hydroxyvitamin D)	Robberecht et al.[300]
	0.07 µM (25-hydroxyvitamin D)	Fedirko et al. [315]

Data represent mean values ($^{\varphi}$ median value) and unit of concentration is standardized (µM) based on values provided by the authors in the paper.
* Values obtained from bioavailability studies of lycopene
$^{\text{ɤ}}$ Values obtained from bioavailability study of resveratrol from red wine (300 ml consumption)
$^{\text{þ}}$ Values obtained from bioavailability study of resveratrol from red wine (600 ml consumption)
$^{\Psi}$ Plasma concentrations of antioxidant compounds with proven biological effects

At physiological concentrations, Zn has been shown to possess antioxidant and anti-inflammatory activities in many *ex vivo* and *in vitro* studies [269, 272, 290]. It was

observed that, the anti-inflammatory of Zn was due after administration of Zn to the cells, neglecting the concentration of Zn intracellularly [269]. Clinton et al. [227] has presented evidence on the concentrations of lycopene in various human tissues including liver, adipose, lung and kidney. For instance, lycopene content in the liver was found to be 0.6 nM/g of tissues. Hence, the study presented by the authors have shown that lycopene is scattered in numerous tissues of human body [227], suggesting the beneficial role plays by lycopene in maintaining human health.

It has been published before, that antioxidant compounds can act as both antioxidant and pro-oxidant under different conditions. Part of the reason is related to the type of cells used and the concentration of the antioxidants use. Resveratrol has been demonstrated to have both actions [349]. In this current study we strongly believe that all the antioxidants used in this study at designated concentrations were possessed antioxidant activities, rather than pro-oxidant activity. As been shown in the Figure 3.3.1 to Figure 3.3.5, it was clear that cells incubated with resveratrol, vitamin D, lycopene and zinc were viable and healthy. Resveratrol was tested at concentrations 25 and 50 µM in a study to determine its antioxidant role against H_2O_2-induced cells cytotoxicity [350]. It was shown that, resveratrol at both concentrations markedly improved cells viability as compared to untreated cells [350]. The observed effect was strongly related to resveratrol antioxidative activity, as H_2O_2-induced cells cytotoxicity is mediated through oxidative stress. Others also have shown that resveratrol at a lower concentration (10 µM) exhibited strong antioxidant activity against cigarette smoke extract-induced ROS in A549 cells [217] . In an experiment done by Coppen et al.[351], Zn at concentrations below than 50 µM exhibited strong antioxidant activity as demonstrated by inhibitory effect of Zn on free radical production and lipid peroxidation in cultured hepatocytes. Vitamin D at a concentration of 0.1 µM exhibited

potent antioxidant effect against oxidative stress induced by H_2O_2 in non-malignant human prostate epithelial cells [319]. Lycopene when tested at a concentration of 50 μM has shown potent antioxidant activity as demonstrated by the decreased in the formation of TBARS in a liposome system [253].

As can be seen in Table 3.4.1, the concentrations we used in this study are in the range of concentrations which have been found to be physiologically relevant in humans and can easily be achieved with nutrient supplementation. Similarly, the concentrations of vitamin D (1μM), zinc (25μM) and lycopene (4μM) that we have used in subsequent experiments, as established previously in the literature, are in the physiologically relevant range.

3.5 Summary

Supplementation of antioxidant compounds (lycopene, vitamin D, zinc and resveratrol) to Calu-3 cells showed no sign of toxicity to the cells. Resveratrol was successfully delivered to Calu-3 cells in the first 6 hours of resveratrol supplementation to the cells.

CHAPTER 4: EFFECTS OF ANTIOXIDANTS ON RHINOVIRUS REPLICATION AND RHINOVIRUS-INDUCED INFLAMMATION IN CULTURED AIRWAY EPITHELIAL CELLS

4.1 Introduction

Emerging evidence has shown that chronic inflammation is an integral part of the pathogenesis of asthma. The 2012 updated Global Initiative for Asthma (GINA) guidelines [352] proposes an operational definition of asthma as a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. Asthma is usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment.

Virus infections, in particular human rhinovirus (RV), are associated with the majority of exacerbations of asthma and chronic obstructive pulmonary disease (COPD). Johnston et al. [3] found that about 80-85% of asthma exacerbations in school age children are associated with upper respiratory viral infections and in particular picornaviruses (mostly RV) accounted for two thirds of these viral infections. An earlier study conducted by Nicholson et al. [4] found that respiratory virus infections commonly cause, or are associated with, exacerbations of asthma in adults.

RV infects airway epithelial cells, and triggers the cells to initiate a defensive response. However, in asthmatic epithelial cells, a defective antiviral response leads to chronic inflammation following RV infection. [141] Monocytes taken from asthmatic subjects and rendered to RV infection also show an impaired immune response, with deficient production of IFN-γ [141]. Others have suggested that asthmatic airway epithelial cells may also have reduced surface expression of TLR3 which is involved in antiviral responses [353]. The chronic inflammation induced by RV infection is believed to be the main mechanism responsible for exacerbations of asthma. Hence, it is believed that airway epithelial cells act as a host for RV and are central to the inflammatory response.

Events occurring in airway epithelial cells once infected with RV have been described in Chapter 1 (Section 1.8 and 1.9). Figure 4.1.1 illustrates the roles played by airway epithelial cells during RV infection, with subsequent initiation of innate and adaptive immune responses. The goal for activation of the host immune response is to eradicate the RV. RV infects airway epithelial cells, replicates and newly synthesized RV is believed to be released via both apical and basolateral membranes of the cells. For example, a study done by Zhang et al. [354] has found that newly synthesized respiratory synctical virus (RSV), after cells are infected with RSV, is primarily released from the apical membranes of the cells. Pro-inflammatory mediators (IL-1β) released by the airway epithelium due to RV infection up-regulate ICAM-1 surface receptor expression thus favouring/increasing susceptibility to RV infection [355]. It has been demonstrated that antibodies against ICAM-1 surface protein prevent RV infection [9, 24, 355, 356].

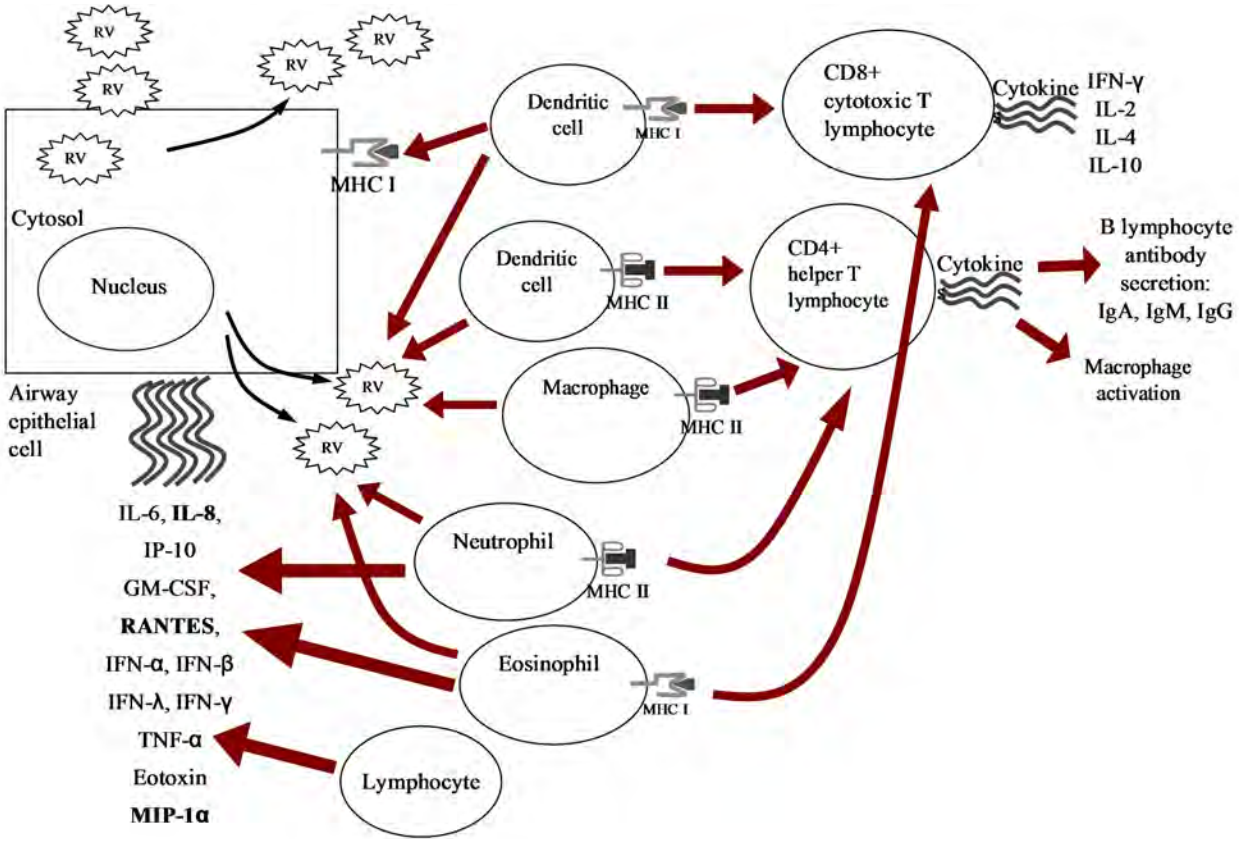


Figure 4.1.1. Airway epithelial cells are the primary target of RV and are implicated in the initiation of innate and activation of adaptive immunity; MHC I – major histocompatibility complex class I; MHC II - major histocompatibility complex class II; IL-6, -8, -4, -10 – interleukin-6, -8, -4, -10; IP-10 - interferon-gamma-induced protein-10; GM-CSF - granulocyte-monocyte-colony stimulating factor; RANTES - regulated upon activation normal T-cell expressed and secreted; IFN-α,-β, -λ,-γ – interferon- α,-β, -λ,-γ; TNF-α – tumor necrosis factor-α; MIP-1α – macrophage inflammatory protein-1α; Ig – immunoglobulin; RV – rhinovirus.

The expression of major histocompatibility complex (MHC) class I and class II molecules on human airway epithelial cells has been previously demonstrated [357]. In a study done by Papi et al. [358], the authors have shown the upregulation of MHC class I and co-stimulatory molecules (B7-2) in RV (major group)-infected airway epithelial cells. Hence the study has shown that airway epithelial cells present infected cells as antigen presenting cells (APC) to dendritic cells (professional APC) for specific activation of CD8+ cytotoxic T lymphocytes, in order to kill the infected cells. The induction of professional APC (dendritic cells) is crucial to prevent release of progeny virus which may infect adjacent cells. Dendritic cells are able to present antigen presenting cells (MHC class I) expressed on other cells, as indicated by infected airway epithelial cells, to cytotoxic T lymphocytes for subsequent termination of infected cells (preventing infected cells from becoming a virus reservoir). The ability of dendritic cells to sense MHC class I on infected cells provides a signal to activate adaptive immunity against infection.

However, RV belong to major group that has been demonstrated to down regulate the T cell stimulatory function of dendritic cells [359]. Co-culture of RV with dendritic cells (from isolated PBMC of normal healthy individuals) results in induction of MHC class I and MHC class II, and normal expression of co-stimulatory molecules including B7-1, B7-2 and B7-H3 on infected dendritic cells. However, inhibition of T cell proliferation following RV co-culture with dendritic cells was demonstrated by upregulation of two inhibitory receptors on dendritic cells, which are B7-H1 (PD-L1) and sialoadhesin [359]. Blocking of B7-H1 and sialoadhesin on infected dendritic cells restored T cell stimulatory capacity to similar levels as uninfected dendritic cells [359]. The inactivation of T-lymphocytes through T cell anergy due to interaction between RV and dendritic cells [359] might explain the delayed RV specific immune response [360],

hence activity against eradicating RV is hindered even though recruitment of leukocytes.

Airway epithelial cells (in particular goblet cells) have the ability to secrete mucus containing β-defensin-2 and viperin, which have bactericidal properties to kill the invading microorganisms [361, 362]. Infected airway epithelial cells produce a wide array of cytokines including IL-6, IL-8, IP-10, GM-CSF, RANTES, IFN-α, IFN-β, IFN-λ, TNF-α, eotoxin and MIP-1α, in order to attract leukocytes (neutrophils, eosinophils, macrophages) and to interfere with viral replication. RV infection leads to a rapid increase in the numbers of neutrophils and lymphocytes in the airway of normal and asthmatic subjects [1, 363, 364]. The secretion of inflammatory cytokines by airway epithelial cells is believed to act as an important mechanism contributing to the pathogenesis of asthma exacerbations [365]. Therefore, inhibition of RV-induced inflammatory cytokines via anti-inflammatory therapy is considered to be an attractive and mainstream treatment for managing and treating asthma. Leukocytes function to eradicate the RV via phagocytosis and endocytosis. Neutrophils and macrophage are capable of presenting peptide antigens on MHC class II to CD4+ helper T lymphocyte for specific activation of adaptive immunity (differentiation of B lymphocyte antibody secretion and macrophage activation) [366, 367]**.** It has been shown that monocytes and airway macrophages can be infected with RV (major group) [368] and this indirectly provides information about the presence of ICAM-1 on the surface of those cells. Additionally, RV has been shown to be unable to replicate on monocytes and macrophages [368] and have limited replication in monocyte-derived macrophages [369], hence strengthening the observation that the primary function of the cells is to eradicating viruses. Recruitment of macrophages is implicated in orchestrating the inflammatory response to RV infection. RV infected macrophages secrete TNF-α via

the Nf-κB pathway [369], and TNF-α is regarded as a strong pro-inflammatory cytokine implicated in the pathogenesis of asthma. Eosinophils co-cultured with RV (major group) have been shown to act as antigen presenting cells (presentation of viral antigen by MHC I) to viral specific-T cells (CD8+ cytotoxic T lymphocytes), hence causing T cell activation and subsequent secretion of antiviral protein IFN-γ [370]. The study also demonstrated that co-culture of eosinophils with RV (major group) increases expression levels of surface receptor of ICAM-1 [370].

There is a paucity of data documenting the effectiveness of secretion of IgG, IgA and IgM by B lymphoctyes after RV inoculation (antibodies-mediated neutralization). Generally, antibodies prevents the extent of infection via blocking the binding of the microbe onto targeted cells and blocking the infection of adjacent cells. It appears that antibodies secreted by B cells are unable to prevent the RV from binding to BEC. This is probably due to the way in which RV binds to the host cells. The atomic resolution structure of RV revealed that the structure has deep depressions known as canyons, which are believed to be the site of receptor binding [371-373]. The sites are conserved and antibodies have difficulty binding to this site [374, 375]. This provides the ability of RV to escape the host immune system surveillance [375]. VP1 proteins on the rhinovirus capsids contain epitopes which are recognized by neutralizing antibodies [376]. Hence, the most likely mechanisms on how antibodies play a preventive role is by acting as opsonins. It remains to be established, however whether secreted antibodies are able to coat viral antigens present on VP1 of viral capsid proteins for subsequent phagocytosis by macrophages.

Therefore, RV infection of airway epithelial cells activates innate immunity and provides a signal for adaptive immunity to participate in clearing the virus. However, due to “intelligent characteristics” possessed by RV, which are able to escape the

continuous surveillance of antibodies and cause defective interaction between professional antigen presenting cells with T lymphocytes, chronic inflammation occurs. By focusing on the airway epithelium, the site of viral infection and replication, our understanding of the inflammatory response to viral replication can be improved. The Nf-κB is one of the pathways that is implicated in RV-induced inflammation. Alteration of this pathway has been shown to reduce the host pro-inflammatory response

Resveratrol, lycopene, zinc and vitamin D possesses strong antioxidant and anti-inflammatory properties *in vitro* and *in vivo*, as described in Chapter I. Those properties, however haven't been studied before in association with RV infection. A few studies have demonstrated the anti-inflammatory effect of resveratrol in lung epithelial cells [43] and alveolar macrophages [377]. Over the last 10 years, vitamin D has gained recognition as a nutrient having important immunomodulatory effects against respiratory tract infection. Co-treatment of primary cells [human tracheobronchial epithelial (hTBE) cells] with the inactive form of vitamin D (25-hydroxyvitamin D_3) and respiratory syncytial virus (RSV) for 24 hours, resulted in a significant increase in secreted antimicrobial protein (cathelicidin) compared to RSV-infected cells [312]. Pre-treating the cells with the active form of vitamin D (1,25-dihydrovitamin D_3) for 18 hours before infection with RSV for 24 hours resulted in significant decreases in RSV induction of IFN-β and interferon stimulated genes (IP-10, interferon stimulated gene-15 and MxA proteins) [311]. Those effects demonstrated by vitamin D were shown to be mediated by its effect on increasing expression of Nf-κB inhibitory protein, Iκβα [311]. Previously, the anti-inflammatory effect of lycopene against RV infection of airway epithelial cells was documented [50] in our laboratory. Lycopene is included in the current study for two reasons: firstly to confirm the published protective effect against RV and secondly to undertake detailed exploration of lycopene anti-rhinoviral

activity. *In vitro* anti-rhinoviral activities of Zn have been documented [178, 273], and it will be very interesting to extend those observations in a model of RV infection of airway epithelial cells.

Based on what has been presented and discussed, airway epithelial cells play very important roles in the activation of innate and adaptive immunity during RV infection. Hence, preventing or limiting airway epithelial cell production of proinflammatory cytokines and at the same time preventing or limiting RV replication are crucial events that will reduce the severity of exacerbations of asthma. Therefore, the aim of the current study is to investigate the role of antioxidants on RV replication and RV-induced inflammation in airway epithelial cells.

4.2 Materials and Methods

Study design:

In this study, confluent Calu-3 cells were incubated with resveratrol, zinc, vitamin D or lycopene for 2, 4, 18 or 24 hours, respectively prior to infection with RV. After removing the media containing aforementioned antioxidants, the cells were incubated with RV1B (minor group) or RV43 (major group) for 1 hour and agitated at room temperature to allow efficient binding of virus. Infection media was then replaced with fresh media, and cells were incubated for 48 hours. Media was collected for measurement of IL-6, IP-10 and IL-8, and viability of cells (consisting viable, apoptotic and necrotic) was assessed at the end of experiment.

Airway epithelial cells:

As described in Chapter 2, human airway epithelial cells (Calu-3, from ATCC, USA) were cultured in 24 well plates in minimum essential medium containing 10% foetal

calf serum (10%FCS/MEM) containing 2% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine, and 2.2g/l $NaHCO_3$ (all were purchased from Invitrogen Corporation, Carlsbad, USA) at 37ºC in the presence of 5% CO_2.

Enriching the airway cells with resveratrol:

As described in Chapter 2 (Section 2.2.9), confluent Calu-3 cells were incubated with resveratrol (5, 10, 25, and 50µM) dissolved in DMSO (0.1% DMSO in cell culture medium). Cells were incubated for 2 hours (as determined in Chapter 3) at 37ºC in the presence of 5% CO_2.

Enriching the airway cells with zinc:

As described in Chapter 2 (Section 2.2.9), confluent Calu-3 cells were incubated with zinc dissolved in sterilized water. The final concentrations of zinc in the media were 10, 25, and 50 µM, based on previously published data [288]. Cells were incubated for 4 hours at 37ºC in the presence of 5% CO_2.

Enriching the airway cells with vitamin D:

As described in Chapter 2 (Section 2.2.9), confluent Calu-3 cells were incubated with vitamin D dissolved in sterilized ethanol (0.1% ethanol in cell culture medium). The final concentrations of vitamin D in the media were 0.01, 0.1 and 1µM, based on previously published data [311, 312]. Cells were incubated for 18 hours at 37ºC in the presence of 5% CO_2.

Enriching the airway cells with lycopene:

As described in Chapter 2 (Section 2.2.9), confluent Calu-3 cells were incubated with lycopene dissolved in THF (0.5% THF in cell culture medium). The final concentrations of lycopene in the media were 2.5, 5 and 10 µg/ml, based on previously published data [50]. Cells were incubated for 24 hours at 37ºC in the presence of 5% CO_2.

Rhinovirus challenge of Calu-3 cells:

As described in Chapter 2, after removing the supplementation media from Calu-3 cells (as mentioned above), they were either infected with (a) RV-1B at multiplicity of infection (MOI) of 20 or (b) RV-43 at multiplicity of infection (MOI) of 50. MOI was calculated using formula as describe below:

$$\text{Volume (ul) of virus stock} = \frac{1000 \times (\text{MOI} \times \text{number of cells})}{\text{TCID}_{50}\ \text{Rhinovirus}}$$

Plates were incubated for 48 hours at 33ºC in the presence of 5% CO_2.

Visual inspection of Calu-3 cells:

Cellular viability of cultured Calu-3 cells was observed before and after supplementation (Olympus microscope, TL4).

