BIOFUNCTIONALITY OF CAFFEIC ACID PHENETHYL ESTER IN THE CONTEXT OF INFLAMMATORY BOWEL DISEASE

JOB OKOKO MAPESA

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DECLARATION AND RECOMMENDATION

This thesis is my original work and has not been presented in this or any other university

Job Okoko Mapesa

Signed: ……..

Date: …….

This thesis has been submitted with our approval as university supervisors:

Prof. D Haller

Technical University Munich, Germany

Signed: ……..

Date: …….

Prof. S.M Mahungu

Egerton University, Njoro, Kenya

Signed: ……..

Date: …….

DEDICATION

To my parents Merab and Wycliffe Anyanje Mapesa, their own commitment to education still inspires me to attempt the same.

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ABSTRACT

Inflammatory bowel disease (IBD) is defined as an inflammatory disorder of the gastrointestinal tract of unknown aetiology that presents in distinct clinical manifestations including Crohn's disease (CD) and ulcerative colitis (UC). Although details concerning the etiology of IBD remain ambiguous, IBD is specifically seen as a representation of abnormal immune responses to microbes in the gut lumen, especially in individuals with a genetic predisposition to develop persistent mucosal inflammation. Among the many factors implicated, activation of the NF-κB pathway is thought to be involved in its pathogenesis in addition to increased production of pro-inflammatory cytokines by lymphocytes and epithelial cells, suggesting that anti-inflammatory compounds may ameliorate this chronic pathological process. Caffeic acid phenethyl ester (CAPE), a bioactive polyphenol from honeybee propolis, has been shown to have anti-inflammatory and cytoprotective effects *in vitro* and *in vivo*. However, this bioactive potential still requires more scientific research that can lead to utilization of CAPE in functional food development. To improve our understanding in the regulatory mechanisms and function of CAPE in disease control, selected *in vitro* cell culture systems including epithelial (intestinal) and immune cells were used to investigate the structure-activity relationship of CAPE with regard to the down-regulation of inflammatory responses and activation of the oxidative stress elements via NF-κB and Nrf2/Keap1 signalling. CAPE significantly inhibited TNF-induced IP-10 expression in intestinal epithelial cells. Using various analogues, it is demonstrated that substitution of catechol hydroxyl groups and addition of one extra hydroxyl group on ring B reversed the functional activity of CAPE to inhibit IP-10 production. The anti-inflammatory potential of CAPE was confirmed in ileal tissue explants and embryonic fibroblasts derived from $TNF^{\Delta ARE/+}$ mice. Interestingly, CAPE inhibited both TNF- and LPS-induced IP-10 production in a dose-dependent manner, independent of p38 MAPK, HO-1 and Nrf2 signalling pathways. In addition it has been found that CAPE did not inhibit TNF-induced IκB phosphorylation/degradation or nuclear translocation of p65/RelA, but targeted downstream signalling events at the level of transcription factor recruitment to the gene promoter as well as IRF1 signalling. Furthermore CAPE protected epithelial cells from oxidative stress through induction of HO-1 via Nrf2 and c-Jun signalling. Although feeding experiments in $TNF^{\Delta ARE/+}$ mice failed to prevent TNF-driven Crohn's disease ileitis, the anti-inflammatory potential of CAPE was confirmed in ileal tissue explants and embryonic fibroblasts derived

from *TNF*$^{\Delta ARE/+}$ mice. This study reveals the structure-activity effects; anti-inflammatory and anti-oxidative potential of CAPE in the context of intestinal inflammation.

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LIST OF ABBREVIATIONS

ActD:	Actinomycin D
ARE:	Antioxidant response element
EGCG:	Epigallocatechin gallate
CAPE:	Caffeic acid phenethyl ester
CARD:	caspase recruitment domain
ChIP:	Chromatin immunoprecipitation
CHOP:	C/EBP homologous protein
CHX:	Cycloheximide
CXCR3:	chemokine (C-X-C motif) receptor 3
CD:	Crohn's disease
DC:	Dendritic cells
EGF:	Epidermal growth factor
ERK:	Extra-cellular signal regulated kinase
GALT:	gut-associated lymphoid tissue
GSH:	Glutathione
HO1:	Heme oxygenase 1
IBD:	Inflammatory bowel disease
IEC:	intestinal epithelial cells
IEL:	intra-epithelial lymphocytes
IFN-γ:	Interferon gamma
IKK:	I-κB kinase
IB:	Immunoblotting
IL:	interleukin
IP:	Immunoprecipitation
IP-10:	Interferon gamma-inducible 10 kDa protein
IRAK:	IL-1-R associated protein kinase
IRF1:	Interferon regulatory factor 1
I-κB-α:	Inhibitory kappa B alpha
KEAP1:	Kelch like-ECH-associated protein 1
LPS:	Lipopolysaccharide
MALT:	mucosa associated lymphoid tissue
MAPK:	Mitogen-activated protein kinase

MIP-2:	Macrophage inflammatory protein 2
Myd88:	Myeloid differentiation primary-response gene 88
NAC:	N-acetylcysteine
NADPH:	nicotinamide adenine dinucleotide phosphate
NF-κB:	Nuclear factor kappa B
NK-T:	Natural killer T cells
NLR:	NOD-like receptors
NOD:	Nucleotide-binding oligomerization domain
NQO1:	NAD(P)H quinone oxidoreductase 1
NRF2:	Nuclear factor-erythroid 2 p45-related factor 2
PI3K:	Phosphatidylinositol 3-kinase
PPAR:	Peroxisome proliferator-activated receptor
PRR:	Pattern recognition receptors
SAPK/JNK:	Stress-activated protein kinase/c-Jun NH2-terminal kinase
SEAP:	Secreted alkaline phosphatase
Rel:	Proteins coded by rel oncogenes
RPL30:	Ribosomal protein L30
TCR:	T-cell receptor
TGF:	transforming growth factor
Th1/2:	T helper 1/2 cells
TLR:	Toll-like receptor
TNF:	Tumour necrosis factor
$TNF^{\Delta ARE}$:	TNF-delta Adenine-Uracil rich element
UC:	Ulcerative colitis
BMDC:	Bone marrow-derived dendritic cells
DMSO	Dimethyl Sulfoxide
BSA	Bovine Serum Albumin
DMEM	Dulbecco’s Modified Eagle Medium

CHAPTER ONE
INTRODUCTION

1.1 Background

Caffeic Acid Phenethyl Ester (CAPE) was first isolated as a bioactive polyphenolic compound from honeybee propolis, and later prepared synthetically to better understand the structural and physiological impact on the immune system. Although it is a non-flavonoid, it shares some structural similarities with flavonoids– a group of polyphenols derived from plant secondary metabolites that have been extensively researched on as a source of potential therapeutic compounds. In fact, we have since come to learn that CAPE is involved in a number of important biological processes, including pathways that regulate cell division and development, inflammation and apoptosis, partly via modulation of NF-κB mediated gene expressions [Abdel-Latif and others, 2005, Nagasaka and others, 2007, Natarajan and others, 1996, Onori and others, 2009, Orban and others, 2000] and the electrophilic/oxidative stress pathway. NF-κB has been implicated in serious and persistent inflammatory diseases, including inflammatory bowel diseases, osteoarthritis, rheumatoid arthritis, atherosclerosis and psoriasis[Baldwin, 2001, Curran and others, 2001, Gilmore, 2008, Yamamoto and Gaynor, 2001]. Inhibition of NF-κB signalling by CAPE has generated interest in the field of therapeutic nutrition [Orban and others, 2000] whereby a quick PubMed search reveals close to 400 research articles.

The inducible regulation of numerous NF-κB responsive genes is through many stimuli including but not limited to Tumour necrosis factor (TNF), Interleukin 1 (IL-1) and Lipopolysaccharide (LPS) [Gilmore and others, 2002, Gilmore, 1999, Hayden and others, 2006, Hoffmann and others, 2003, Ting and Endy, 2002] that results in distinct and diverse signal transduction pathways. The diversity of the physiological roles of NF-κB raises some interesting questions about how a set of signal transduction molecules both upstream and downstream of NF-κB pathway are regulated by CAPE. Besides, NF-κB plays an important role in the immune system, regulating the expression of inducer and effector proteins at many points in different disease pathologies and as a consequence our understanding of NF-κB signalling is based on studies derived from these pathways (Figure 1). However, the increasing number of disease conditions and disorders in which CAPE deregulates NF-κB does not show target selectivity or specificity. In fact there is no clear evidence that CAPE indeed, has a unique mechanism of action. This is in light of some ambiguous observations made in studies geared towards understanding the regulatory mechanisms and functionality