Analysing the inflammatory biomarkers concentration of media:

As described in Chapter 2, interleukin-6 (IL-6) and interferon-gamma induced protein-10 (IP-10) concentrations of medium were measured using BD CBA Soluble Protein Flex Set System [BD™ Cytometric Bead Array (CBA) Human IL-6 Flex Set and BD™ Cytometric Bead Array (CBA) Human IP-10 Flex Set (BD Bioscences, San Jose,

USA)] according to manufacturer's instructions and analysed using flow cytometer (BD FACSCanto^{TM} II Flowcytometer, BD Biosciences, San Jose, CA, USA); and interleukin -8 (IL-8) concentration of medium were measured by ELISA (R&D Systems Minneapolis, MN, USA) according to manufacturer's instructions. The assays were undertaken in duplicate for each cell culture assay.

Flow cytometric analysis of viable, apoptotic and necrotic cells:

Flow cytometry was used to quantify the number of viable, apoptotic and necrotic cells after 48 hours of viral infection, as described in Chapter 2 (Section 2.3.3).

Viral titration assay:

As described in Chapter 2, $TCID_{50}$ experiments were performed using near confluent RD-ICAM-1 cells seeded in 96-well tissue culture plates (NUNC, Roskilde, Denmark). Cells were infected by either media alone or virus containing media at varying dilutions. Serial ten-fold dilutions of the samples were prepared and 4 individual wells were infected with each dilution. For titration of samples 7 dilutions were prepared, and 4 control wells were prepared with media alone. After five days the plates were read and $TCID_{50}$ calculated. Infected wells were scored based on the cytophatic effect (CPE) seen, >50% CPE was demonstrated by light microscopy was considered a positive result. Viral titers of the samples determined by cell titration assay using RD-ICAM-1 cells and the viral titer was calculated and expressed as tissue culture infectious dose at 50% in log value ($TCID_{50}$ log_{10}) [328] and using the Karber formula for the tissue culture infective dose 50% ($TCID_{50}$) [329].

Statistical analysis:

The results are presented as mean ± SEM values or as medians with interquartile range. Differences between antioxidant supplemented and un-supplemented airway epithelial cells prior to RV infection were analysed using either one-way ANOVA test, followed by Dunnet's multiple comparison test (for normally distributed data) or Kruskal-Wallis test, followed by Dunn's multiple comparison test (for not normally distributed data) by Graphpad Prism® Version 6.00 for Windows, GraphPad Software, La Jolla California, USA). A P value of 0.05 or less was considered statistically significant.

4.3 Results

Visual inspection of Calu-3 cells:

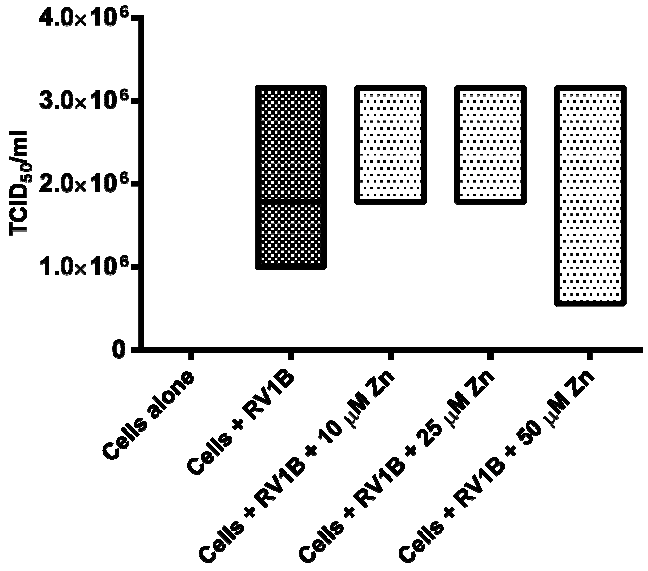
Visual inspection of the cultured Calu-3 cells during the experiment (before and after) confirmed that cells were healthy.

Virus titre of rhinovirus-43 (RV43) and rhinovirus-1B (RV1B) in infected Calu-3 cells enriched with and without antioxidant compounds:

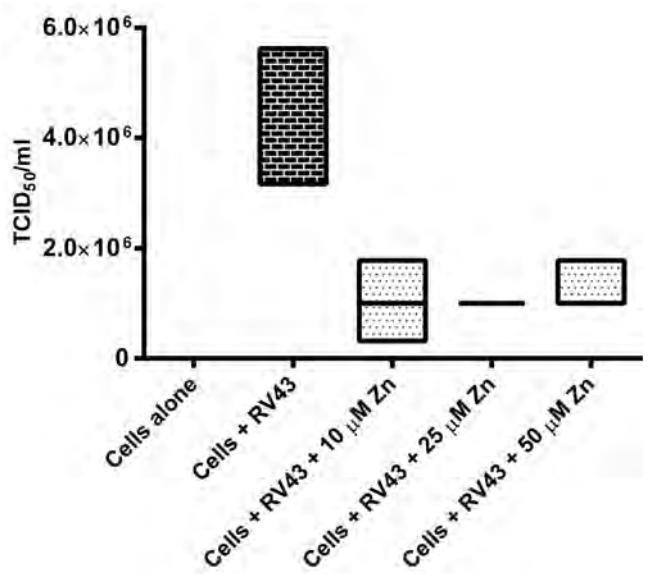
Various concentrations of antioxidants were used to identify antiviral effects against RV-43 and RV-1B. Both serotypes were used to investigate whether the effect was serotype specific. Figures 4.3.1 to 4.3.5 show effects of antioxidants on rhinovirus replication as determined by $TCID_{50}$/ml. Pre-treatment of the cells with different concentrations of zinc (Zn) for 4 hours failed to demonstrate significant effects against replication of RV-1B, however, Zn (at all concentrations tested) resulted in slight inhibition of replication of RV-43 [Figure 4.3.1 (a), (b)]. Pre-treatment of the cells with different concentrations of vitamin D for 18 hours also revealed similar effects as Zn on replication of RV-1B, while only 1µM of vitamin D (pre-treatment) showed slight

inhibition of replication of RV-43 [Figure 4.3.2 (a), (b)]. Pre-treatment of the cells with different concentrations of lycopene for 24 hours showed a tendency to inhibit replication of RV-1B but the effect was not statistically significant [Figure 4.3.3 (a)], and pre-treatment of the cells with 5.0 µg/ml of lycopene significantly inhibited replication of RV-43 [(Figure 4.3.3 (b)]. Pre-treatment of the cells with 2.5 µg/ml of lycopene resulted in slight inhibition of replication of RV-43. Pre-treatment of the cells with different concentration of resveratrol also revealed no inhibition of replication of RV1B [Figure 4.3.4 (a)], however pre-treatment of the cells with 5 and 50 µM of resveratrol significantly inhibited replication of RV-43 [(Figure 4.3.4 (b)]. It was shown that, pre-treatment of the cells with various concentrations of Zn resulted in a similar trend (slight inhibition) against replication of RV43. We believed that Zn at a concentration of 25µM (instead of at 10 or 50µM) is worthy of investigation against replication of RV43. Previously, it was clearly demonstrated that incubation of airway epithelial cells with 25µM of Zn resulted in more concentration of Zn intracellularly (more than double), compared to incubation of the cells with 10µM of Zn [288]. Pre-treatment with Lycopene at a 2.5µg/ml shows a similar magnitude as treating the cells at higher concentration (5µg/ml) against RV43. Based on the results presented in Figures 4.3.1 to 4.3.4, it was concluded that 25µM of Zn, 5µM of resveratrol, 1µM of vitamin D and 2.5µg/ml of lycopene were able to limit the replication of major group rhinovirus (RV-43). Hence, confirmation experiment (n=6) were done with 25µM of Zn, 5µM of resveratrol, 1µM of vitamin D or 2.5µg/ml of lycopene against replication of RV1B and RV43, to confirm the inhibitory effect. Figure 4.3.5 (a) and (b) shows the experimental design used to confirm the inhibitory effects.

(a)



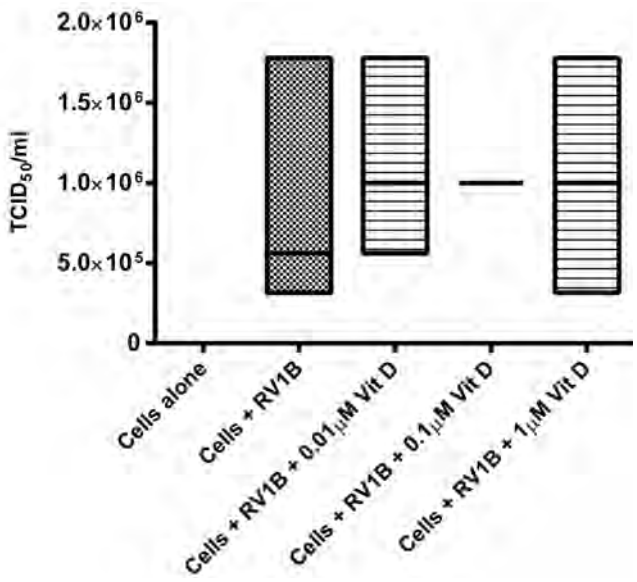
(b)



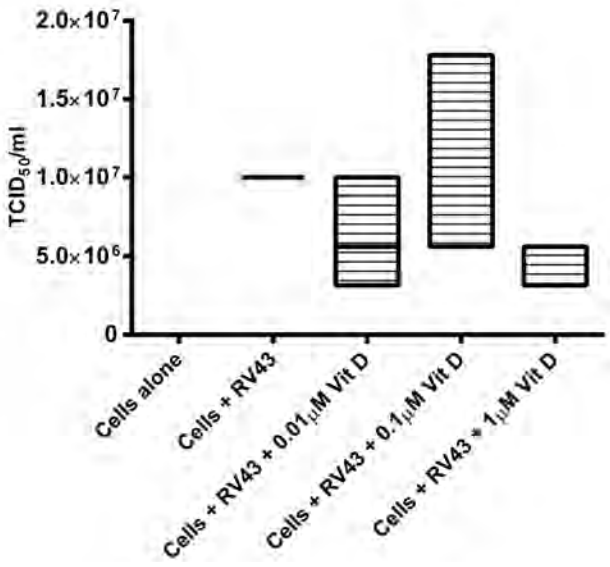
Calu-3 cells were pre-treated with individual antioxidants prior to infection with RV1B (MOI=20) or RV43 (MOI=50) as described in Chapter 2. Viral titers in the supernatant of infected cells were determined after 48hrs of infection by employing $TCID_{50}$/mL assay using RD-ICAM-1 cells as described in Chapter 2. Data are summarized by floating bars (min and max values): the central line represents the median, (n=3).

Figure 4.3.1. Effect of zinc at various concentrations on (a) rhinovirus-1B (RV-1B) and (b) rhinovirus-43 (RV43) replication in Calu-3 cells.

(a)



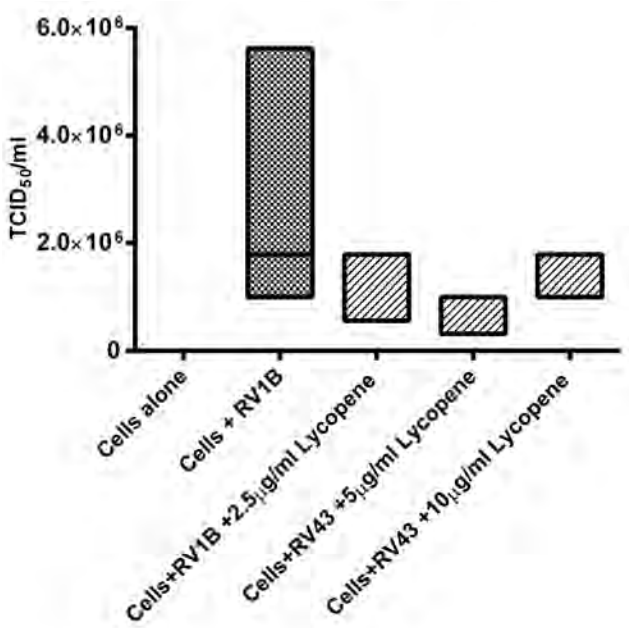
(b)



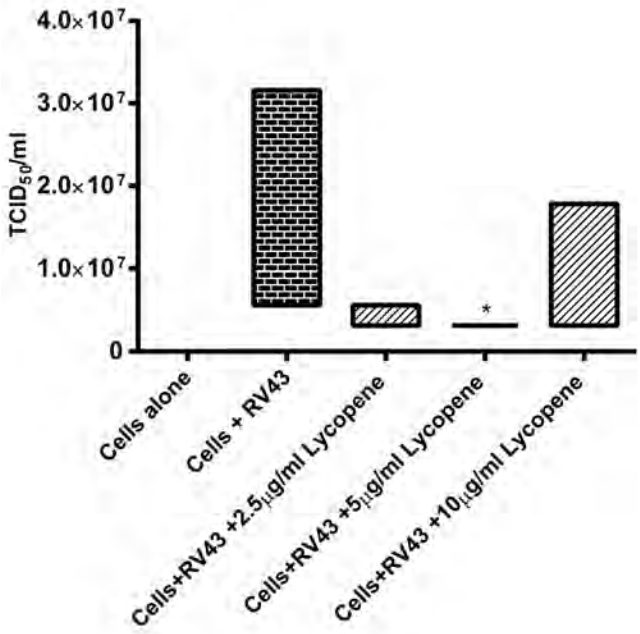
Calu-3 cells were pre-treated with individual antioxidants prior to infection with RV1B (MOI=20) or RV43 (MOI=50) as described in Chapter 2. Viral titers in the supernatant of infected cells were determined after 48hrs of infection by employing $TCID_{50}$/mL assay using RD-ICAM-1 cells as described in Chapter 2. Data are summarized by floating bars (min and max values): the central line represents the median, (n=3).

Figure 4.3.2. Effect of vitamin D at various concentrations on (a) rhinovirus-1B (RV1B) and (b) rhinovirus-43 (RV43) replication in Calu-3 cells.

(a)



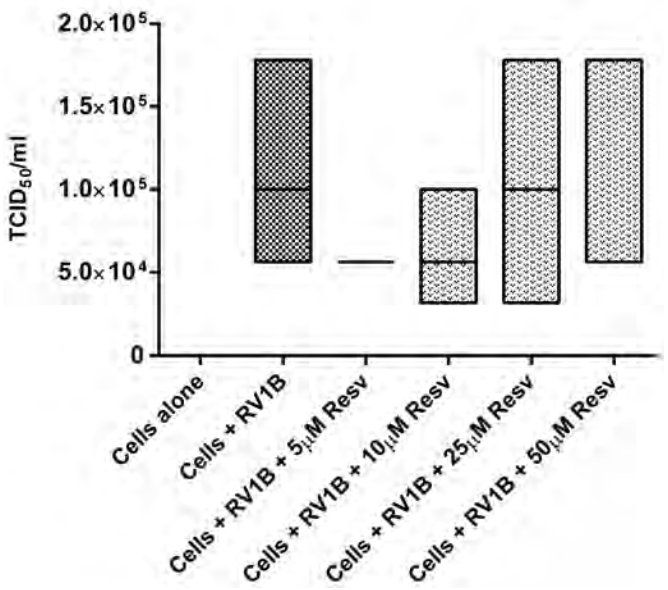
(b)



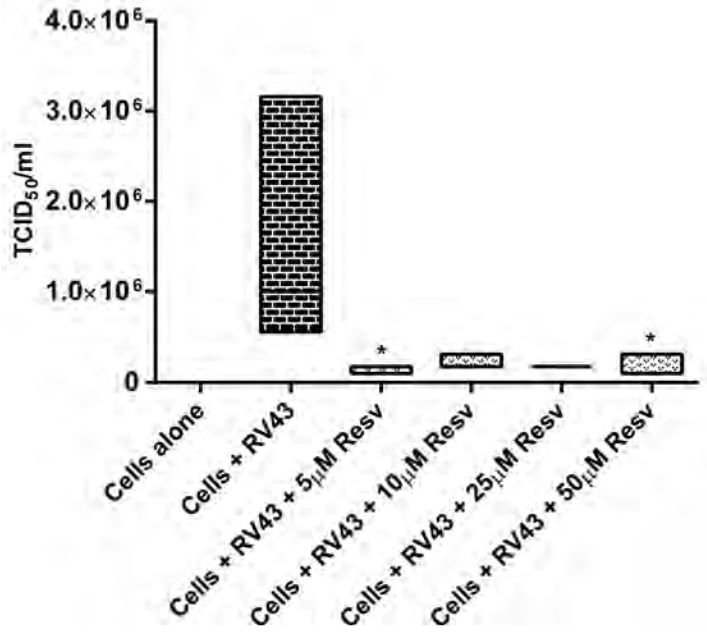
Calu-3 cells were pre-treated with individual antioxidants prior to infection with RV1B (MOI=20) or RV43 (MOI=50) as described in Chapter 2. Viral titers in the supernatant of infected cells were determined after 48hrs of infection by employing $TCID_{50}$/mL assay using RD-ICAM-1 cells as described in Chapter 2. Data are summarized by floating bars (min and max values): the central line represents the median, (n=3).
*$p<0.05$ compared to cells + RV43

Figure 4.3.3. Effect of lycopene at various concentrations on (a) rhinovirus-1B (RV1B) and (b) rhinovirus-43 (RV43) replication in Calu-3 cells.

(a)



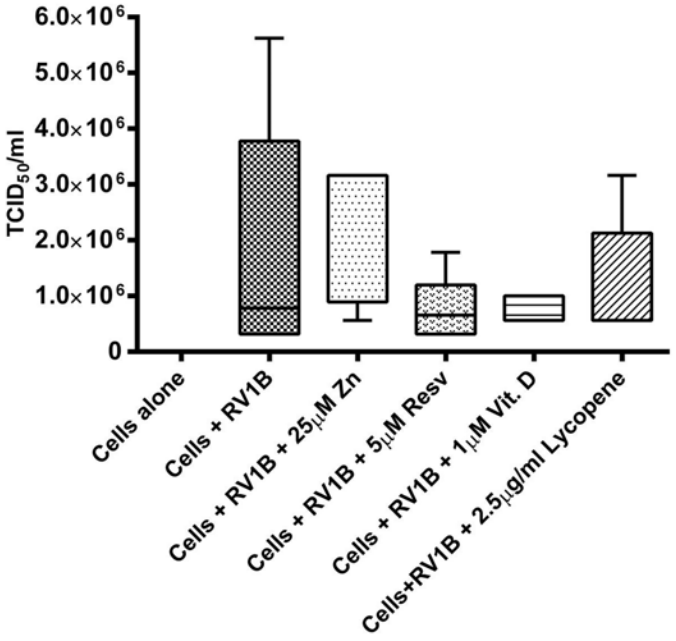
(b)



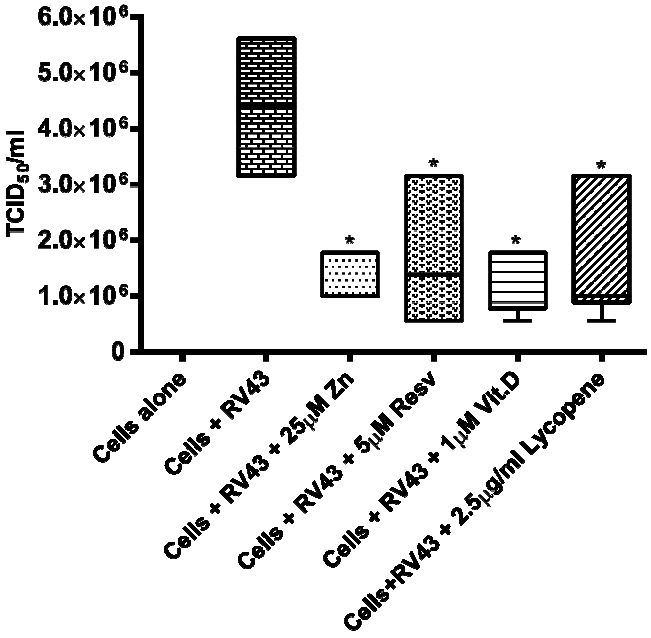
Calu-3 cells were pre-treated with individual antioxidants prior to infection with RV1B (MOI=20) or RV43 (MOI=50) as described in Chapter 2. Viral titers in the supernatant of infected cells were determined after 48hrs of infection by employing $TCID_{50}$/mL assay using RD-ICAM-1 cells as described in Chapter 2. Data are summarized by floating bars (min and max values): the central line represents the median, (n=3).
*$p<0.05$ compared to cells + RV43

Figure 4.3.4. Effect of resveratrol at various concentrations on (a) rhinovirus-1B (RV1B) and (b) rhinovirus-43 (RV43) replication in Calu-3 cells

(a)



(b)

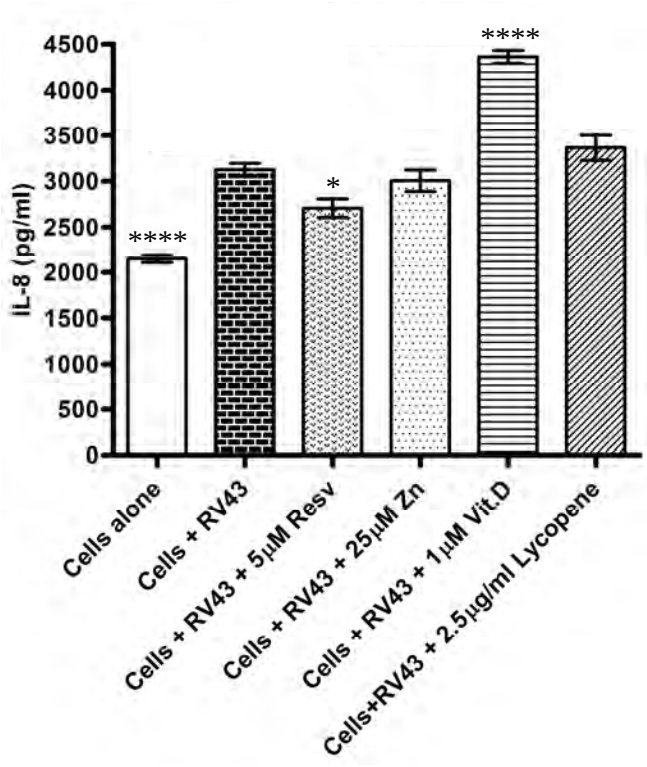


Calu-3 cells were pre-treated with individual antioxidants prior to infection with RV1B (MOI=20) or RV43 (MOI=50) as described in Chapter 2. Viral titers in the supernatant of infected cells were determined after 48hrs of infection by employing $TCID_{50}$/mL assay using RD-ICAM-1 cells as described in Chapter 2. Data are summarized by box and whiskers (min and max values): boxes represent the 25th and 75th percentiles, the central line represents the median, (n=6).
*$p<0.05$ compared to cells + RV43

Fig. 4.3.5. Combined experiment on the effect of antioxidants on (a) rhinovirus 1B (RV1B) and (b) rhinovirus 43 (RV43) replication in Calu-3 cells.