of CAPE in disease control [Lee and others, 2008, Natarajan and others, 1996, Onori and others, 2009, Toyoda and others, 2009, Wang and others, 2009]. Evidence shows that dietary changes and food preparation methods can influence intestinal microflora, however, the relationship between the pathogenic (*Mycobacterium avium*, *Escherichia coli* and *Campylobacter jejuni)* and commensal (*Streptococcus mutans*, *Lactococcus lactis* and *Lactobacillus acidophilus*) microbial communities in the gut and IBD is not well described.

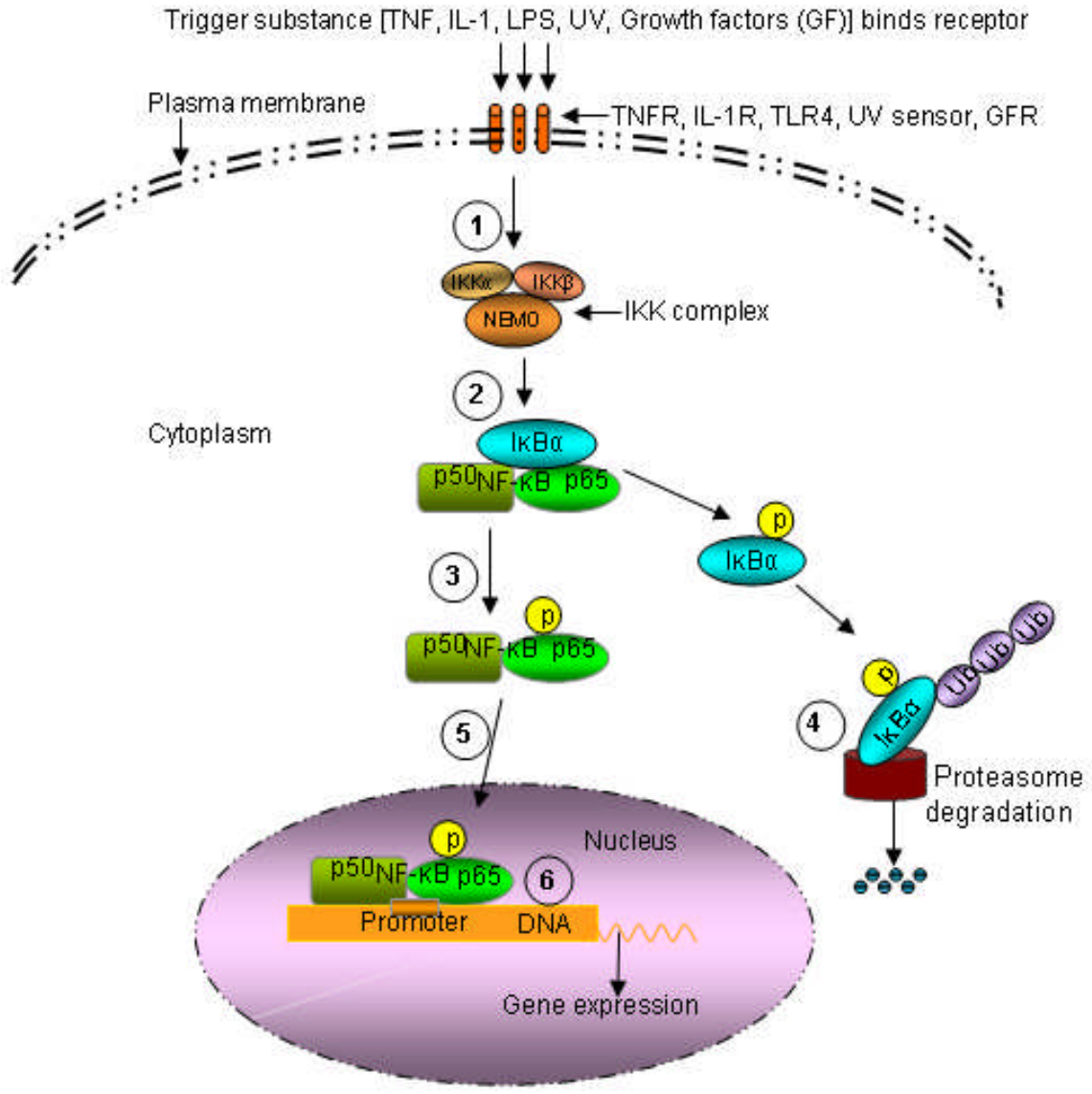

Figure 1. NF-KB Signalling Pathway Target Inhibition Points

TLR4, TNFR and Antigen receptors are activated in the presence of ligands (LPS, TNF, Antigens) leading to recruitment of adaptor (TRAF and RIP) proteins that subsequently stimulates the IKK complex. (1) Activated IKK results in the phosphorylation of IκBα and

release of NF-κB. (3) Free and unbound NF-κB is phosphorylated on the p65 subunit either in the cytoplasm by IKK or PKAc. (4) Phosphorylated IκBα is marked for degradation by the ubiquitin- proteasome system. (5) NF-κB translocates into the nucleus (free NF-κB can also be phosphorylated in the nucleus by MSK1/2 or RSK1). (6) Phosphorylation of p65 optimizes NF-κB facilitating the recruitment of various transcriptional cofactors (CBP/p300), acetylation of histone and p65, promoting target gene transcription. TLR4, toll like receptor; TNFR, TNF Receptor; TRAF, TNF Receptor Associated Factor; Rip, Receptor Interacting Protein; IKK, Inhibitor of κB Kinase; PKAc, Protein Kinase A catalytic subunit; MSK1/2, Mitogen- and Stress-Activated Protein Kinases 1/2; RSK1, Ribosomal S6 Kinase 1; CBP, Creb- Binding Protein; ub, Ubiquitin; p, Phosphorylated. For detailed schematics on NF-κB signalling [Hayden and others, 2006, Perkins, 2007].

Improved understanding of the importance of biologically active dietary food components of plant, animal, and microbial origin, has significantly changed the role of diet in health. The development of functional foods has directly been linked to the increasing knowledge in food and nutrition science that has advanced beyond the management and treatment of disease deficiency syndromes using micronutrients to reduction of disease risk and health promotion. It is common knowledge that foods can no longer be evaluated only in terms of macro- and Micro-nutrient content alone. Biological activity and physiological information is equally important, especially health promoting capacity. Although it is obvious that all foods have some physiological effects, it is desirable that functional foods when consumed as part of a regular diet should have potential health benefits. The health benefits may result through increased consumption of substances already part of a diet or from adding new substances to the diet. However, the bioactive potential of CAPE still requires more scientific research that can lead to its utilization in functional food development

In this study attention is drawn to aspects of NF-κB and HO1 regulation and function that reflect the broad physiological significance of CAPE with a focus on areas I feel have not been given much attention. Using selected in vitro cell culture systems that include both epithelial (intestinal) and immune cells the structure-activity-relationship of CAPE with regard to the down-regulation of inflammatory responses and activation of the oxidative stress elements via NF-κB and Nrf2/Keap1 signalling pathways were investigated respectively. Various analogues were synthesized using CAPE as the lead compound to explore the functional consequences of structural modifications with regard to expression of Interferon gamma-inducible 10 kDa protein (IP-10), a chemokine that is thought to be highly

up-regulated in Inflammatory Bowel Disease (IBD). In addition, new insights into the mechanistic significance of CAPE in the regulation of NF-κB activity under inflammatory conditions are provided and more importantly, the physiological relevance of *in vitro* findings of CAPE was tested using the $TNF^{\Delta ARE/+}$ mouse model of ileitis.

1.2 Statement of the problem

Given the cellular and physiological effects exhibited by most polyphenols in immunological, inflammatory and oxidative responses including TNF, NF-κB and HO1 signalling, there is need to determine the mechanisms of action of CAPE under conditions of chronic inflammation in the gut.