IL-8 released by Calu-3 cells enriched with and without antioxidant compounds prior to infection with RV-43:

Based on the results of $TCID_{50}$ (viral replication), it was concluded that the antioxidants used in the study have limited effect on RV infection, suggesting that the effect of antioxidants on RV was serotype specific. Hence prompt us to investigate whether inhibition of viral replication was associated with significant reduction in RV-induced inflammation. Calu-3 cells infected with RV43 show significant ($p<0.0001$) inductions of IL-8 release (Figure 4.3.6). In addition, pre-treated the cells with 5μM of resveratrol for 2 hours prior to RV43 infection, the release of IL-8 was significantly ($p<0.05$) reduced. However, pre-treating the cells either with 25μM of Zn or 2.5μg/ml of lycopene failed to elicit a statistically significant effect. Interestingly, vitamin D significantly ($p<0.0001$) increased the release of IL-8 by Calu-3 cells infected with RV43.

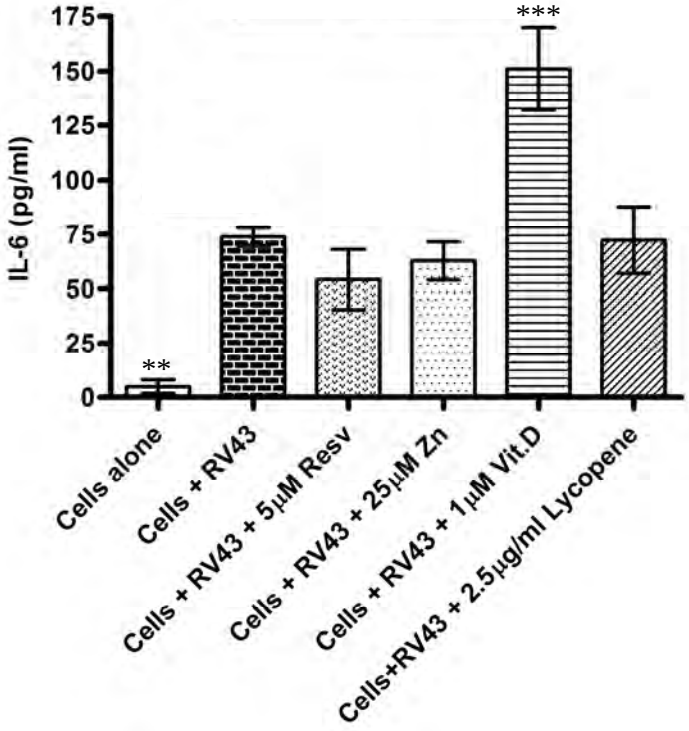


Calu-3 cells were pre-treated with individual antioxidants and infected with RV43 at a MOI=50. IL-8 concentrations were measured by ELISA at 48 hrs post-infection. Data is mean ± SEM (n=6).
*p<0.05 compared to cells + RV43
****p<0.0001 compared to cells +RV43

Figure 4.3.6. Effects of antioxidants on rhinovirus 43 (RV43)-induced production of pro-inflammatory cytokine, IL-8.

IL-6 released by Calu-3 cells enriched with and without antioxidant compounds prior to infection with RV-43:

Figure 4.3.7 shows the release of IL-6 in cultured media of Calu-3 cells enriched with and without antioxidant compounds before RV-43 infection. Calu-3 cells infected with RV43 showed significant ($p<0.01$) increase in IL-6 release. However, no significant effect was observed when the cells were pre-treated with resveratrol, zinc and lycopene. Cells pretreated with 1μM of vitamin D showed significant ($p<0.001$) induction of IL-6.

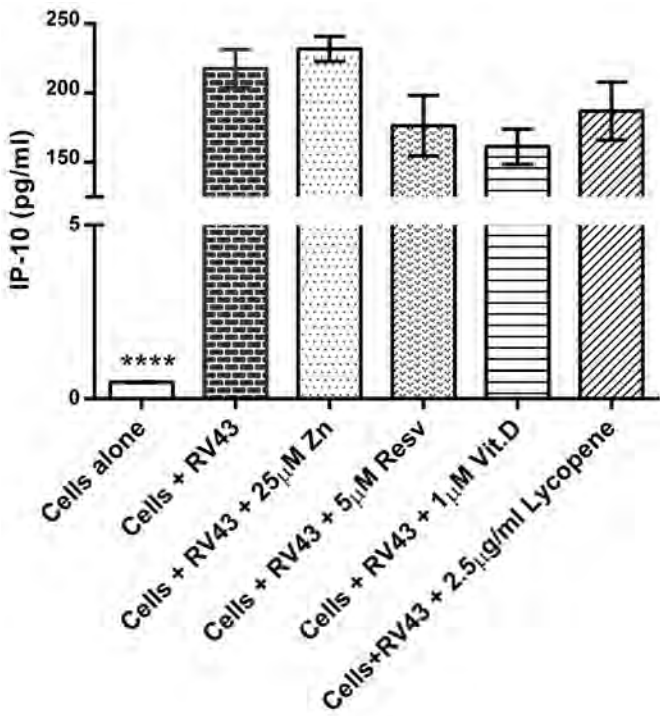


Calu-3 cells were pre-treated with individual antioxidants and infected with RV43 at a MOI=50. IL-6 concentrations in cultured media were measured using flow cytometry bead array at 48 hrs post-infection. Data is mean ± SEM (n=6).
**p<0.01 compared to cells + RV43
***p<0.001 compared to cells +RV43

Figure 4.3.7. Effects of antioxidants on rhinovirus 43 (RV43)-induced production of the pro-inflammatory cytokine, IL-6.

IP-10 released by Calu-3 cells enriched with and without antioxidant compounds prior to infection with RV-43:

Figure 4.3.8 shows the release of IP-10 in cultured media of Calu-3 cells enriched with and without antioxidant compounds before RV-43 infection. Calu-3 cells infected with RV43 showed significant ($p<0.0001$) inductions of IP-10 released. However, no significant effect was observed when pre-treated the cells with resveratrol, zinc, vitamin D and lycopene before RV43 infection.



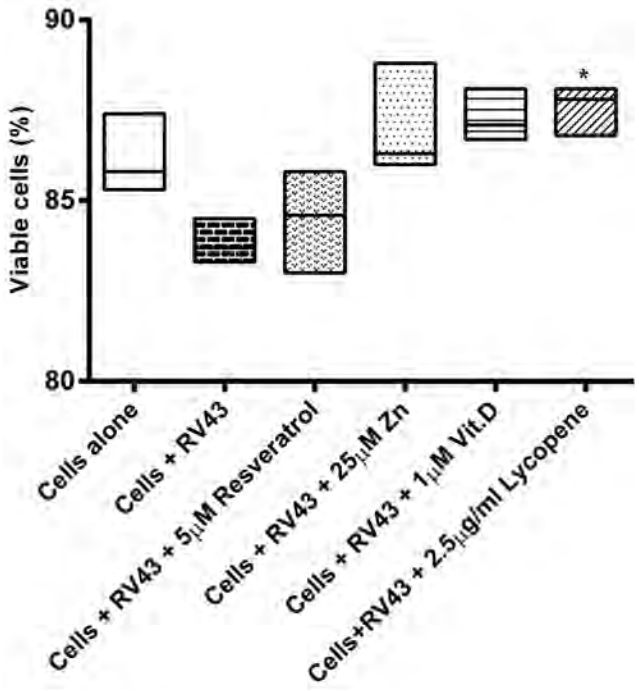
Calu-3 cells were pre-treated with individual antioxidants and infected with RV43 at MOI=50. IP-10 concentrations in cultured media were measured by flow cytometry bead array at 48 hrs post-infection. Data is mean ± SEM (n=6).
****$p<0.0001$ compared to cells + RV43

Figure 4.3.8. Effects of antioxidants on rhinovirus 43 (RV43)-induced production of the pro-inflammatory cytokine, IP-10.

Antioxidant promotes viability of infected Calu-3 cells:

Robust assessment of the viability of cells is achieved by employing specific staining (PE Annexin V and 7-amino-actinomycin) to distinguish between viable and dead (apoptotic or necrotic) cells and those cells are quantified by flow cytometry. At the end of study (48 hours post infection), more than 80% of cells were healthy (Figure 4.3.9). Calu-3 cells infected with RV43 showed slight reduction in viability when compared to uninfected Calu-3 cells. Contrary, pre-treatment of the cells either with Zn or vitamin D prior to RV43 infection slightly increased viability of the cells when compared to untreated infected cells (Figure 4.3.9). Pre-treatment of the cells with

lycopene significantly ($p<0.05$) increased viability of the cells when compared to untreated infected cells (Figure 4.3.9).



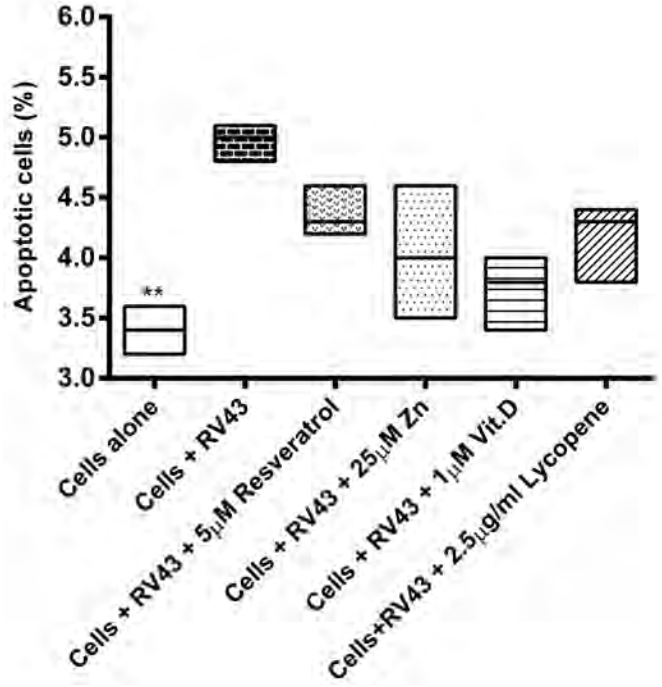
Calu-3 cells were pre-treated with individual antioxidants and infected with RV43 at a MOI=50. Cell viability was assessed by Annexin V-PE and 7-AAD staining and analyzed by flow cytometry at 48 hrs post-infection. Data are summarized by floating bars (min and max values): the central line represents the median, (n=3).
*$p<0.05$ compared to cells + RV43

Figure 4.3.9. Effects of antioxidants on viability of Calu-3 cells infected with rhinovirus 43 (RV43).

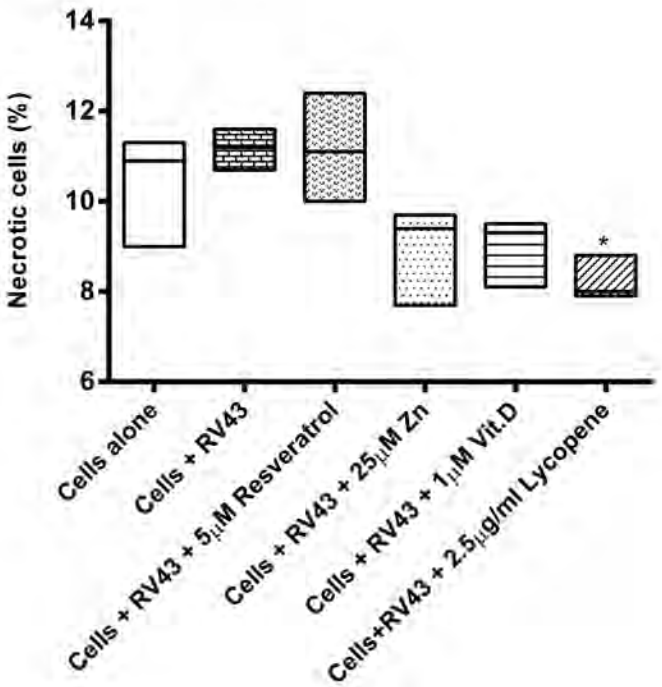
The viability of infected Calu-3 cells significantly decreased at the end of experiment possibly due to cells undergoing apoptosis [Figure 4.3.10 (a)]. The percentage (%) of cells undergoing apoptosis is significantly ($p<0.01$) higher in untreated infected cells when compared to uninfected cells. Pre-treatment of the cells with antioxidant compounds prior to RV43 infection, shown slight decreased the percentage (%) of apoptotic cells when compared to untreated infected cells. However, the percentage (%) of necrotic cells revealed no statistically significant difference between untreated infected cells and uninfected cells [Figure 4.3.10 (b)]. Additionally,

pre-treatment of the cells with lycopene prior to RV43 infection significantly ($p<0.05$) decreased necrotic cells when compared to untreated infected cells [Figure 4.3.10 (b)].

(a)



(b)



Calu-3 cells were pre-treated with individual antioxidants and infected with RV43 at a MOI=50. Apoptotic and necrotic cells were assessed by Annexin V-PE and 7-AAD staining and analyzed by flow cytometry at 48 hrs post-infection. Data are summarized by floating bars (min and max values): the central line represents the median, (n=3).
*$p<0.05$ compared to cells + RV43
**$p<0.01$ compared to cells + RV43

Figure 4.3.10. Effects of antioxidants on (a) apoptotic and (b) necrotic/late apoptotic Calu-3 cells infected with rhinovirus 43.

4.4 Discussion

This study was conducted to find a suitable antioxidant, which is abundantly available through dietary sources, to counteract inflammation induced by RV infection associated with respiratory disorders. In this study, two serotypes of human rhinovirus were used, RV-1B and RV-43, representing minor and major group rhinoviruses respectively. In contrast with other studies [181, 378-380], high multiplicity of infection (MOI) on both rhinoviruses serotypes (MOI=50 for RV43; and MOI=20 for RV1B) was used to ensure that the maximum number of cells became infected. In the preliminary study, (data not shown) to determine the best MOI for optimum level of viral replication (as determined by $TCID_{50}$ assay), it was established that MOI=50 for RV43 and MOI=20 for RV1B were optimal. Additionally, with the MOI used in the studies, the cells are still considered healthy. The experimental conditions mimic the conditions of rhinovirus infection in humans, showing that RV infection does not cause morphological changes in the airway epithelial cells but the pathogenesis associated with rhinovirus is due to the host cell response against viral infections. MOI refers to the number of virions that are added per cell during infection. Ideally, with MOI=1, one cell is infected with one virion of virus. We found that antioxidant compounds (resveratrol, zinc, vitamin D and lycopene) exhibit antiviral activity against the major group rhinovirus, RV-43.

In airway epithelial cells, infection with RV leads to a cytopathic effect (CPE). RV infection of airway epithelial cells ($16HBE14o^-$) induces the self death programme (apoptosis) via the mitochondrial pathway [155]. The induction of apoptosis via the mitochondrial pathway is exemplified by the release of cytochrome c into the cytoplasm, due to permeabilization of the outer mitochondrial membrane, activation of caspase-9 and caspase-3. RV induced apoptosis is believed to be mediated by oxidative

stress. Since RV infections cause an elevated level of the superoxide anion [381] hence could trigger the release of cytochrome c. Many studies have demonstrated that reactive oxygen species (ROS) cause cell apoptosis [349, 350]. It has been demonstrated that apoptosis induced by RV infection has an effect in promoting the release of virus from the cells [155]. Pre-treatment of the cells with the known caspase inhibitor, zVAD.fmk prior to RV infection reduced the amount of virus released at the end of infection [155]. In the current study, cellular enrichment with resveratrol, zinc, vitamin D and lycopene were found to have antiviral activity against RV-43. However, antioxidant-enriched cells infected with minor group of RV, failed to show significant effect on viral replication. Based on these results, it would appear that antiviral activity of the tested antioxidant compounds is limited to the major group of RV that use ICAM-1 receptors on the host cell to gain entry and replicate. The antioxidant-enriched cells promote the viability of infected cells, and decrease the numbers of cells undergoing apoptosis during RV infection. As reported by others, oxidative stress is induced during RV infection [142, 158], hence can favour activation of the apoptosis intrinsic pathway, and the presence of resveratrol, zinc, vitamin D and lycopene intracellularly may neutralize oxidative stress-induced by RV, thus limiting apoptosis and reducing viral titre.

Studies have reported that airway epithelial cells taken from asthmatics have deficient innate immunity against RV infection [141], but treatment with exogenous administration of IFN-β limited viral replication [169]. Therefore, administration of IFN-β seems to rectify the deficient innate immunity experienced by asthmatics. In the present study, we found antioxidants were able to significantly reduce RV replication. Hence, administration of antioxidants to asthmatic patients might provide benefit against RV infections due to deficient innate immunity against viral infections as found in asthmatics.

The antiviral properties of antioxidants were investigated to determine whether reduction in viral replication leads to a decreased inflammatory response in Calu-3 cells. To do this, protein levels of IL-6, IL-8 and IP-10 were determined at 48 hours in RV43 infected antioxidant-enriched Calu-3 cells. Asthma patients who develop symptomatic colds after infection with RV have higher levels of IL-6, IP-10 in the nasal lavage fluids [12, 16]. It is believed that RV replication and its replication intermediate (dsRNA) is strongly involved in pro-inflammatory cytokine induction of host cells. UV-inactivated RV has impaired ability to replicate (as assessed by $TCID_{50}$, shown by inability to cause cytopathic effects in RD ICAM-1 cells), and is widely used in studies of RV infection. Infection induced with UV-inactivated RV in airway epithelial cells demonstrates significant abrogation of IP-10, IL-6 and IL-8 proteins [16, 105, 168]. Beas-2B cells treated with polyrIC, a synthetic analogue of dsRNA resulted in robust inductions of IL-6 and IL-8 proteins compared to untreated cells [105]. Several studies have reported the association between viral replication and induction of inflammatory cytokines. Sanders et al. [382] reported that potent glucocorticoid budesonide did not affect viral replication but modestly inhibited IL-6 and IL-8 proteins. Additionally, when nitric oxide donor (NONOate) was used in the experiment, viral replication and viral induced IL-6 and IL-8 proteins were significantly abrogated [382]. Similarly, carbocisteine (mucolytic drug) significantly inhibited RV replication and viral induced IL-6 and IL-8 proteins [143]. In another study, macrolide (azithromycin) was found to significantly inhibit RV replication, however no significant changes were noted in RV induced IL-6 and IL-8 proteins [383]. As shown in the current study, levels of IL-6, IL-8 and IP-10 proteins secreted by antioxidant-enriched cells prior to RV infection, did not have direct association with RV43 replication. The different pathways involved in viral replication and viral-induced pro-inflammatory cytokine production might be responsible for the

observed results. Furthermore, the disagreement between viral replication and viral induced proinflammatory results observed in antioxidant-enriched cells as compared to the above mentioned studies may be explained by differences in the experimental model. In the current study, the cells was pre-treated with antioxidants at the indicated time, the media containing antioxidants was removed and then the infections were carried out without any addition of the antioxidant until the experiment finished. In contrast, in one of the previously mentioned studies, the cells were pre-treated with carbocisteine for 3 days prior to RV infection, and the infection experiment was done with the addition of carbocisteine until the experiment finished [143]. It is possible that in our study, the antioxidant compounds in the Calu-3 cells were exhausted during 48 hours of RV infection, which may explain the observed results.