1.3 Broad objective

To establish the bio-functional effects of CAPE and its analogues in intestinal epithelial and immune cells in the context of inflammatory bowel disease.

Specific objectives

1. To determine the function and structure-activity relationship of CAPE and related analogues in intestinal epithelial and immune cells.
2. To determine the mechanisms of action of CAPE in the NF-κB signalling pathway in Mode-K cells.
3. To identify the molecular mechanisms governing Nrf2/Keap1 system in the presence of CAPE.
4. To establish the physiological relevance and function of CAPE in a cytokine environment i.e. IP-10, IL-6, MIP-2 and TNF.
5. To determine the biological effects of CAPE in the $TNF^{\Delta ARE/+}$ mouse model of experimental ileitis.

1.4 Hypothesis

1. There is no significant difference in CAPE and its analogues in function and structure-activity relationship.
2. CAPE has no influence on the NF-κB signalling pathway.
3. CAPE has no influence on the Nrf2/Keap1 system.
4. CAPE has no physiological relevance a cytokine environment.
5. CAPE has no biological effect in the $TNF^{\Delta ARE/+}$ mouse model of experimental ileitis.

1.5 Justification

Polyphenols are increasingly being recognized as the major active components in dietary fruits and vegetables. Structural modification of plant based molecules has led to the development of useful inhibitors in the treatment and management of chronic illnesses. However, the huge and complex structural diversity within the polyphenols family has made it difficult to clearly define the structure/activity relation and by extension the underlying mechanisms of individual compounds. Hence, an understanding of how most of these compounds modulate signal transduction pathways seems to be the best suited approach of analysis. To expand or discover new antagonists of pro-inflammatory proteins, there is need to search for molecules with a realistic potential for disease control.

1.6 Expected output

This study is expected to reveal the structure-activity effects and anti-inflammatory potential of CAPE in the context of IBD. In addition the mechanistic readouts will give more insights on how CAPE can be applied as a therapeutic in nutritional and dietary related disorders. All the research outputs will generate a PhD thesis and at least two publications in referred journals.

CHAPTER TWO
LITERATURE REVIEW

2.1 Functional Foods

Functional foods are defined as foods and food components that provide additional health benefits that may reduce disease risk and/or promote optimal health over and above the conventional physiological functions of providing macronutrients and micronutrients to the body when consumed [Kavanaugh and others, 2007, Kris-Etherton and others, 2008, Lambert and others, 2007]. Functional foods include conventional foods, modified foods (fortified, enriched, or enhanced), medical foods, and foods with specialized dietary needs [Lambert and others, 2010]. The health and nutrition model has changed significantly in the last 20 years. Today, food is not merely viewed as a source of essential nutrients to ensure proper growth and development, but as a route to good health [Nagini, 2008]. Increasing identification of bioactive food constituents is greatly enhancing the chances of producing more functional foods. Hence, foods that naturally contain bioactive molecules may be enhanced to increase the amounts present in the food or fortify alternative foods with low concentrations to provide consumers with a broader food choice [Kavanaugh and others, 2007, Moriarty and others, 2007]. However, poor dietary practices have been linked to the emergence of lifestyle diseases including cardiovascular disease, hypertension, type-2 diabetes, overweight and obesity, as well as osteoporosis. Nonetheless, the ability to identify and quantify the components of interest in functional foods is an important first step in the determination of efficacy.

Nanofoods are foods that have been processed or packaged using nanotechnology, or to which nano-materials have been added. Nanofood has, in fact, been part of food processing for centuries, since many food structures naturally exist at the nanoscale. The benefits of nanofood, for example, include health-promoting additives, improved food safety or new flavor varieties. However, it is well known that the nano-particles with new chemical and physical properties that vary from normal macro particles of the same composition may be physiologically toxic to the body. In addition we also have nutraceuticals which are complementary to functional foods as well. Health Canada officially defines nutraceuticals as “a product isolated or purified from foods, and generally sold in medicinal forms not usually associated with food and demonstrated to have a physiological benefit or provide protection against chronic disease”.

2.2 Influence of polyphenols on cell signalling

Polyphenols are non nutritive phytochemicals with substantial anti-carcinogenic, anti-mutagenic and anti-inflammatory properties. Despite the progress in our understanding of the above mentioned physiological processes, the mechanistic effects of many of the polyphenols have not been elucidated completely. The huge and complex structural diversity within the polyphenolic family has made it difficult to clearly define the structure/activity relationships and by extension the underlying molecular mechanisms of action of individual compounds [Bereswill and others, 2010]. Hence, an understanding of how most of these compounds modulate signal transduction pathways seems to be the best suited approach of analysis. For instance, many molecular alterations associated with polyphenols affect the cellular response pathways involved in growth, differentiation and apoptosis [Bereswill and others, 2010, Rice-Evans and Miller, 1996, Toyoda and others, 2009]. Examples include the MAPK, NF-κB Nrf2 and AP1, which elaborately shape the transcription processes.

Unlike other nutritive components, polyphenols influence several cell signalling pathways and molecules, making them important inflammatory and immune system modulators [Bereswill and others, 2010, Rice-Evans and Miller, 1996, Toyoda and others, 2009]. Most polyphenols interact directly with protein receptors or enzymes triggering intracellular signalling pathways that work in synergy enhancing their potency. For example isoflavones (genistein and daidezein) interact with oestrogen receptors in brain cells leading to changes in gene expression patterns [Carreau and others, 2008, Raffaelli and others, 2002]. There is abundant scientific literature that very strongly implicates polyphenols in improved human health as highlighted in the areas of cardiovascular disease, immune system regulation, nervous system health and longevity, and blood glucose regulation. Some of the more highly publicized polyphenols include quercetin, hesperidin, resveratrol, epigallocatechin-3-gallate ("EGCG"), caffeine, genistein, anthocyanins and isoflavones. However in the recent past there is an emergent of new classes of polyphenols including fisetin, hydroxycinnamic acid, CAPE and galangin. These are just a handful of the thousands of polyphenols that have been isolated. While polyphenols serve important roles in plants as a means of defence from predators, scientific research is now finding that most of these compounds have significant cellular pharmacological effects [Bergman Jungestrom and others, 2007, Dinkova-Kostova, 2002, 2007, Grunberger and others, 1988]. Polyphenols are increasingly being recognized as the major active components in dietary fruits and vegetables. As the individual polyphenols have been studied in detail, and compared to each other, it has become clear that each class of

polyphenols acts slightly distinctly with regard to cellular responses. Dietary polyphenols can exert their effects on intracellular signalling pathways separately, sequentially or synergistically. In fact, many reports show that polyphenolic mixtures have profound effects on the cardiovascular and immune systems [Inoue and others, 2001, Yao and others, 2007].

There is good evidence that polyphenols present in tea, red wine, fruit juices, and olive oil influence the process of cancer. For instance, the phenolic compounds may interact with reactive intermediates of carcinogens and mutagens, modulate activity of important cell cycle progression proteins and influence the expression of many cancer-associated genes [van Erk and others, 2005, van Erk and others, 2005]. Perhaps most notably, the anticancer properties of tea polyphenols have been reported in animal models, human cell lines, as well as in human intervention studies [Inoue and others, 2001]. Polyphenols are ubiquitously found in plants and are therefore consumed in relatively high quantities in the diet. There is a significant amount of information about the potential health effects of several classes of polyphenols particularly flavonoids. In addition, our understandings about the bioavailability of polyphenols and the mechanisms by which they exert such benefits are being determined. These mechanisms are believed to involve interactions with a number of cellular signalling pathways that are essential in the normal cell functions. It is assumed that such interactions appear to modulate these pathways in a way that acts to control various immunological responses relevant to progression chronic inflammation or diseases. Although we have gained a better understanding of how polyphenols interact with cells, there is still much to unravel before the specific cellular targets and mechanisms of action can be established through novel techniques[Rist and others, 2006]. While various lines of pharmacological evidence targeting several protein kinases, cell surface receptors and cytokines in vitro indicate several potential mechanisms of action, a comprehensive proof and conclusive understanding is yet to be established. This relates mainly to noteworthy limitations with regard to current data from in vitro experiments, which aims at determining the mechanistic effect by which polyphenols exert their bioactivity in vivo. In most cases, these polyphenol-bioactivity in vitro data is derived by the direct use of plant or food ‘crude’ extracts or pure isolates, a process that does not take into account the absorption and metabolism processes that polyphenols undergo [Dinkova-Kostova, 2002, Inoue and others, 2001, Russell and others, 2007, Sun-Waterhouse and others, 2009, Yao and others, 2007].