The pro-inflammatory proteins IP-10, IL-6, and IL-8 are robustly secreted by infected (RV) airway epithelial cells, as demonstrated in studies conducted *in vitro* and *in vivo* [12, 15, 16]. Consistent with these observations, we have shown that Calu-3 cells infected with RV, significantly increased secretion of IL-6, IL-8 and IP-10 proteins compared to uninfected cells. In this study, RV infections of Calu-3 cells produced more IL-8 protein than IL-6, which is in agreement with others [9, 384]. RV infection caused optimum increases in viral titer [9] and levels of IL-6, IL-8, and IP-10 at 48 hours post infection [12, 16, 105]. Resveratrol, zinc, vitamin D and lycopene, all possess antiviral activity against RV-43 as shown in the current study, but do not inhibit inflammation induced by RV infection. Only resveratrol has shown anti-inflammatory effect against RV-induced IL-8, but not IP-10 and IL-6. Pre-treating the cells with resveratrol prior to RV43 infection, reduced viral induced IP-10 and IL-6 proteins, however failed to reach statistically significant levels. Interestingly, the current study showed that vitamin D significantly promoted RV induced IL-6 and IL-8 proteins. The

biological action of vitamin D is mediated through gene expression. The biologically active form of vitamin D binds to its intracellular receptor (vitamin D receptor) in the nucleus, and then binds to vitamin D response element (VDRE) located in the promoter region of IL-8 gene [385]. RV is known to induce IL-8 as one of the mechanism of cellular infection to eradicate the virus. Based on the observed result, it is likely that vitamin D increased the production of IL-8 to assist infected host cells in eradicating the virus. The effect of vitamin D on RV-induced IL-6 could be explained via a similar mechanism, however no study to date has shown whether VDRE is also present on the promoter region of the IL-6 gene.

Cells infected with RV have shown activation of several intracellular signalling pathways causing an inflammatory response in host cells. RV infection of airway epithelial cells has been shown to activate three notable intracellular signalling pathways i.e. Nf-κB, mitogen-activated protein kinase (MAPK) and protein kinase R (PKR). RV-induces the inflammatory proteins IL-6, IL-8 and IP-10 in airway epithelial cells, dependent on the Nf-κB pathway [12, 15, 142, 386]. However Kim et al. [384] has reported that induction of IL-6 and IL-8 proteins by RV infections in airway epithelial cells (Beas-2B cells) was independent of Nf-κB activation. The use of known inhibitors of NF-κβ, either sulfasalazine (interfering with phosphorylation of IκBα) or calpain inhibitor I (inhibits proteosomal degradation of IκB) in RV infection experiments leads to inhibition of the activation of NF-κB, without affecting mRNA levels for IL-6 and IL-8 [384]. These findings clearly show that activation of Nf-κB is not absolutely essential for RV induced induction of IL-6 and IL-8 gene expression [384]. Interesting to note, sulfasalazine significantly inhibited RV-induced IL-6 and IL-8 protein production, which strongly suggests that sulfasalazine has post-transcriptional effects on IL-6 and IL-8 protein production [384].

4.5 Conclusions

In conclusion, antioxidant-enriched cells have a multitude of effects against RV-induced inflammation on airway epithelial cells. In airway epithelial cells infected with RV, resveratrol was found to significantly reduce IL-8, but not IP-10 and IL-6; zinc and lycopene were found not to have any significant effect on IL-8, IL-6 and IP-10; and interestingly vitamin D was found to significantly increase IL-6 and IL-8. In addition, we have shown that antioxidant compounds have the properties to reduce the replication of RV. The effect of antioxidants on RV43-induced inflammation is independent of its effects on viral replication. The prevention of infected cells to undergo apoptosis might explain the antioxidant compound antiviral activity against major group rhinovirus, RV43. Resveratrol, zinc, vitamin D and lycopene possesses antiviral activity towards major group rhinovirus and this offers an opportunity for the development of anti-rhinovirus agents.

CHAPTER 5: MECHANISMS BY WHICH ANTIOXIDANTS REDUCE VIRAL REPLICATION IN RHINOVIRUS-INFECTED CULTURED AIRWAY EPITHELIAL CELLS

5.1 Introduction

The inflammatory response to RV infection was heavily suggested in causing asthma exacerbations, hence RV infection posing a serious threat to asthmatics. It has been reported that more than one million working people and school students miss work or school every year due to common cold [387], and RV was implicated in majority of common cold [6]. It was discovered that two membrane bound receptors on epithelial cells are responsible for the uptake of RV [18, 143-145, 388]. Since then, many studies have been carried out which employ the knowledge that the ICAM-1 and LDL-r families of receptors, allow RV to gain entry into epithelial cells [143, 389].

To date, more than 100 serotypes of RV have been discovered. Theoretically, development of individual vaccines for each serotype is impractical. In general, a specific antibody can be produced by B-lymphocytes during RV infection, however this initial strategy in response to RV infection is inadequate and ineffective in preventing RV entry into airway epithelial cells. It has been hypothesized that, a specialized and conserved region on RV's capsid (what is known as canyon) is a site where the virus binds to the ICAM-1 receptor on host cells, and it was shown that the binding sites of the receptor (ICAM-1) and for the antibody [390] were at different location on the virus. RV are the most thoroughly studied viruses, but as yet no antiviral remedies are available to the community and no antiviral agent has been approved for the treatment of RV infection. However, many antiviral compounds have been synthesized and evaluated for prevention of RV replication. For example, pleconaril (also called WIN compound), developed by the Sterling-Winthrop company and the ViroPharma company has been shown to possess broad antiviral activity against enterovirus [391]. Pleconaril mode of action against RV is by binding onto the virus capsid (located beneath the canyon floor) and as a result it induces conformational changes in the floor

of RV, thus making the virus unable to attach to the cells and also increasing virion rigidity for preventing virus uncoating and subsequent release of viral RNA [392-396]. Although, the drug was shown to be a very promising candidate against RV infection, United States Food and Drug Administration (USFDA) has not approved the use of the drug because of concerns of viral resistance and side effects in patients [397]. Indeed Pevear et al. [398] demonstrated the emergence of virus resistance or reduced susceptibility to pleconaril treatment. Therefore, approved anti-viral agents are in high demand for either the prevention or treatment of RV-associated diseases.

Viral infections result in the production of oxidative stress in the infected cells [25, 29, 142, 158, 399]. It has been shown that the life cycle of several viruses is influenced by host cell redox state. A number of experimental systems involving studies on influenza A virus [27], Sendai virus [400], HIV [401], herpes simplex virus type 1[399], coxsackievirus (strain B3, B4) and rhinovirus type 14 [402] replication, have shown that administration of exogenous GSH efficiently inhibits viral replication. Additionally, several other antioxidant compounds including pyrrolidine dithiocarbamate (PDTC), vitamin C, the vitamin E derivative Trolox, 2-mercaptoethanol, and N-acetyl-L-cysteine (NAC) were investigated for infection of epithelial cells with several serotypes of RVs [110]. PDTC was found to have strong antiviral activity against minor and major groups of RV. However, the infection experiment was conducted with both RV and PDTC compound, and after removing the culture medium containing RV and PDTC compound, fresh culture medium containing PDTC was added to the cells until the completion of the infection experiment [110]. It is reasonable to hypothesize that PDTC might have a direct effect against RV (virus inactivation and or virus adsorption) in the cultured medium rather than a direct effect intracellularly, as pre-treatment of the cells with PDTC before infection did not protect

the cells against RV infection [110]. Other antioxidant compounds (vitamin C, the vitamin E derivative Trolox, 2-mercaptoethanol and N-acetyl-L-cysteine (NAC)), however failed to demonstrate antiviral effects against RV. This is possibly due to the concentration of antioxidants used in the study [110]. However, to date no study has reported the actual relationship or association between viral replication cycle and level of oxidant stress in the infected cells.

Previously, we have demonstrated that Calu-3 cells enriched either with resveratrol, zinc, vitamin D or lycopene possesses antiviral activity against RV43. Therefore, in this current chapter we hypothesized that enrichment of airway epithelial cells with antioxidant compounds and their intracellular utilization will have antiviral effects toward RV infectious cyles, viz viral attachment, viral internalization, viral RNA replication and/or viral polyprotein processing. Hence, in this study the aim was to provide an insight into the mechanisms by which antioxidants modulate viral replication.

5.2 Materials and Methods

Study design:

Confluent Calu-3 cells were incubated with resveratrol (2 hours), zinc (4 hours), vitamin D (18 hours), lycopene (24 hours) or wortmannin (PI3-kinase inhibitor) (2 hours), prior to infection with RV43 (major group). After removing the media containing the aforementioned compounds, the cells were incubated with RV43 for 1 hour and agitated at room temperature to allow efficient binding of the virus. The infection media was then replaced with fresh media, and cells were incubated for up to 48 hours of infection. Viral internalization was assessed by confocal microscopy, PI3-kinase activation and cleavage of eIF4GI was determined by Western blot, viral RNA

level was assessed by RT-qPCR, viral titer was assessed by $TCID_{50}$ assay using RD-ICAM-1 cells, cell viability was assessed by Annexin V-PE and 7-AAD staining and analysed by flow cytometry, and ICAM-1 surface protein expression was also determined by flow cytometry.

Airway epithelial cells:

As described in Chapter 2, human airway epithelial cells (Calu-3, from ATCC, USA) were cultured in 24 well plates in minimum essential medium containing 10% foetal calf serum (10%FCS/MEM) containing 2% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine, and 2.2g/l $NaHCO_3$ (all were purchased from Invitrogen Corporation, Carlsbad, USA) at 37ºC in the presence of 5% CO_2.

Enriching the airway cells with resveratrol:

As described in Chapter 2 (Section 2.2.9), confluent Calu-3 cells were incubated with 5µM resveratrol dissolved in DMSO (0.1% DMSO in cell culture medium). Cells were incubated for 2 hours at 37ºC in the presence of 5% CO_2.

Enriching the airway cells with zinc:

As described in Chapter 2 (Section 2.2.9), confluent Calu-3 cells were incubated with 25µM zinc dissolved in sterilized water. Cells were incubated for 4 hours at 37ºC in the presence of 5% CO_2.

Enriching the airway cells with vitamin D:

As described in Chapter 2 (Section 2.2.9), confluent Calu-3 cells were incubated with 1µM vitamin D dissolved in sterilized ethanol (0.1% ethanol in cell culture medium). Cells were incubated for 18 hours at 37ºC in the presence of 5% CO_2.

Enriching the airway cells with lycopene:

As described in Chapter 2 (Section 2.2.9), confluent Calu-3 cells were incubated with 2.5µg/ml lycopene dissolved in tetrahydrofuran (THF) (0.5% THF in cell culture medium). Cells were incubated for 24 hours at 37ºC in the presence of 5% CO_2.

Rhinovirus challenge of Calu-3 cells:

As described in Chapter 4 (Section 4.2), after removing the supplementation media from Calu-3 cells (as mentioned above), they were infected with RV43 at multiplicity of infection (MOI) of 50. Plates were incubated for up to 48 hours at 33ºC in the presence of 5% CO_2. To confirm that virus required PI3-kinase for efficient replication, the cells was pre-treated with 0.1, 1 or 5 µM of wortmannin, dissolved in DMSO (final concentration of DMSO in culture medium is 0.1%) for 2 hours prior to viral infection as described.

Visual inspection of Calu-3 cells:

Cellular viability of cultured Calu-3 cells was observed before and after antioxidant supplementation (Olympus microscope, TL4).

Western blot analysis:

Whole cell lysate was recovered for determination of protein AKT (phosphorylated form) and protein eIF4GI at 0 hours and at 6, 24, 48, 72 hours after infection respectively, and was normalized to protein GAPDH. Western blot analysis was carried out as described in Chapter 2 (Section 2.3.6).

Flow cytometric analysis of membranous-ICAM-1 surface expression:

Flow cytometry was used to quantify the level of membranous- intercellular adhesion molecule-1 (m-ICAM-1) surface expression on rhinovirus-infected Calu-3 cells and rhinovirus-infected antioxidant-enriched Calu-3 cells. Analysis of m-ICAM-1 surface expression was determined at 6 hours and 48 hours after viral infection, as described in Chapter 2 (Section 2.3.4).

Flow cytometric analysis of viable, apoptotic and necrotic cells:

To determine that treating Calu-3 cells with wortmannin does not cause cell toxicity, flow cytometry was used to quantify the number of viable, apoptotic and necrotic cells. Cellular apoptosis, necrosis and viability was determined at 48 hours after viral infection and was analysed by flow cytometry, as described in Chapter 2 (Section 2.3.3).

Reverse transcriptase-quantitative chain reaction (RT-qPCR) analysis of viral RNA:

RT-qPCR was used to quantify viral RNA copy number on rhinovirus-infected Calu-3 cells. At 0, 2, 4, 6, 8, 12, and 24 hours after infection, cellular RNA was harvested for analysis of viral RNA as described in Chapter 2 (Section 2.3.7). Viral RNA copy

numbers was derived from extrapolation of a standard curve of known RV copies numbers.

Viral titration assay:

As described in Chapter 2 (Section 2.2.2), $TCID_{50}$ experiments were performed using near confluent RD-ICAM-1 cells seeded in 96-well tissue culture plates (NUNC, Roskilde, Denmark). Cells were infected by either media alone or virus containing media at varying dilutions. Serial ten-fold dilutions of the samples were prepared and 4 individual wells were infected with each dilution. For titration of samples 7 dilutions were prepared, and 4 control wells were prepared with media alone. After five days the plates were read and $TCID_{50}$ calculated. Infected wells were scored based on the cytophatic effect (CPE) seen, >50% CPE demonstrated by light microscopy was considered a positive result. Viral titer of the samples was determined by cell titration assay using RD-ICAM-1 cells and the viral titer was calculated and expressed as tissue culture infectious dose at 50% in log value ($TCID_{50}$ $\log_{10}$) [328] and using the Karber formula for the tissue culture infective dose 50% ($TCID_{50}$) [329].

Rhinovirus-43 labelling and confocal microscopy:

The virus was purified as described in Chapter 2 (section 2.2.3.1) before label with AlexaFluor 555 carboxysuccinimidyl ester (Molecular Probes™) as described in Chapter 2 (Section 2.2.3.2). Confocal microscopy was done to visualize entry of labelled RV into Calu-3 cells as described in Chapter 2 (Section 2.2.4).

Statistical analysis:

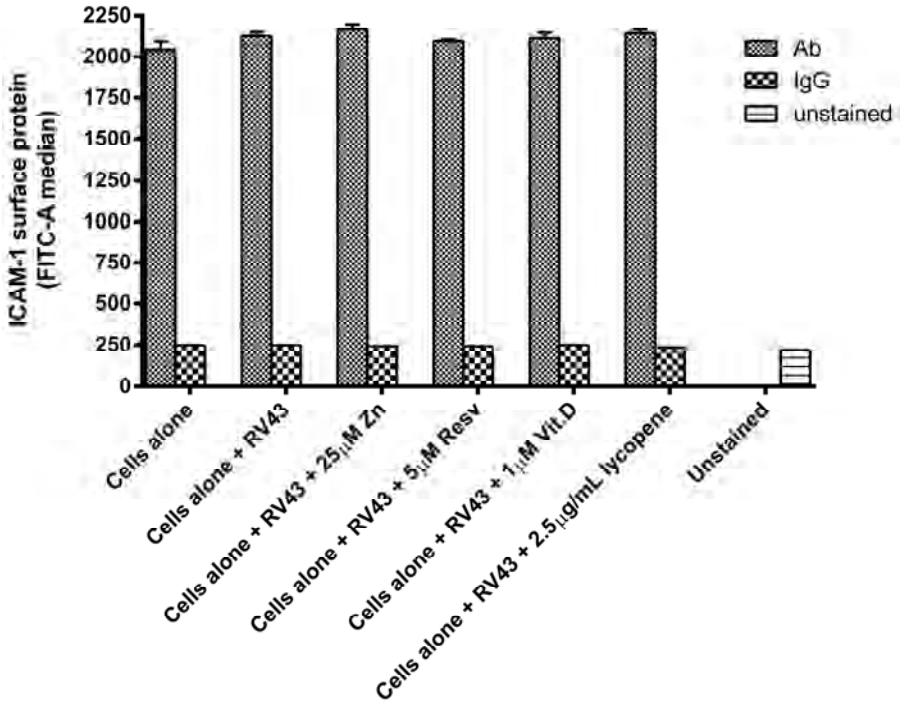
Differences between antioxidant supplemented and un-supplemented airway epithelial cells prior to RV infection were analysed using Kruskal-Wallis test, followed by Dunn's multiple comparison test (un-supplemented RV infection cells set as control) or 2-way ANOVA followed by Sidak's multiple comparison test performed by Graphpad Prism® Version 6.00 for Windows, GraphPad Software, La Jolla California, USA). A P value of 0.05 or less was considered statistically significant.

5.3 Results

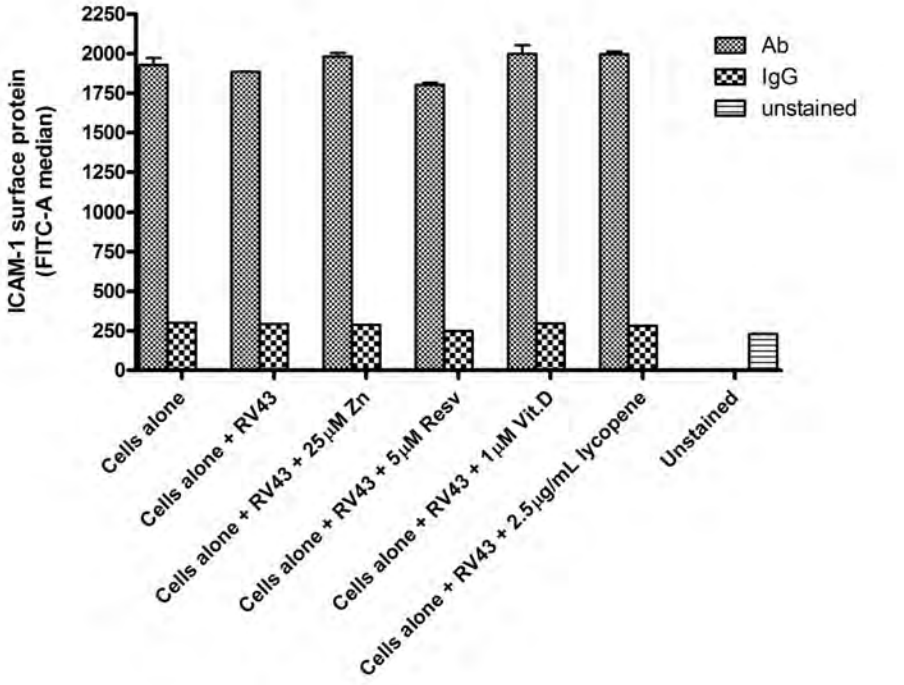
Membranous-ICAM-1 (m-ICAM-1) expression: response to RV infection and antioxidants:

Expression of m-ICAM-1 of Calu-3 during early infection (6 hours) and at the peak of infection (48 hours) was not different to baseline (Figures 5.3.1 a and b). In addition, at 6 and 48 hours post-infection, cells pre-treated with each of the antioxidants before virus infection gave similar results to rhinovirus infection of untreated cells.

(a)



(b)

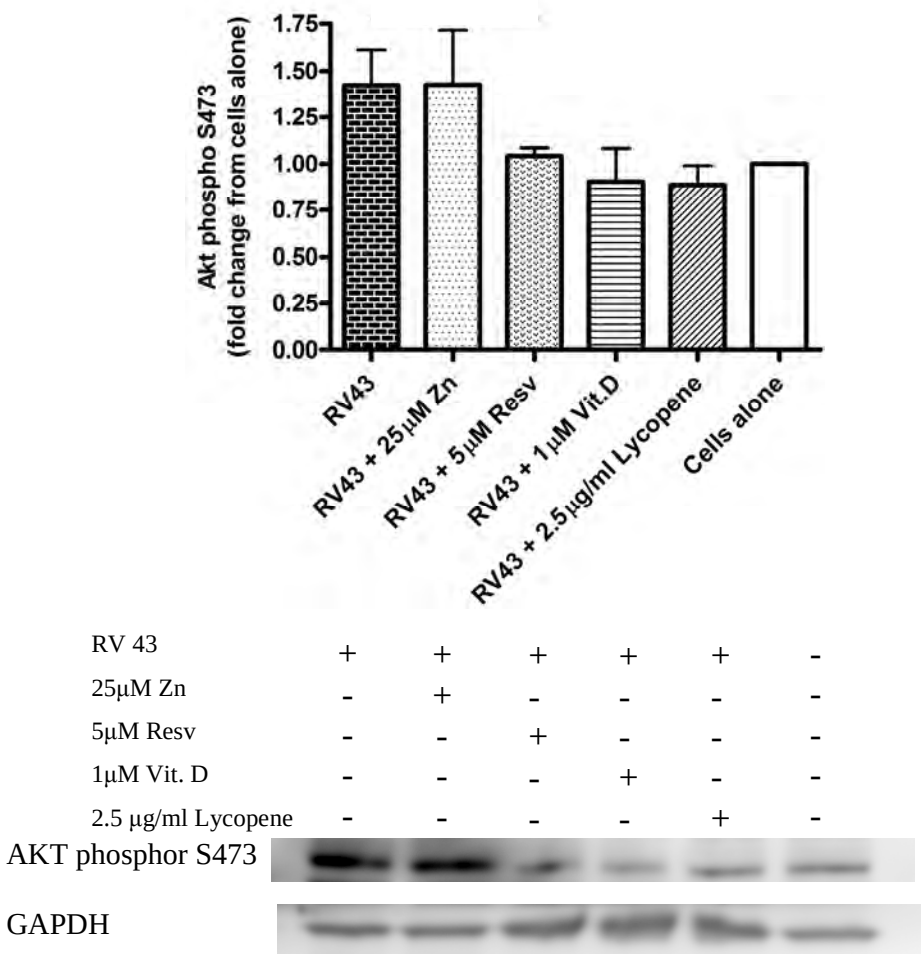


Calu-3 cells were pre-treated with individual antioxidants and infected with RV43 at a MOI=50. After 6 and 48 hours of viral infection, Calu-3 cells were incubated with either FITC-labelled ICAM-1 antibody (Ab) or IgG isotype control to determine the level of ICAM-1 surface protein expression as described in Chapter 2. Data is mean ± SEM (n=3).

Figure 5.3.1. Membranous ICAM-1 protein expression at (a) 6 hours, (b) 48 hours in Calu-3 cells pre-supplemented with antioxidants and then infected with RV43

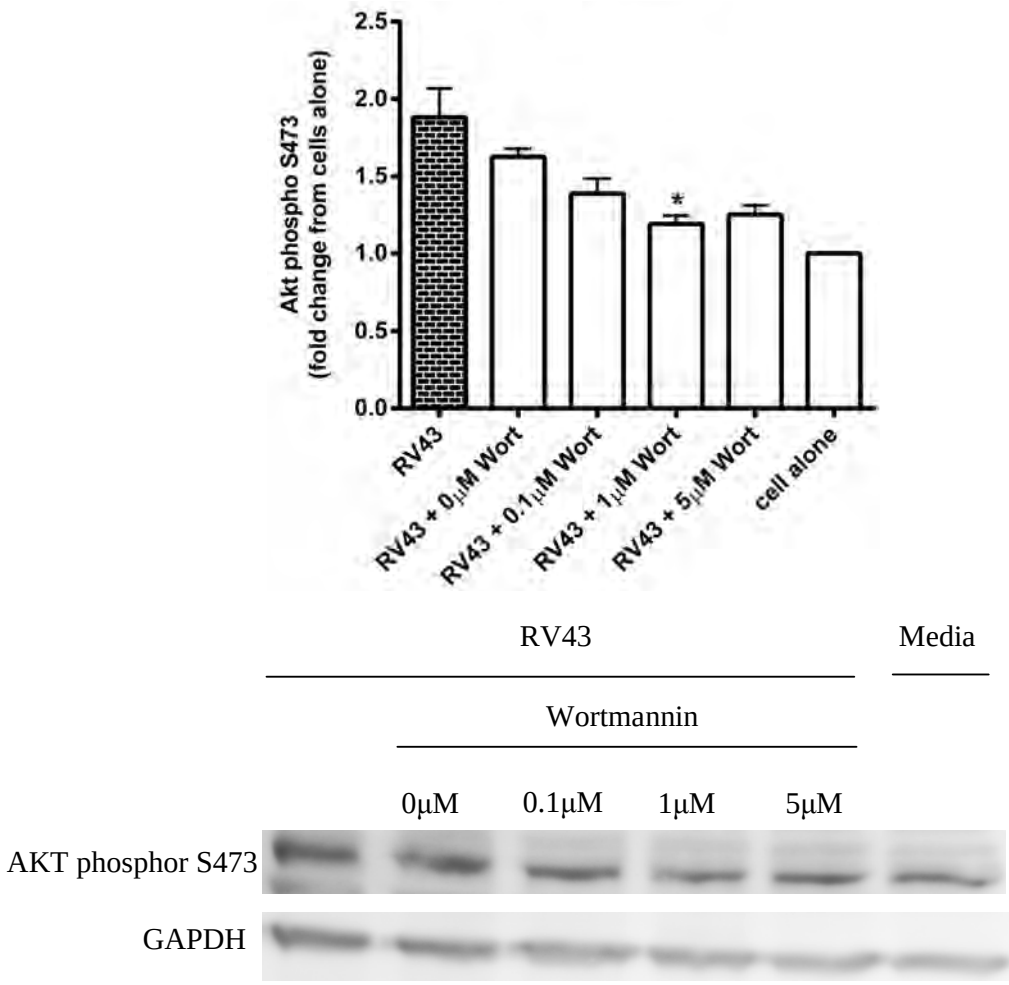
The involvement of PI3-kinase during RV infections: effect of antioxidants and wortmannin:

The activation level of PI3-kinase was assessed by measuring the protein AKT (phosphorylated form), the substrate of PI3-kinase. Western blotting was performed to determine the level of protein AKT (phosphorylated form, activated). RV infection markedly increased the activation level of PI3-kinase (Figure 5.3.2). Pre-treatment of the cells with resveratrol, vitamin D and lycopene, but not with zinc, prior to RV infection, resulted in 27, 37, and 38% decrease in PI3-kinase activity respectively. Pre-treatment of the cells with various concentrations (at increasing doses) of wortmannin for 2 hours prior to RV infection, showed marked reduction in the activation of PI3-kinase (Figure 5.3.3). The inhibition of PI3-kinase by wortmannin during RV infection significantly affects the infectivity of RV as demonstrated by the decreased RV titer as shown in Figure 5.3.4 (a). Additionally, wortmannin not only caused decreases in RV titer, but also affected the intracellular replication of RV as shown by RV43 copy number (FAM), measured by RT-qPCR [(Figure 5.3.4 (b)]. Wortmannin at the concentrations used in this study does not markedly alter cellular viability as shown in Figure 5.3.5, hence suggesting that effect of wortmannin on viral replication is mediated specifically by targeting on PI3-kinase activation.



Calu-3 cells were pre-treated with individual antioxidants and infected with RV43 at a MOI=50. Whole cells lysate was harvested at 6 hours post infection for determination of PI3-kinase, by measuring pAKT using Western blotting as described in Chapter 2. Upper panel, densitometry of the phosphorylation AKT (pAKT) normalized to GAPDH, expressed as the mean fold change ± SEM (n=6) when compared with cells alone; Bottom panel, representative blot showing inhibition of pAKT by antioxidants (similar results was obtained from 5 independent experiments).

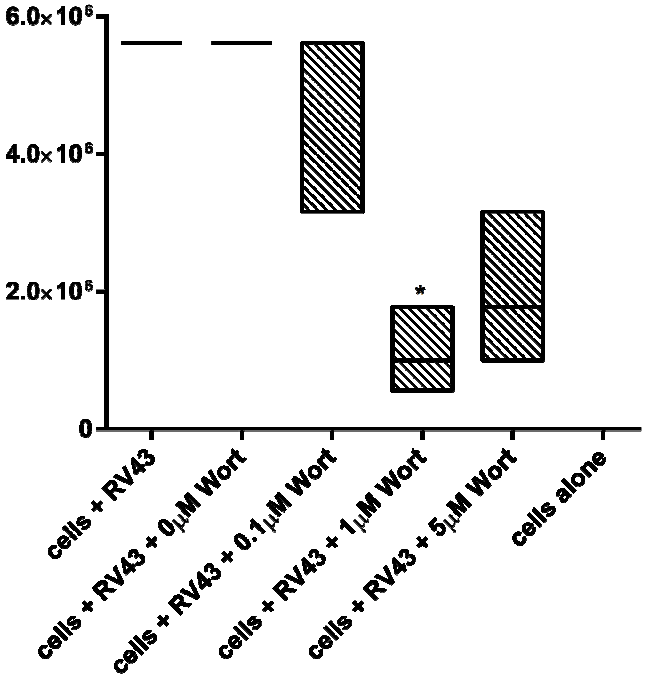
Figure 5.3.2. The activation of PI-3 kinase in RV43 infected Calu-3 cells is inhibited by resveratrol, vitamin D and lycopene.



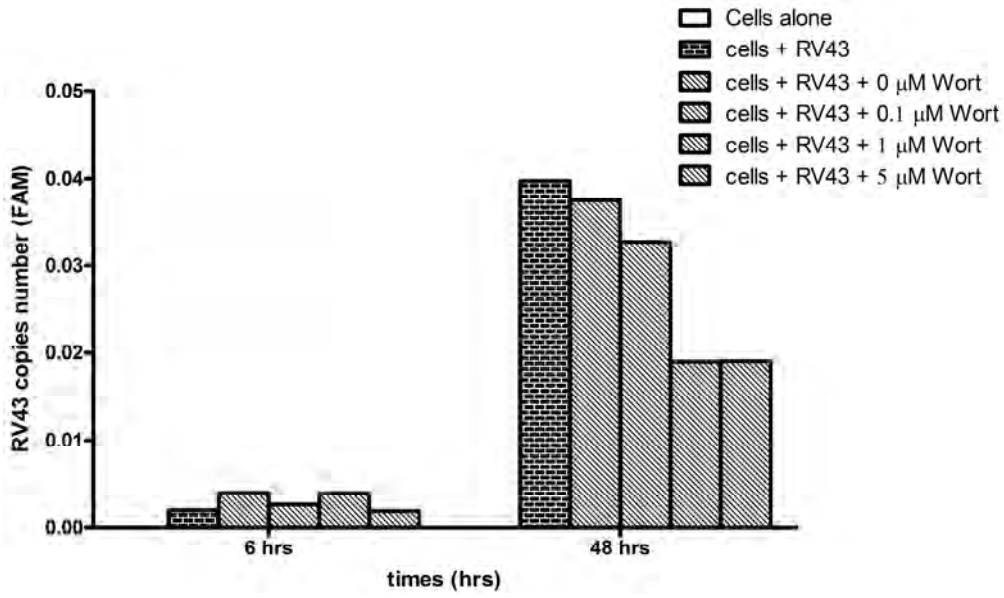
Calu-3 cells were pre-treated with different concentrations of wortmannin for 2 hours prior to RV43 infection. Whole cell lysate was harvested at 6 hours of infection to determine the level of PI3-kinase activation, by measuring pAKT as described in Chapter 2. Upper panel, densitometry of the phosphorylation AKT (pAKT) normalized to GAPDH expressed as fold change when compared with cells alone; Bottom panel, representative blot showing inhibition of pAKT by wortmannin (Wort).
*$p<0.05$ versus RV43

Figure 5.3.3. Inhibition of PI3-kinase by wortmannin on RV43 replication in Calu-3 cells.

(a)

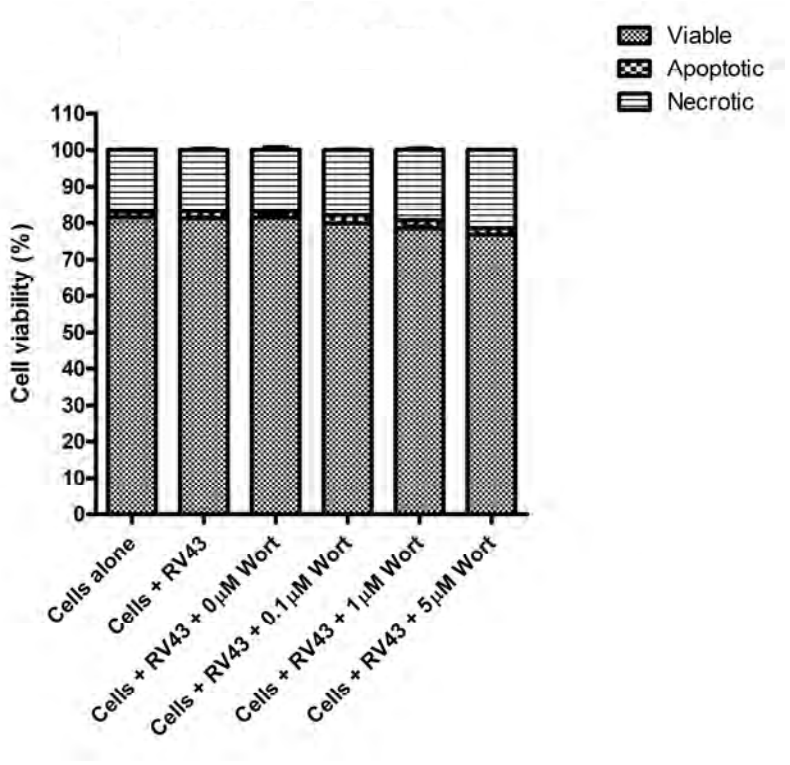


(b)



Calu-3 cells were pre-treated with different concentrations of wortmannin for 2 hours prior to RV43 infection. (a) Supernatant was collected at 48 hours of infection to determine viral titer using $TCID_{50}$ assay as described in Chapter 2. $TCID_{50}$ Data are summarized by floating bars (min and max values): the central line represents the median, (n=3). (b) Cellular RNA was harvested at 6 and 48 hours of infection to determine RV43 copy number by RT-qPCR as described in Chapter 2.
*$p<0.05$ versus cells + RV43 group

Figure 5.3.4 Wortmannin reduces RV43 replication in Calu-3 cells



Calu-3 cells were pre-treated with different concentrations of wortmannin for 2 hours prior to RV43 infection. Viable, apoptotic and necrotic cells were assessed by Annexin V-PE and 7-AAD staining and analyzed by flow cytometry at 48 hours post-infection. Data is mean ± SEM (n=3).

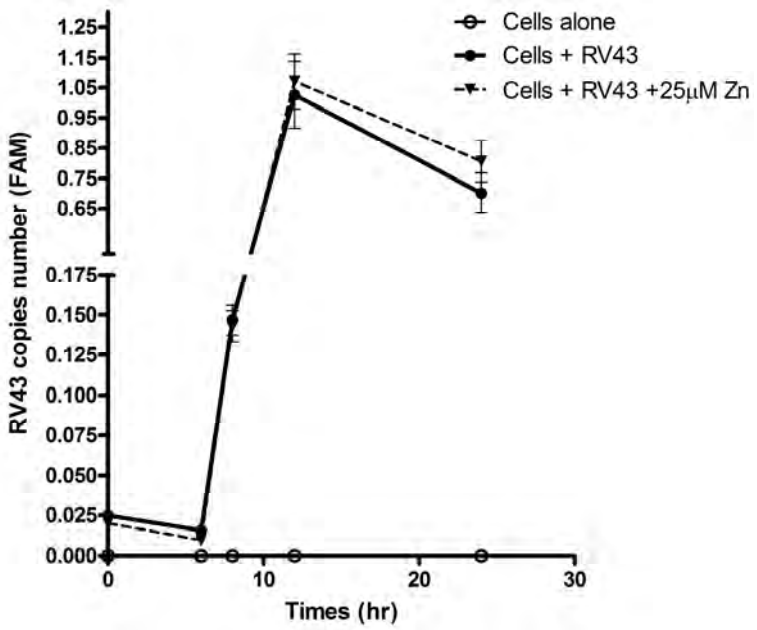
Figure 5.3.5. Effects of wortmannin (Wort) on viability of Calu-3 cells infected with RV43.

Effects of antioxidant compounds on viral RNA replication:

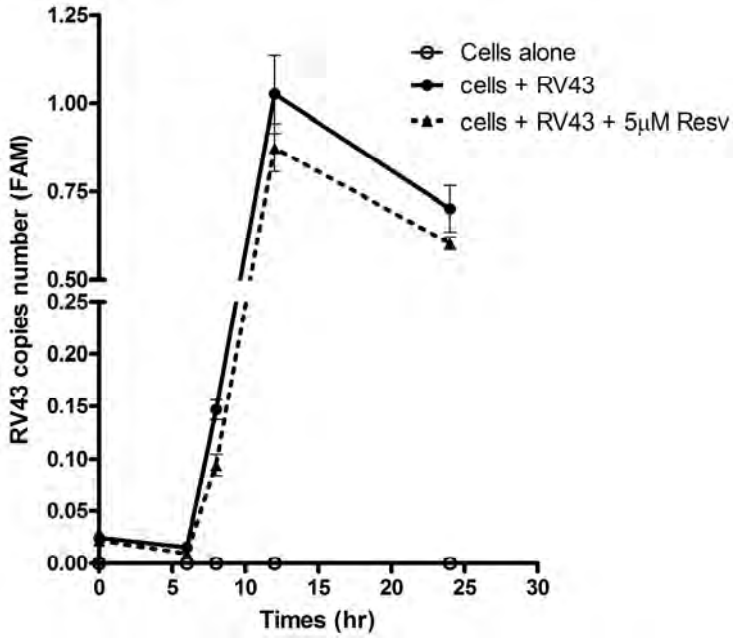
RV43 internalization by Calu-3 cells was shown to occur within the first 6 hours of viral infection based on the number of viral RNA determinations (Figure 5.3.6, Figure 5.3.7). Figures 5.3.6 (a,b) and 5.3.7 (a,b) show that viral replication was actively occuring in Calu-3 cells between 6 to 12 hours of infection. At 24 hours of infection, the level of viral replication was slightly reduced, indicating that the first cycle of viral replication in Calu-3 cells occurred in the first 12 hours of infection. Pre-treatment of the cells with resveratrol before viral infection, shown reduced viral RNA replication at

all time points investigated [Figure 5.3.6 (b)], however failed to reach statistical significant. Pre-treatment of the cells with either zinc or vitamin D before infection did not have any significant effect on viral RNA replication at all time points investigated [Figure 5.3.6 (a); 5.3.7 (a)]. On the other hand, at 12 hours of viral infection, lycopene significantly ($p<0.01$) reduced viral RNA replication [Figure 5.3.7 (b)].

(a)



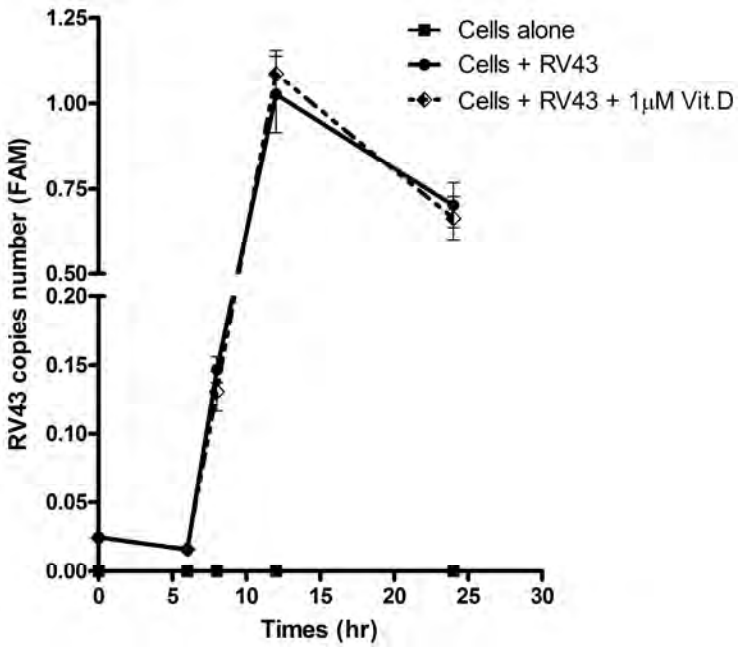
(b)



Calu-3 cells were pre-treated with (a) 25μM zinc for 4 hours, (b) 5μM resveratrol for 2 hours and infected with RV43 at a MOI=50. Cellular RNA was harvested at 0, 6, 8, 12 and 24 hours of infection to determine RV43 copy number by RT-qPCR as described in Chapter 2.