As such, caution should be expressed when interpreting in vitro data linking many polyphenols to activity in vivo and effects against a range of diseases, especially when data of

the resultant metabolic intermediates of the polyphenols is missing. For example, it is difficult to gain meaningful insights into the biological effects exerted by polyphenols via *in vitro* exposure if there is no evidence for the absorption of a particular polyphenol *in vivo*, especially when the cells come from regions which do not have a direct contact with the compounds, with the exception of the gut. Within the gut the polyphenolic compounds may directly interact with cells without having undergone absorption and metabolism. Therefore, this makes it more relevant to perhaps investigate the effects of botanicals on gut-derived cells, although the gut microbiota may metabolize them before they are completely taken up. These and many other limitations significantly hinder the translation of in vitro data in terms of bioactivity of botanicals into meaningful mechanistic understanding of the ultimate in vivo effects.

2.3 Caffeic Acid Phenethyl Ester (CAPE)

CAPE is highly concentrated in propolis, a resin-like compound collected by honeybees from plants (Figure 2). The accumulated propolis is used by bees to seal the hives and comb borders in addition to its protective effect against pathogenic micro-organisms. Propolis has been used in folk medicine since ancient times a cross many cultures in disease management due to the broad spectrum bio-activities it posses [Banskota and others, 2001].

2.3.1Chemical properties

CAPE is chemically known as 3-(3,4-Dihydroxy-phenyl)-acrylic acid phenethyl ester. The Molecular formula of CAPE is $C_{17}H_{16}O_4$ while the Molecular weight is 284.3. It is an off-white powder with a melting point of about 128°C.Due to its hydrophobicity it easily dissolves in organic solvents including DMSO, acetonitrile, acetone and ethanol with a ChemDraw ultra software calculated LogP (CLogP) value of 3.298.The catechol ring is highly photo-oxidative thus CAPE should be protected from light during storage and use.

2.3.2 Biological functions

Although many dietary constituents can increase the risk of cancer development i.e. carcinogens, several lines of compelling evidence from clinical and laboratory studies show a somewhat inverse relationship between regular consumption of nutritional factors and the risk of specific cancer progression. In fact several health-related organisations like WHO have established dietary guidelines to help people reduce cancer risks.