Figure 5.3.6. Effect of zinc and resveratrol on RV43 replication

(a)



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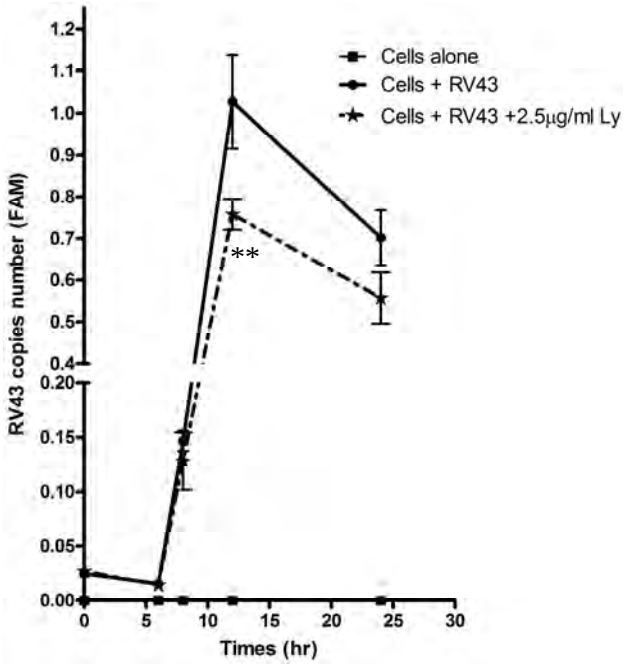
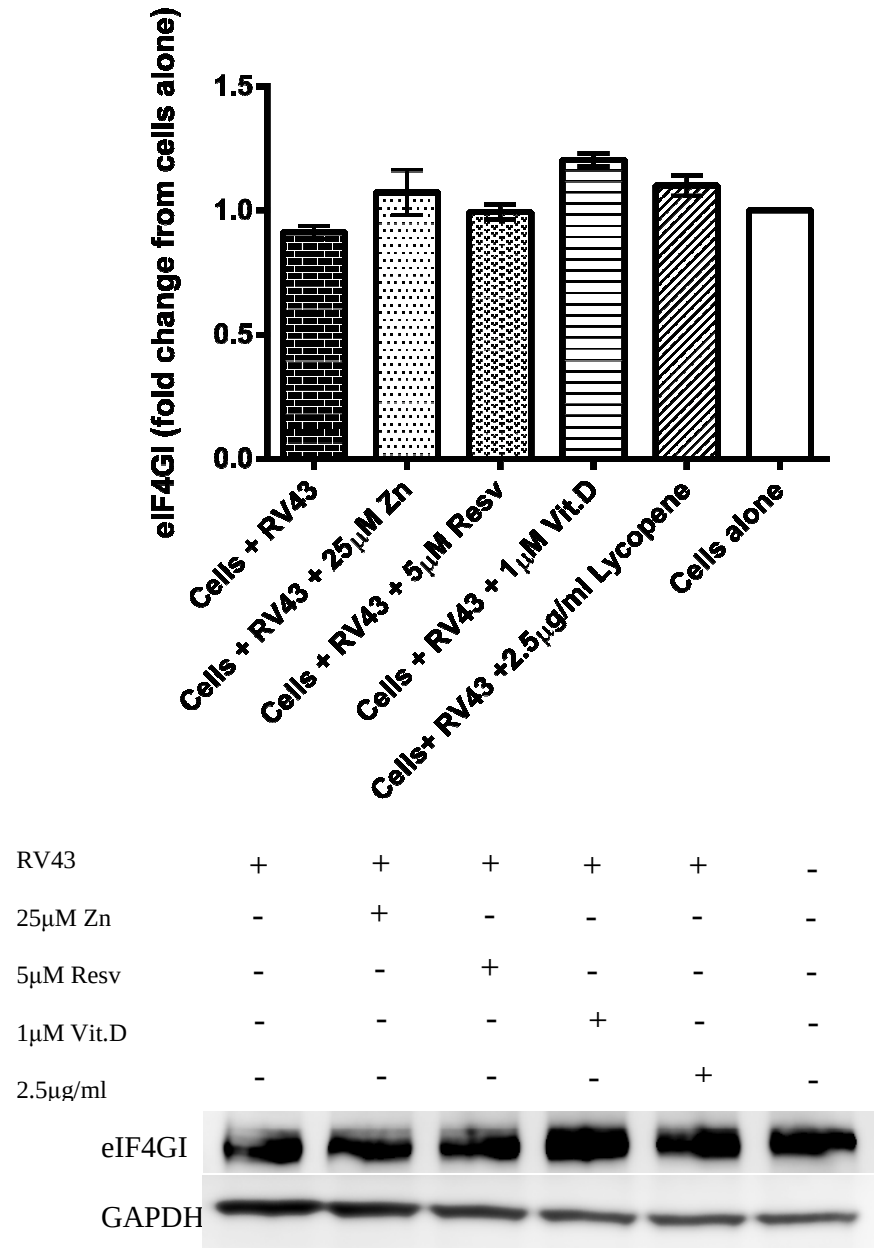


Calu-3 cells were pre-treated with (a) 1μM vitamin D for 2 hours, (b) 2.5μg/ml lycopene for 24 hours and infected with RV43 at a MOI=50. Cellular RNA was harvested at 0, 6, 8, 12 and 24 hours after infection to determine RV43 copy number by RT-qPCR as described in Chapter 2. **$p<0.01$ versus Cells + RV43 group

Figure 5.3.7. Effect of vitamin D and lycopene on RV43 replication

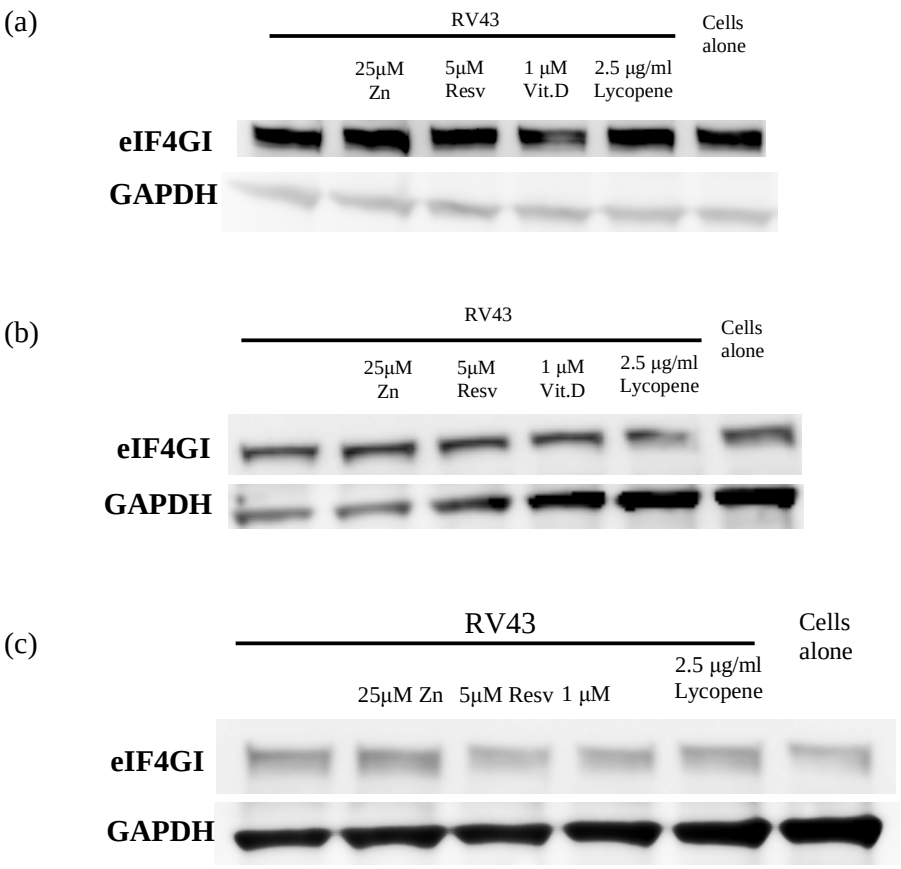
Effects of antioxidants on cleavage of cellular substrates during RV infection:

The investigation of the effect of antioxidant compounds on viral replication was continued by determination eIF4GI protein, to ascertain whether antioxidant compounds play a role in viral RNA genome translation, a process to produce mature viral polypeptide. During the translation step, viral protein 2A proteinase is produced, and this viral protein has been shown to effectively cleave the cellular translation initiation factors eIF4GI [403-405] leading to the shutoff of host cell protein synthesis. Hence, measurement of eIF4GI, can be an indirect tool to investigate the influence of antioxidant compounds has on viral RNA translation. Whole cells lysate was recovered at 6, 24, 48 and 72 hours of infection, to study the influence of antioxidant compounds on the cleavage of this cellular 2A proteinase substrate in Calu-3 cells. At 6 hours after viral infection, no change in the level of eIF4GI in Calu-3 cells was observed, suggesting that no cleavage had occurred (Figure 5.3.8). Similar results were evident at 24 hours of viral infection. As duration of viral infection increased (48 and 72 hours), the intensity of the eIF4GI band decreased, indicating cleavage of eIF4GI at these later time points, due to cell death (Figure 5.3.9). Based on both Figure 5.3.8 and Figure 5.3.9, RV43 did not cleave the eIF4GI at 6 hours post infection as expected. Hence, we can't determine the effect of antioxidants on this translational process. Therefore it can be concluded that antioxidant compounds are not interfering on late stages of viral replication in the Calu-3 cells used in the experiments in this thesis.



Calu-3 cells were pre-treated with individual antioxidants and infected with RV43 at a MOI=50. Whole cell lysate was harvested at 6 hours post infection and Western blot was performed to detect cleavage of eIF4GI as described in Chapter 2. Upper panel, densitometry of the eIF4GI normalized to GAPDH, expressed as the mean fold change ± SEM (n=3) when compared with cells alone; Bottom panel, representative blot showing protein eIF4GI (similar results was obtained from 3 independent experiments).

Figure 5.3.8. Effect of antioxidant-enriched cells on RV43 mediated cellular cleavage of eIF4GI at 6 hours of infection

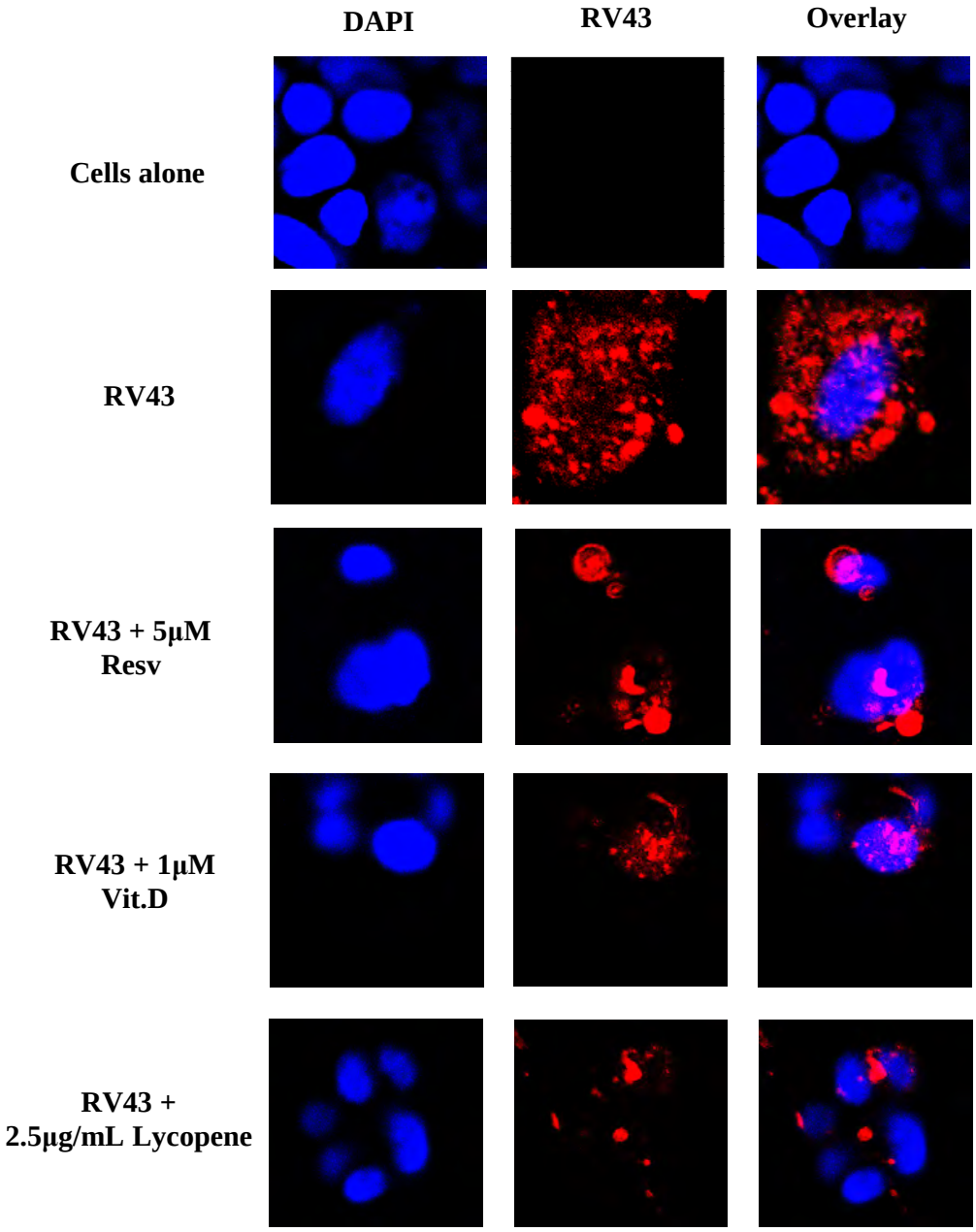


Calu-3 cells were pre-treated with individual antioxidants and infected with RV43 at a MOI=50. Whole cell lysate was harvested at (a) 24 hours, (b) 48 hours and (c) 72 hours post infection and Western blot was performed to detect cleavage of eIF4GI as described in Chapter 2. Representative blot showing protein eIF4GI (similar results was obtained from 3 independent experiments).

Figure 5.3.9. Effect of antioxidant-enriched cells on RV43 mediated cellular cleavage of eIF4GI at 24, 48 and 72 hours of infection

Viral entry inhibition: effects of resveratrol, vitamin D and lycopene:

Figure 5.3.10 demonstrates that resveratrol, vitamin D and lycopene inhibited the viral internalization step (less labelled-RV43 was observed in the Calu-3 cells when compared to untreated-infected cells).



Confocal fluorescent microscopy images demonstrating RV43 infection (red) with and without antioxidant pre-treatment of Calu-3 cells. The nucleus was stained with DAPI (blue). Calu-3 cells were grown on coverslips until 70% confluent, then treated either with resveratrol, vitamin D or lycopene and challenged with AlexaFluor 555-labelled RV-43 at MOI 0.5 for 1 hour at 33ºC. Then cells were mounted with ProLong® Gold Antifade Reagent with DAPI and visualized using an Olympus IX81 Fluoview FV1000 Confocal Laser Scanning Biological Microscope and the FV10-ASW Version 2.0 image software.

Figure 5.3.10. Inhibition of RV43 entry by resveratrol, vitamin D and lycopene.

5.4 Discussion

As presented in the previous chapter, antioxidant compounds, except resveratrol (which significantly reduced IL-8) didn't have the expected effect of down regulating the induction of inflammatory cytokines induced by RV infection in Calu-3 cells. However, they were able to modulate viral replication. This chapter demonstrates a mechanism by which antioxidants were able to limit the viral replication. The effect wasn't due to up-regulation of innate immunity of the infected cells, as evident from the level of IP-10 secretion in the cultured media.

Recently, Edlmayr et al. [406] described the use of recombinant VP1 of RV capsid proteins as a vaccine against RV infection. Host cellular receptor, ICAM-1, that binds to the RV receptor and is located at the canyon or depression site on RV, is implicated in avoidance of immune surveillance [146, 375]. The justification provided by Edlmayr et al. [406] was based on the evidence that the VP1 proteins are the most immunodominant and exposed proteins. In addition, the canyon encircled the fivefold axes of the viral capsid within VP1 proteins [407]. Another study reported the effectiveness of using specific antibodies on RV capsid against RV infection [408]. Edlmayr et al. [406] have shown that rabbit serum (antisera) containing specific antibodies raised against purified VP1 of RV89 and RV14, inhibited either RV89 or RV14 infection of cultured HeLa cells. This appears promising, however it should be noted that the effects might be due to the effect of antisera induced toxicity on the cells leading to a reduction in CPE. In addition they used HeLa cells, while similar studies on airway epithelial cells may be more relevant for the *in vivo* condition.

Papi et al. [18], has shown that major group RV infection of primary bronchial epithelial cells and A549 cells caused significant induction of ICAM-1 protein expression and pre-treatment of the cells with GSH or 2% DMSO prior to infection,

resulted in significant decrease in ICAM-1 (both mRNA and protein levels). It was shown that RV induction of ICAM-1 was critically dependent on the activation of Nf-κB [18]. Hence, it is suggested that antioxidant compounds like GSH (but not the oxidized form of GSH, GSSG) can successfully prevent RV infection by inhibiting RV-induced ICAM-1 protein expression which was shown to be necessary for RV to bind to and to gain entry into the targeted cells. Contrary to this, in the current study we found that RV43 failed to induce expression of ICAM-1 protein expression in Calu-3 cells, neither at initial time of infection (6 hours) nor at peak time of infection (48 hours). An innate property of the Calu-3 cell line is constitutive activation of the Nf-κB pathway and this might explain why RV43 in the current study failed to induce up-regulation of ICAM-1 protein expression compared to uninfected cells. On the other hand, others have suggested the ratio of membranous to soluble forms of ICAM-1 (receptor) is an important determinant of RV infection. For example, Whiteman et al. [409] reported that RV-type 14 significantly induced the membranous form of ICAM-1 while at the same time down-regulated soluble form of ICAM-1 gene expression in normal human bronchial epithelial cells. The use of monoclonal antibody to ICAM-1 in the RV experiment significantly down regulated the membranous ICAM-1 gene expression (while significantly up-regulating the soluble form of ICAM-1 gene expression) and resulted in significantly decreased viral titre [409]. Hence, these results suggest that measurement of the ratio of membranous to soluble ICAM-1 may be most informative.

The engagement of RV to the ICAM-1 receptor on host cells initiates a viral internalization pathway which is responsible for the successful delivery of viral RNA genome. PI3-kinase was shown to play a role during RV internalization. Newcomb et al. [184] have demonstrated that the inhibition of PI3-kinase results in significant impairment of viral internalization of labelled RV-39 into human bronchial epithelial

cells. In agreement with this observation, the current study showed that inhibition of PI3-kinase resulted in significant reduction of RV43 viral titer and lesser viral RNA detected in Calu-3 cells. The effect on viral replication was not due to the toxicity effect that can be caused by the administration of wortmannin to the cells, as shown in the result section (Figure 5.3.5). The current study is the first to demonstrate the effect of zinc, resveratrol, vitamin D and lycopene on activation of PI3-kinase induced by RV on airway epithelial cells. Pre-treatment of the cells with resveratrol, vitamin D or lycopene prior to RV infection, resulted in minimal (27-38%) decrease in PI3-kinase activation. Based from this observation, it can be hypothesized that the antiviral activity of the antioxidant compounds (except zinc) used in this study can be explained by their role in interfering with viral internalization. To confirm this finding, RV43 was purified and then labelled with alexa-fluor 555 in order to visualize the entry of virus into antioxidant-enriched Calu-3 cells (pre-treated with resveratrol, vitamin D and lycopene before infection). As expected (due to inhibition of PI3-kinase activation), lesser RV43 was noted in the cells which were pre-treated with resveratrol, vitamin D or lycopene before infection with labelled-virus (Figure 5.3.10). Together these findings (PI3-kinase and confocal microscopy images), provide novel evidence that antioxidant compounds play a useful role in limiting viral internalization of airway epithelial cells.

Resveratrol, vitamin D and lycopene reduce the viral internalization process as indicated by decreased PI3-kinase activation; therefore reducing the successful rate of uncoating viral RNA. Copy numbers of intracellular viral RNA were determined using RT-qPCR. In the study, however, it was noted that lycopene significantly reduced viral RNA at 12 hours post-infection during viral RNA replication. However, zinc, resveratrol and vitamin D were not able to significantly reduce viral RNA during RNA replication (at all time points studied). In conclusion, lycopene did influence the rate of

viral replication in Calu-3 cells and this may partly provide an explanation for the antiviral activity demonstrated by the compound.

As described in Chapter 1 (Section 1.9), viral RNA is also used to produce (via translation) viral proteins which consist of enzymes needed for viral RNA replication and viral protein coatings (VP1, VP2, VP3, VP4). In this aspect, eIF4GI has been used as an indirect marker to assess viral protein translation. Gaudernak et al. [110] have found during RV infection on HeLa cells, as early as 4 hours after infection, eIF4GI was cleaved and the protein was totally cleaved at 6, 8 and 24 hours of infection with significant synthesis of rhinoviral proteins (VP1, VP2, and VP3). In another study done by Khan et al. [410], eIF4GI was cleaved after 3 hours of RV (type 2 and type 14) infection on RD-ICAM-1 cells. Both studies have shown cleavage of eIF4GI during RV infection. However, in the current study, even at 6 and 12 hours of infection, the protein was still intact, and only by the 72 hours of infection, significantly lesser amount of eIF4GI is detected. The latter observation is probably due to cell death. The use of different cells might provide an explanation for the inconsistent observations regarding viral induced cleavage of the protein. Hence, due to the lack of effect of RV43 on cleavage of eIFGI in Calu-3 cells, we conclude that antioxidant compounds don't interfere with the viral translation process in Calu-3 cells.

5.5 Conclusion

The antiviral activity of the antioxidant compounds studied was shown to be mediated by interfering at the viral internalization step. The present chapter demonstrates the potential to utilise antioxidant compounds which are highly available and accessible as a preventive or treatment strategy for limiting virus-induced asthma exacerbation.

CHAPTER 6: EFFECTS OF ANTIOXIDANT TREATMENT ON RHINOVIRUS INTERNALIZATION IN INFECTED CULTURED AIRWAY EPITHELIAL CELLS AND THE ROLE OF PI3-KINASE

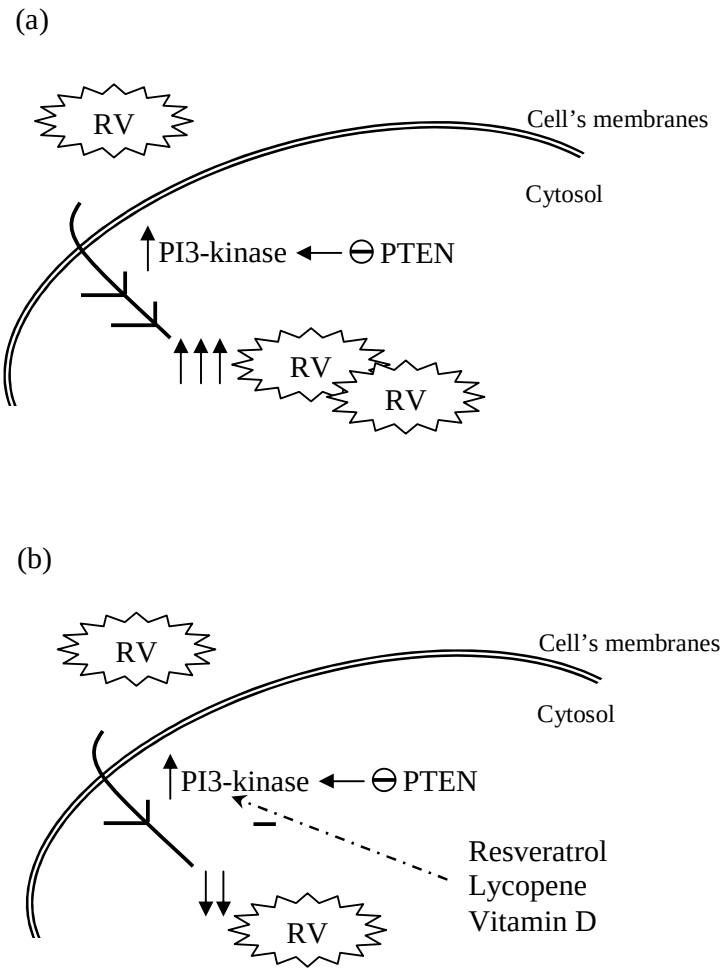
6.1 Introduction

In the previous study (Chapter 4), we have shown that antioxidants had antiviral effects on RV infection in human airway epithelial cells. In this chapter, we have done a series of experiment (mechanism study) to further understand how these antioxidant compounds may influence RV replication in this model (Chapter 5). In chapter 4, we have shown that antioxidants reduced RV replication primarily through preventing RV internalisation into the airway epithelial cells. In this chapter, we determine that the antioxidant compounds (resveratrol, vitamin D and lycopene) specifically inhibit RV induced activation of cellular PI3-kinase and this has a direct effect on reducing RV internalisation.

The specific target of the antioxidants on PI3-kinase during virus replication (entry and uncoating) could be investigate by suppressing the main regulator of PI3-kinase, which is tumor suppressor protein PTEN [PtdIns(3,4,5)P_3 phosphatase]. It is known that activation of PI3-kinase leads to synthesis of phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3) that is dephosphorylated by PTEN to phosphatidylinositol-4,5-biphosphate (PtdIns(4,5)P_2) [411, 412]. PTEN cleaves the 3'phosphate from PtdIns(3,4,5)P_3 to generate PtdIns(4,5)P_2. Works done by Leslie et al.[191] have demonstrated the inactivation of PTEN leads to increase cellular PtdIns(3,4,5)P_3 and activation of downstream PtdIns(3,4,5)P_3 target, PKB/Akt.

In this study, we hypothesized that by silencing the protein PTEN, the expression of PI3-kinase will be increased in the course of RV infection and hence, favouring RV internalisation. As a result, RV replication on Calu-3 cells will be increased (hypothesis). Additionally, we would like to demonstrate that enrichment of cells with the antioxidant compounds will halted the PI3 kinase activation, thus impairing RV entry and replication. (Figure 6.1.1). Therefore, this study is conducted to confirm that

the antioxidant compounds specifically target PI3-kinase during RV internalisation (as shown in the previous chapter). A preliminary experiment was conducted to validate the effectiveness of employing small interfering RNA in silencing a gene on Calu-3 cells and these results are presented in this chapter.



(a) Knock down PTEN will sustain activation of PI3-kinase, thus favouring viral replication, (b) PTEN is knock down, however as evidenced in the previous chapter (Chapter 5) that antioxidant had inhibits the activation of PI3-kinase, hence antioxidant is hypothesized to inhibits viral replication.

⊖ Knock down
– Inhibit activation
↑ Increase
↓ Decrease

Figure 6.1.1. Hypothesis on the direct effect of antioxidant on PI3-kinase during viral internalization via silencing regulatory protein of PI3-kinase, PTEN.

6.2 Materials and methods

Study design:

In this study, Calu-3 cells were incubated with small interference RNA (siRNA) PTEN for total duration of 96 hours (Figure 6.2.1). The first 24 hours, the cells were solely incubated with siRNA PTEN, then the cells were supplemented with either 2.5 µg/ml of lycopene or 1µM of vitamin D or 5µM of resveratrol for 24, 18, and 2 hours respectively before RV infection (MOI=50). After 48 hours of infection, media and RNA were harvested to determine $TCID_{50}$/ml and to assess the rate of PTEN gene knockdown. Cellular viability, including assessment of apoptosis and necrosis were also carried out at 48 hours post-infection to assess and verify that the administration of siRNA PTEN does not induce toxicity.

Airway epithelial cell culture:

As described in Chapter 2, human airway epithelial cells (Calu-3, from ATCC, USA) were cultured in minimum essential medium containing 10% foetal calf serum (10%FCS/MEM) containing 2% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine, and 2.2g/l $NaHCO_3$ (all were purchased from Invitrogen Corporation, Carlsbad, USA) at 37ºC in the presence of 5% CO_2. For transfection experiments, cells density at 4.5 x 10^4/well was used.

RNA interference:

As described in Chapter 2, RNA interference on Calu-3 was done via mixing of siRNA PTEN (Ambion) diluted to 60nM in OPTI-MEM1 with transfection reagent consist of diluted siPORT NeoFX transfection agent in OPTI-MEM1. This transfection complex was mixed with freshly trypsinized Calu-3 at a density of 1 x 10^5/ml and was added to

the tissue culture 24-well plate. The transfectant cells were incubated at 37ºC and 5% CO_2 incubation for 48 hours before RV infection. GAPDH was silenced with siRNA in a separate well as a positive control. The cells were administered with lycopene, vitamin D and resveratrol for 24, 18 and 2 hours, respectively, before RV infection. Freshly prepared siRNA against PTEN was then added to the cells for duration of 48 hours of RV infection.

Lycopene enrichment of cultured epithelial airway cells:

As described in Chapter 2 (Section 2.2.9), Calu-3 cells were incubated for 24 hours with 2.5µg/ml of lycopene dissolved in 0.5% tetrahydrofuran (THF) in cell culture medium.

Vitamin D enrichment of cultured epithelial airway cells:

As described in Chapter 2 (Section 2.2.9), Calu-3 cells were incubated for 18 hours with 1µM of 1α,25-dihydroxyvitaminD3 dissolved in 0.1% ethanol in cell culture medium.

Resveratrol enrichment of cultured epithelial airway cells:

As described in Chapter 2 (Section 2.2.9), Calu-3 cells were incubated for 2 hours with 5µM of resveratrol dissolved in 0.1% DMSO in cell culture medium.

Rhinovirus challenge of Calu-3 cells:

After removing the supplementation (antioxidant) media (containing siRNA) from Calu-3 cells, they were infected with RV43 at MOI of 50 (as described in Chapter 4, Section 4.2). Plates were incubated with media containing siRNA for 48 hours at 33ºC in the presence of 5% CO_2.

Visual inspection of Calu-3 cells:

Cellular viability of cultured Calu-3 cells was observed during the experiments (Olympus microscope TL4).

Flow cytometric analysis of viable, apoptotic and necrotic cells:

To determine that treating Calu-3 cells with siRNA against PTEN does not cause toxicity, flow cytometry was used to quantify the number of viable, apoptotic and necrotic cells after 48 hours of viral infection, as described in Chapter 2 (Section 2.3.3).

Viral titration assay:

As described in Chapter 2, $TCID_{50}$ experiments were performed using near confluent RD-ICAM-1 cells seeded in 96-well tissue culture plates (NUNC, Roskilde, Denmark). Cells were infected by either media alone or virus containing media at varying dilutions. Serial ten-fold dilutions of the samples were prepared and 4 individual wells were infected with each dilution. For titration of samples, 7 dilutions were prepared, and 4 control wells were prepared with media alone. After five days the plates were read and the $TCID_{50}$ calculated. Infected wells were scored based on the cytopathic effect (CPE) seen by light microscopy, >50% CPE was considered a positive result. Viral titer was calculated and expressed as tissue culture infectious dose at 50% in log value ($TCID_{50}$ log_{10})[328] and using the Karber formula for the tissue culture infective dose 50% ($TCID_{50}$) [329].

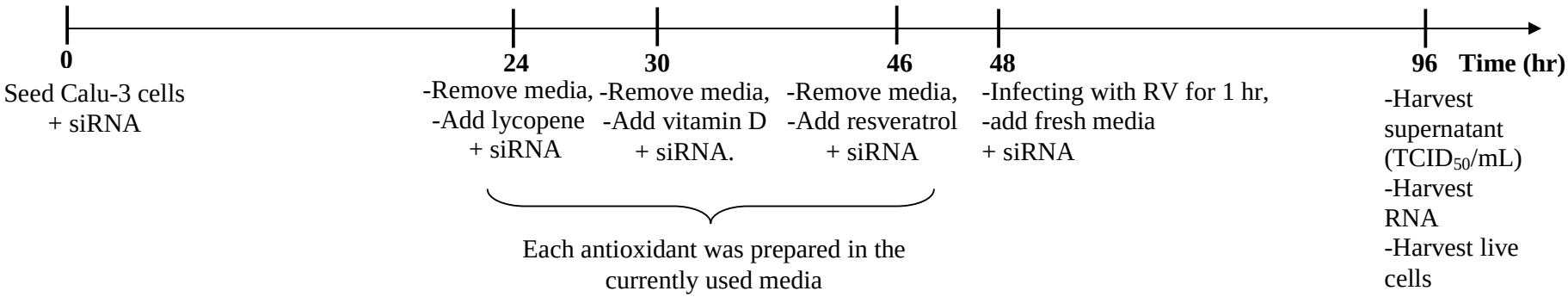


Figure 6.2.1. Schematic diagram of the study design to assess role of antioxidants in inhibiting activation of PI3-kinase via silencing PTEN on RV replication

6.3 Results

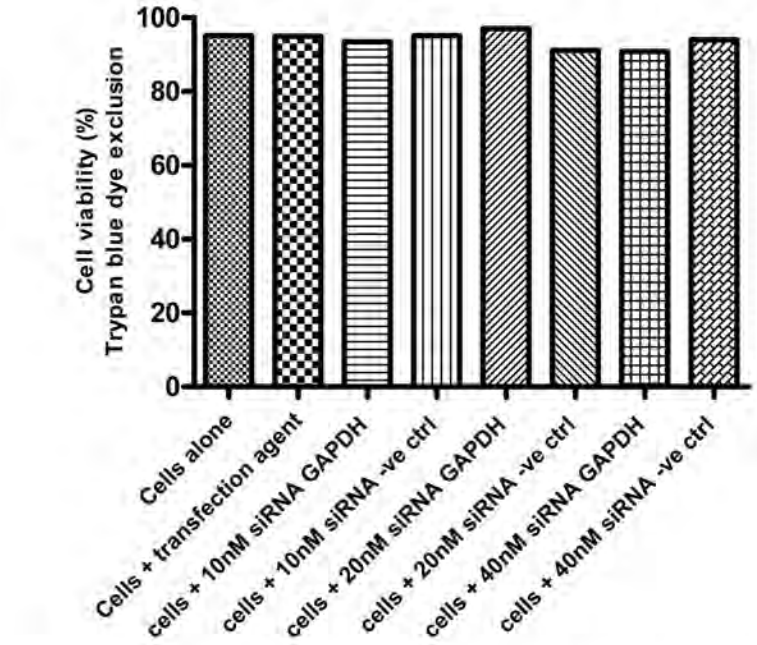
Visual inspection of Calu-3 cells:

Visual inspections of the cultured Calu-3 cells during the experiments confirmed that cells were healthy.

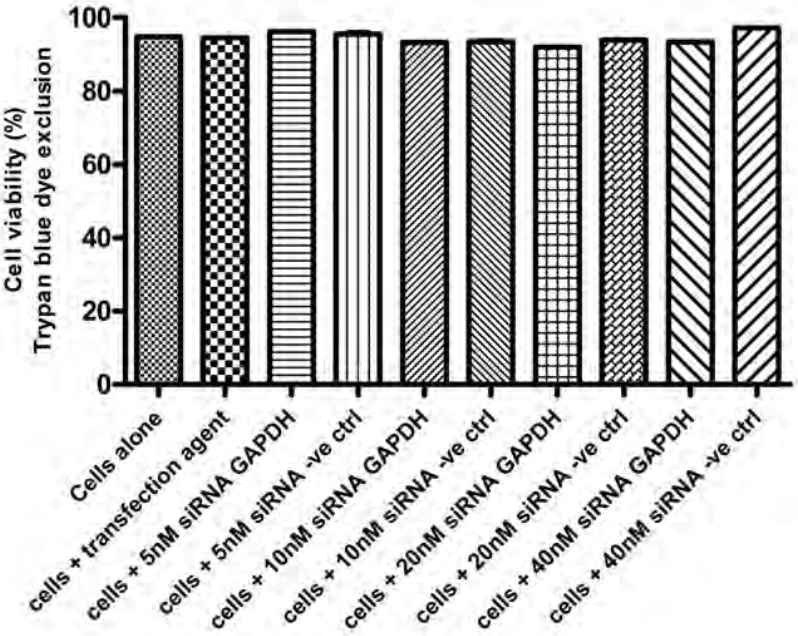
The administration of siRNA against GAPDH to Calu-3 cells:

We first need to determine if siRNA could successfully be delivered to Calu 3 cells without inducing toxicity. The cells were seeded together with the siRNA, and siRNA was delivered to the cells using siPORT NeoFx. As shown in Figure 6.3.1 (a) and (b), administration of siRNA against GAPDH mRNA at increasing doses does not cause cells toxicity. It was observed that administration of siRNA GAPDH at concentration of 40nM to Calu-3 cells (seeded at $4.5x10^4$ cells density) resulted in about 60% of mRNA GAPDH was successfully silenced [figure 6.3.2 (a)].

(a)



(b)

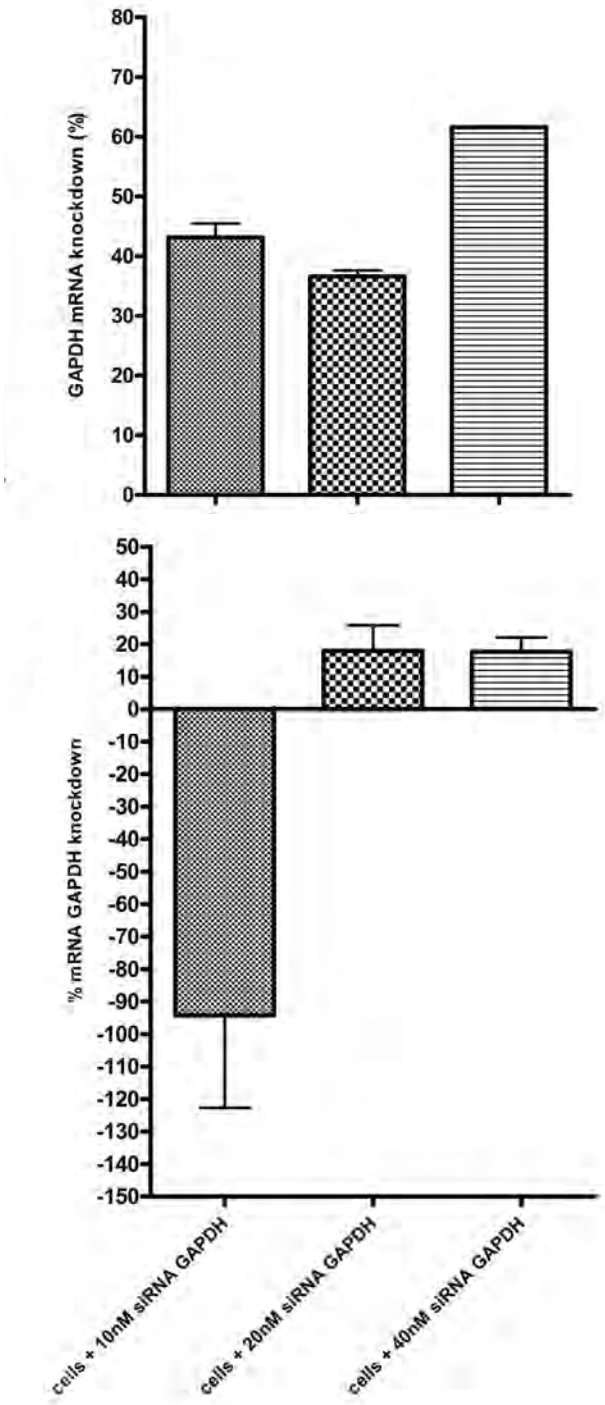


Calu-3 cells were seeded at (a) 4.5 x 10^4 or (b) 9 x 10^4 cells density with different concentrations of siRNA against GAPDH as indicated in Materials and Methods. The toxicity of siRNA against GAPDH was then determined by Trypan blue dye exclusion staining technique after 48 hours of incubation.

Figure 6.3.1. The used of siRNA at concentrations below or at 40nM does not cause cells toxicity

(a)

(b)



Calu-3 cells were seeded at (a) 4.5 x 10^4 or (b) 9 x 10^4 cells density with different concentrations of siRNA against GAPDH as indicated in Materials and Methods. RNA was harvested and RT-qPCR was run as indicated in Materials and Methods to determine the knockdown of mRNA GAPDH. Comparitive Ct value ($2^{-\Delta\Delta Ct}$) related to an internal control gene (18S rRNA) was then generated to assess the knockdown of mRNA PTEN.

Figure 6.3.2. Percentage (%) of mRNA GAPDH knocked down when siRNA to GAPDH was administered to Calu-3 cells at 48 hours of incubation times

Effect of siRNA against PTEN on Calu-3 cells:

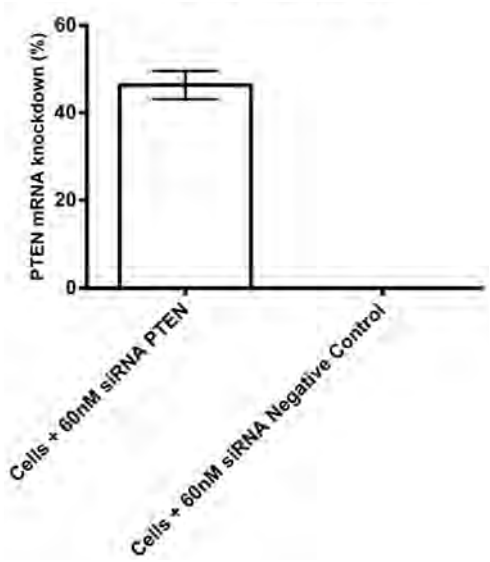
At 40nM of siRNA, it was observed that about 60% of mRNA GAPDH was successfully silenced. In order to increase the rate of gene knock down in Calu-3 cells, siRNA (against PTEN) at higher concentration (60nM) was administered to Calu-3 cells. Administration of 60nM of siRNA against PTEN on Calu-3 cells, resulted in about 50% of mRNA PTEN being silenced (Figure 6.3.3). RV infection on Calu-3 cells was observed to decrease mRNA PTEN (Figure 6.3.4). siRNA against PTEN mRNA in Calu-3 cells does not influenced by the antioxidant compounds (resveratrol, lycopene and vitamin D) (Figure 6.3.4)

Effect of silencing PTEN on Calu-3 cells on viral replication:

In the introduction of this chapter, we hypothesized that continuous activation of PI3-kinase by silencing its negative regulator, PTEN, will increase RV replication.. While siRNA to PTEN was only successful in a 50% knock down of the protein, this appeared to be sufficient to lead to a, marked decrease in RV43 titre (Figure 6.3.5).

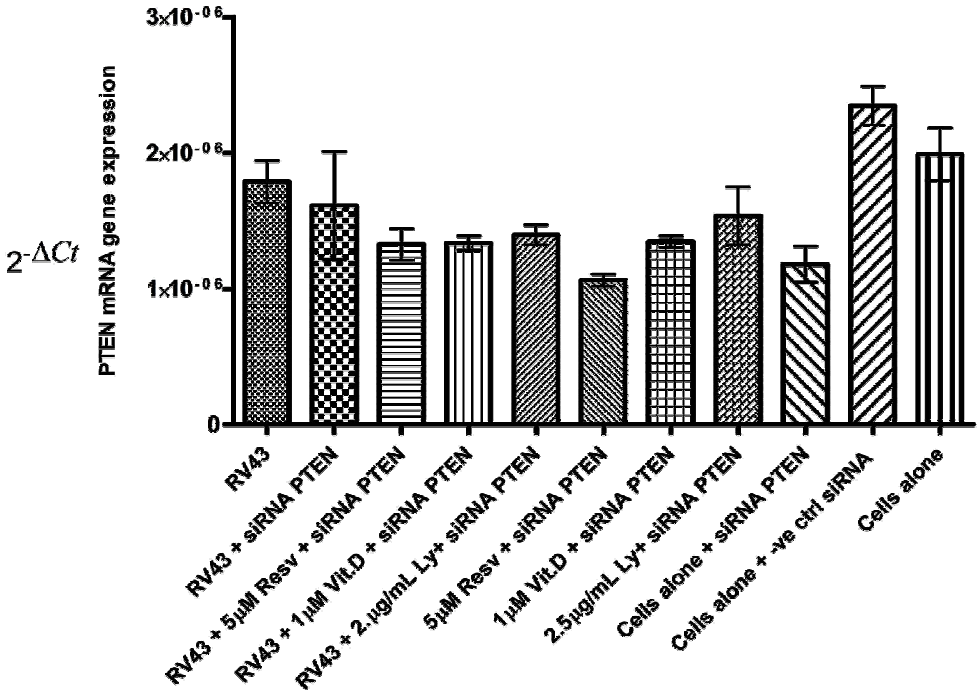
Effect of silencing PTEN on Calu-3 cells viability:

Figure 6.3.6 (a), (b), (c) shows cellular viability of Calu-3 cells were transfected with siRNA to PTEN. Transfection of Calu-3 cells with siRNA against PTEN mRNA resulted in a large decrease in viability (about 20%), with a large increase in necrotic (18%), and very small increase in apoptosis (1%) of apoptotic Calu-3 cells. The effect of siRNA PTEN on causing Calu-3 cells death (necrotic) could provides an explanation on why knocked down of PTEN decreases viral infectivity in the cells.



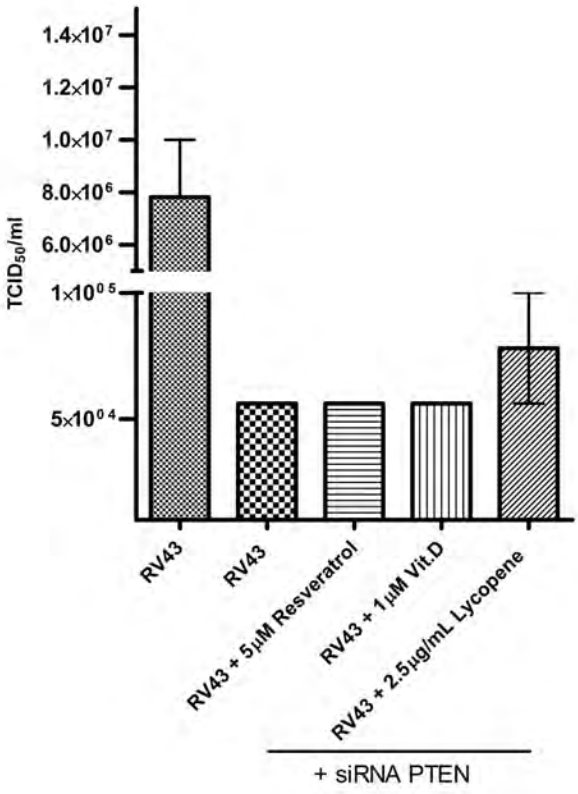
Calu-3 cells were transfected with either 60nM siRNA against mRNA PTEN or 60nM siRNA negative control for 48 hours of incubation times as indicated in material and methods. RNA was harvested and RT-qPCR was run as indicated in Materials and Methods to determine the knock down of mRNA PTEN. Comparative Ct value ($2^{-\Delta\Delta Ct}$) related to an internal control gene (18S rRNA) was then generated to assess the knock down of mRNA PTEN. Data represent means ± SEM (n= 4).

Figure 6.3.3. Percentage (%) of PTEN mRNA knocked down in Calu-3 cells



Calu-3 cells were transfected with either either 60nM siRNA against mRNA PTEN or 60nM siRNA negative control for 48 hours before RV infection. Antioxidant (resveratrol, vitamin D, or lycopene) was administered to the cells as indicated in Materials and Methods prior to RV infection. siRNA containing media was then added during 48 hours of viral infection. RNA was harvested and RT-qPCR was run as indicated in Materials and Methods, $2^{-\Delta Ct}$ value was generated to determine PTEN mRNA gene expression normalized to 18S rRNA. Data represent means ± SEM from 2 experiments.

Figure 6.3.4. PTEN mRNA gene expression in RV43 infected-antioxidant enriched-Calu-3 cells



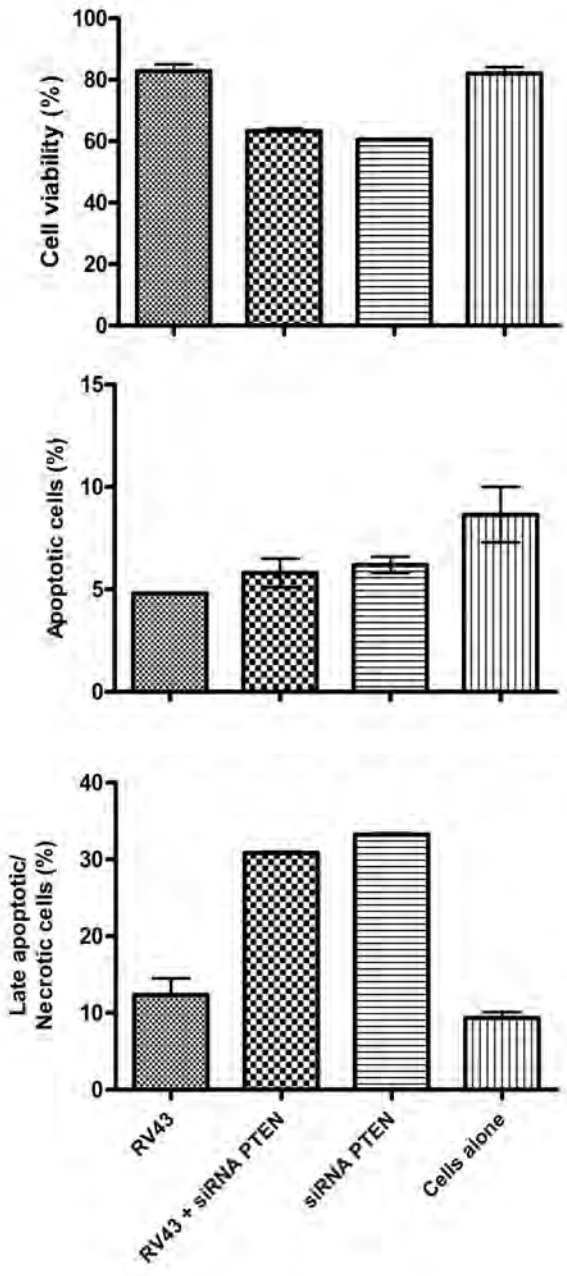
Calu-3 cells were transfected with 60nM siRNA against mRNA PTEN for 48 hours before RV infection. Antioxidant (resveratrol, vitamin D, or lycopene) was administered to the cells as indicated in Materials and Methods prior to RV infection, siRNA containing media was then added during 48 hours of viral infection. Viral titer in the supernatant of infected cells were determined at 48 hours of infection by employing $TCID_{50}$/ml assay using RD-ICAM1 cells as described in Chapter 2. Data represent means ± SEM from 2 experiments.

Figure 6.3.5. PTEN mRNA knocked down decreases the rate of viral titer in Calu-3 cells

(a)

(b)

(c)



Calu-3 cells were transfected with 60nM siRNA against mRNA PTEN for 48 hours before RV infection. Antioxidant (resveratrol, vitamin D, or lycopene) was administered to the cells as indicated in Materials and Methods prior to RV infection, siRNA containing media was then added during 48 hours of viral infection. (a) Viable, (b) apoptotic, (c) necrotic (late apoptotic) cells were assessed by Annexin V-PE and 7-AAD staining and analysed by flow cytometry at 48 hours of infection. Data represent means ± SEM from 2 experiments.

Figure 6.3.6. siRNA against PTEN markedly affects Calu-3 cells viability

6.4 Discussion

The preliminary experiment (by knocked down GAPDH mRNA) has demonstrated the use of Calu-3 cells for the silencing experiment of PTEN mRNA. Others have also shown that Calu-3 cells can be used as a model in silencing experiment [413, 414].

Using siRNA to PTEN we achieved 50% mRNA knock down, however this resulted in significant cellular toxicity. While we demonstrated that siRNA to PTEN was associated with reduced RV43 replication, we cannot determine that this effect was not simply due to the reduced cellular viability that resulted. PTEN functions to negatively regulate PI3-kinase. The loss of PTEN results in an increased concentration of PtdIns(3,4,5)$P_{3,}$ and hence increases Akt activation, preventing apoptosis. Activated AKT is well established survival factor. As explained in the earlier chapters (Chapter 2 and Chapter 5), the newly synthesized RV virion rely upon the use of host cells machinery (ribosome) to produce viral polyproteins (viral capsid) and viral replication enzymes (copying of viral genome). It is believed that the cells are induced to undergone apoptosis at earlier time point (we found marked increment number of cells undergone late apoptotic/necrotic induced by siRNA to PTEN at the end of the study), result in cellular protein synthesis being shut off and cause significant effect for the invaded virus to use the machinery for its replication. Hence, could explained why PTEN silencing in Calu-3 cells prior to RV infection leads to marked decrease of viral titer at the end of study. The impairment of RV entry by siRNA to PTEN still could be determined if administration of siRNA PTEN to the cells is not causing toxicity at earlier time point, and simultaneously is able to silenced the gene (PTEN).

Experimental design (times needed for the antioxidants pre-treatment) was based on the previous chapter. Hence, justify why the antioxidant compounds required

different incubation time points prior to RV infection. Previously, it was shown that all antioxidants were given at specific times prior to RV infection, and resulted in decreased PI3-kinase activation, hence could provide an explanation of anti-RV replication demonstrated by resveratrol, lycopene and vitamin D. Based on that observation, in this current chapter we would like to investigate the direct effect of all the antioxidants (except zinc) on the RV-induced PI3-kinase activation. However, we do not aim to find which is the best antioxidant compound; rather we aim to investigate the direct effect of each antioxidant compound on RV-induced PI3-kinase activation.

The aim to verify that antioxidant does inhibits activation of PI3-kinase during viral internalization could not be achieved due to the observed toxicity effect on Calu-3 cells caused by knocked down (about 60% is remain) of PTEN mRNA. Therefore an alternative approach (such as by targeting other gene that also responsible in regulating PI3-kinase, or any approach that could enhance the expression of PI3-kinase) could be considered and used to achieve this aim. However, the study does provides useful findings in term of gaining information that Calu-3 cells can be used in silencing experiment study and that PTEN maintenance of cellular viability.

6.5 Conclusion

In summary, PTEN plays an important role in cells viability, and even partial siRNA knocked down resulted in reduced cellular viability.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

7.0 General Discussion

Airway inflammation is central to asthma pathogenesis. The current thesis examined the effects of antioxidant compounds on the inflammatory responses induced by RV infection in airway epithelial cells. It was found that, antioxidant compounds do not possess anti-inflammatory effects against RV-induced inflammation. However, antioxidants were shown to possess other properties that have a major impact on the response to RV infection. Antioxidants (resveratrol, lycopene, zinc and vitamin D) were investigated in this thesis and were found to decrease RV replication, by limiting RV endocytosis. This is a particularly novel and interesting finding. An enzyme, PI3-kinase, was demonstrated to play a significant role during RV internalisation and cells enriched with antioxidant compounds (resveratrol, vitamin D and lycopene, but not zinc) were found to inhibit viral replication due to their inhibitory effect on this enzyme.

It was noted that various vehicles (0.1% DMSO, water, 0.5% THF, 0.1% ethanol) are used as a carrier for resveratrol, lycopene, zinc and vitamin D. In agreement with that, others also have used the mentioned vehicles at similar concentration to deliver many compound of interest against many viral infections. The vehicles that we have used in the study are within the range neither causing toxicity nor protecting the cells against viral infection. For example, it was shown that DMSO at a concentration of 0.1% does not cause any effect against RSV and RV infection in airway epithelial cells [415, 416]. Similarly the use of THF at a concentration of 0.5% revealed non-significant effects in the course of RV infection [50]. Ethanol when used at a concentration of 0.5% has shown no significant effect against herpes simplex virus infection [417].

Resveratrol was among the antioxidant compounds studied in this thesis, which has been widely studied in regard to viral infection on human diseases. Notably, resveratrol was found to exhibit antiviral effect in various *in vitro* studies setting

against influenza A [28] human immunodeficiency virus-1[418] and human cytomegalovirus [419], and *in vivo* studies of herpes simplex virus-1, herpes simplex virus-2 [420-422]. These studies have indicated that, resveratrol possesses antiviral activities against broad spectrum of viruses including DNA and RNA viruses, hence suggested the compound selectively targeted host cells rather that the virus itself. Evers et al. [419] has demonstrated that resveratrol antiviral activity against human cytomegalovirus was due to the inhibition of the viral attachment and entry which shown the PI3-kinase involvement. Therefore, finding from our study on resveratrol (and other antioxidants) on RV (inhibition on PI3-kinase) was in fact in agreement with study done by Evers et al [419].

However, it was also reported that resveratrol significantly enhanced replication of hepatitis C virus [423]. The observation demonstrated by Nakamura et al. [423] on effect of resveratrol against replication of hepatitis C virus was contrary to the numerous anti-viral activities exhibited by the compound. We believed that, the methodology used by the author to investigate role of resveratrol on hepatitis C virus was responsible to the contrary results. Nakamura et al. [423] deal with a virus (hepatitis C virus) which is known extremely elusive to culture *in vitro*, and hence provide the reason why the authors used hepatits C virus replicon cell culture system to investigate anti viral activity of resveratrol. In this system, specifically the replication stage of the virus is investigated [424], rather than investigating into the whole stages of viral replication cycle. In our study, we found that the antioxidant compounds used in the study did not effecting RV replication (RNA replication). It is justifiable that compound possessing antioxidant activity unanimously targeted host cells, leading to altering the favorable intracellular environment needed for effective viral replication.

Lack of anti-viral agents and vaccinations against RV resulting in the evolution of numerous RV serotypes prompted a surge in antiviral research to tackle both the economical and medicinal problems caused by RV infection. This thesis has examined the roles of four antioxidant compounds on RV-induced airway inflammation in BEC, which is heavily implicated in the inception and exacerbations of asthma.

Oxidative stress has been strongly implicated in the pathogenesis of airway diseases such as asthma and COPD. For example, Wood et al [116, 127, 425], Kirkham and Rahman [337], Riedl and Nel [426] and Nadeem et al. [427] have demonstrated the involvement of oxidative stress in asthma, and hence antioxidants have been suggested as a potential therapeutic strategy [129, 428, 429]. Many mechanistic pathways have been proposed. Among the pathways include up-regulation of the transcription factor Nrf2, which responsible for expressing molecules with antioxidant and anti-inflammatory actions. Additionally, preventing oxidative stress will have beneficial effects on the activation of the pro-inflammatory transcription factor, NF-κB.

The "new" role of antioxidant as a promising candidate in many viral pathogenesis is well explained. RV infection *in vitro* was demonstrated to induce oxidative stress in the infected cells as was reviewed earlier (Chapter 1, Section 1.7). In addition to RV infection, other lethal viruses such as influenza and human immunodeficiency virus were also shown to induce oxidative stress *in vivo* [430, 431]. Therefore, having common feature as an oxidative stress agent; and it was demonstrated that oxidative stress is involved in the pathogenesis of the human diseases due to viral infection, provides a "new" role of antioxidant compound.

Considering the inconclusive findings on the beneficial effects of antioxidants in asthma in human/clinical trials (as reviewed by us and others), we have utilised *in vitro* studies to understand the potential mechanisms of antioxidant action. This thesis has

demonstrated that the antioxidants tested in these study are worthy of investigation in a clinical trial studying their protective effect against viral respiratory infection. This may establish a role for these nutrients in preventing or managing people with asthma who are at high risk of experiencing worsening of the disease in response to RV infection.

7.1 Thesis limitations

This thesis employed Calu-3 cells, a cell line originating from the upper respiratory tract of the human airway, as a working model to study the roles of antioxidant compounds on viral infection. Though many other studies have successfully used this cells to study respiratory viral infections, a few limitations of this cells are noticed. ICAM-1 protein expression, activation of Nf-κB (investigated but data not shown), cleavage of eIF4GI, oxidative stress induced by H_2O_2 (investigated but data not shown), are amongst the processes that cannot be fully investigated in this cell line, due to constitutive cellular activation of Calu-3 cells. Those markers have been investigated by others (using different available cell lines), and have been shown to be modulated by RV-infection. For example, RV-16 infection has been shown to induce ICAM-1 up-regulation in primary bronchial and A549 respiratory epithelial cells [18], RV-2 infection has been shown to successfully cleave eIF4GI protein in HeLa cells [110] and RV-14 infection has been shown to induce activation of Nf-κB in A549 cells [12, 15]. Hence the use of alternate cell lines would be necessary to explore the role of antioxidant compounds on these pathways following RV infection.

Time of treatment and treatment doses used for various experiments are critical and may have played a role in cells's metabolic regulation that could affect the observed findings in the course of RV infection on airway epithelial cells. A number of pathways

were involved during RV infection and we have discussed those pathways as described in the thesis, may partly be responsible to the observed findings.

7.2 Future directions

An important future direction is examination of the benefit of antioxidant combinations, to determine whether additive or synergistic effects can be achieved. The antioxidant compounds that were used in this study may have different properties due to differences in cellular uptake and interaction with cell surface receptors/proteins. Many studies have shown synergistic effects of compounds/molecules in association with disease (pathogenesis) prevention or as adjuvant treatment.

It was noted in the thesis that RV infection causes oxidative stress. This condition was shown previously in different types of airway epithelial cells, however this was not investigated or demonstrated directly by us in this thesis (which used Calu-3 cells). Hence, it is worthwhile to explore or investigate, the indicators used to measure oxidative stress including cytochrome C and ROS induced by RV on Calu-3 cells, to strengthen the observed effects of antioxidant compounds on RV infection.

The results presented in this thesis would be strengthened by conducting the experiments using primary airway epithelial cells. Among the challenges will be the development of methodology to successfully deliver the antioxidant compounds into cells without causing toxicity. Determination of the appropriate concentrations and carriers for delivery of antioxidants to the cells and understanding the kinetics of the antioxidants once administered to the cells while maintaining a low toxicity level will be needed. We have observed that different airway epithelial cell lines (for example Beas-2B cells) acts differently while incubated with the antioxidant (for example resveratrol). We have found that it is very difficult to demonstrate the bioavailability of

resveratrol in this type of cells. We believed that this was due to the differences in the cells metabolic regulation. A similar condition is expected to occur if we wanted to extend the observation using primary airway epithelial cells. The bioavailability of the antioxidant using primary airway epithelial cells could be design by using a different carrier to the cells, for instance using a liposome. The concentration of the antioxidant compound, and together with the carrier (liposome) should be carefully determined, to avoid causing toxicity to the cells while successfully deliver the antioxidant.

Another important avenue for further work in this area would be to compliment the *ex vivo in vitro* findings by conducting an *in vivo* study. Antioxidant delivery may be oral, by nasal spray, nebulizer or through intravenous administration. The delivery of antioxidants in an oral form is very relevant and achievable in human subjects. The delivery of antioxidants (as part of the human diet) via the digestive tract (from mouth to stomach and continue to intestinal lumen) offers a few challenges. Antioxidants might not be well absorbed possibly due to food matrix effects and the gastrointestinal tract needs to be conducive for the absorption of the nutrient. For example, a lipid soluble or organic antioxidants including lycopene, resveratrol and vitamin D are able to be absorbed from the intestinal lumen after incorporating in the micelles. Additionally, it is quite difficult to ascertain the actual concentration of antioxidant at the intended destination, in this case, the airway epithelium.

The administration of antioxidants can also be done via nebulizer/nasal spray. However, it has been shown that when a nebulizer/nasal spray containing GSH (one of the potent scavengers of oxidant substances) was delivered to mild asthmatic subjects, it caused major airway narrowing, induced cough or breathlessness [432]. If these side effects can be resolved, this delivery approach can be considered as very promising

because it allows the antioxidant to be delivered directly to the site of interest (airway epithelium).

Once the delivery route has been identified, the next step would be to demonstrate that the uptake of antioxidants has been achieved. This can be confirmed in several ways, including measurement of antioxidant levels in plasma, urine or tissue. Alternatively, the level of oxidative stress (for example lipid peroxidation, LDL oxidation or DNA damage) can be measured. This is considered an indirect, yet functional, demonstration of the successful delivery of antioxidants.

7.3 Conclusions

The antioxidant and anti-inflammatory properties of the compounds used in this study have been demonstrated previously and we have extended these findings by examining the use of these nutrients in airway diseases/disorders associated with RV infection. We found that not all the antioxidants used in this study exhibit anti-inflammatory properties in the system/model tested. The major finding from this thesis is that all the antioxidants (resveratrol, zinc, vitamin D and lycopene) have anti-rhinoviral activity. We explored the mechanism by which this occurs and have shown that the effect is due to the suppression of the activation of PI3-kinase, associated with RV internalization. Resveratrol, lycopene, vitamin D and zinc, at physiologically relevant concentrations, were demonstrated to have beneficial roles in limiting RV replication in Calu-3 cells. Preventing or ameliorating RV replication will hopefully bring significant impact toward managing asthmatic patients who are at high risk of suffering RV-induced asthma exacerbations.

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