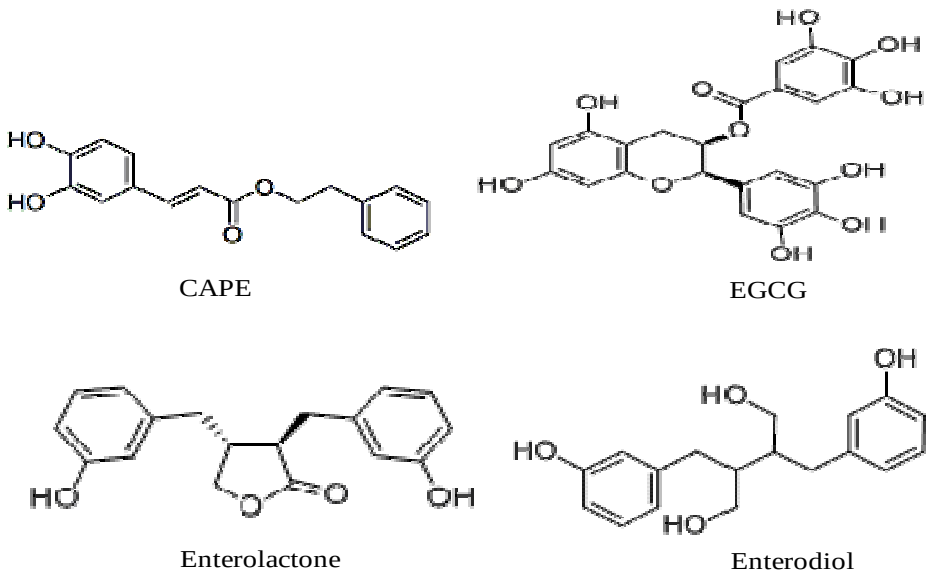

Figure 2. Structures of CAPE, EGCG, Enterolactone and Enterodiol

2.4 Epigallocatechin gallate (EGCG)

Epigallocatechin Gallate (EGCG) is an anti-oxidant polyphenol flavonoid isolated from green tea (Figure 2). Its possible benefit as a nutritional chemopreventive agent for cancer, atherosclerosis, and neurodegenerative diseases is generating increased scientific interest. EGCG has demonstrated chemopreventive and chemotherapeutic actions in cellular and animal models of cancer [Yang and Wang, 2011, Yang and others, 2011]. EGCG treatment of various cell lines indicates that it targets many pathways, including AP1, NF-kappa, B AKT and PI3K. EGCG selectively induces apoptosis in human carcinoma cell lines, inhibits MAPk mediated signalling pathways. EGCG blocks the activation of EGF receptors and HER-2 receptors which are over-expressed or constitutively active in many human malignancies. It interferes with angiogenesis by suppressing VEGF activity, VE-cadherin phosphorylation and matrix metalloproteinase activity. EGCG inhibits telomerase and DNA methyltransferase, two enzymes involved in cancer gene expression and cellular immortality. EGCG's anti-oxidant action protects cells from lipid peroxidation and DNA damage induced by reactive free radicals [Yang and Lambert, 2011, Yang and Wang, 2011].

2.5 Enterolignans

Flaxseed is commercially produced as a source of essential fatty acids as flaxseed oil. On the other hand studies have revealed in the recent past presence of lignans within the flaxseed which have potential medicinal benefits[Fuchs and others, 2007]. Lignans are a group of phytochemicals found in seeds and grains, with flaxseed being the richest source

[Raffaelli and others, 2002]. Polyphenolic lignan in flaxseed is called secoisolariciresinol glucoside (SDG). Within the gut SDG is metabolised and converted by commensal bacteria to [enterodial (ED) and enterolactone (EL) Figure 2] [Clavel and others, 2005]. These enterolignans have been shown to enhance the immune system functions, being effective against many different diseases. Their anti-proliferative or anti-angiogenic properties have been referred to as being responsible for the anti-cancer effects of lignans both *in vivo* and *in vitro* [Bergman Jungestrom and others, 2007, Thompson, 1998]. The health beneficial effects of ED and EL is thought to be phytoestrogenic because they mimic the hormone estrogen, and individuals who consume foods (flaxseed and sesame) rich in lignan have lower incidences of breast and colon cancers. It has been reported that structurally distinct phytoestrogens, including enterolignans exert their estrogenic effects through direct binding and activation of the estrogen receptor. However, the extent to which these enterolignans exert weak estrogenic and/or anti-estrogenic effects in humans is not well described, especially their mechanisms of action on the estrogen receptor. In addition, several lines of evidence show that, although ED and EL are structurally and functionally similar they have overlapping and yet distinct effects on endogenous cellular targets [Carreau and others, 2008].

2.6 Structure–activity relationship

In creating a relation between chemical structures and activity, Structure-activity relationship (SAR) analyses are in principle aimed at developing an understanding of what constitutes a class of molecules that are active. In addition it seeks to determine what gives the compounds ‘relative activity’ and how this class of active compounds can be distinguished from groups of inactive members. Taken together SAR is more of an interaction of different arms of science namely chemistry, biology and mathematics (Figure 3). To best address this scientific association, computational models have been developed. These include the Quantitative Structure activity relationship (QSAR) and proteochemometrics (the most recent one). Both of these computational approaches explore the ligand-based and target-based interactions, depended on the chemical features of the ligand and biological effects exhibited by the target protein. Hence, making it possible to now study the ligands of all known targets and how they relate to each other improving the possibilities of developing multi-target drugs [Doddareddy and others, 2009]. The main assumption in QSAR is that analogous compounds have common mechanistic elements that define the rate-determining step with similar energy requirements for activity (only ligand features are considered and the activity is modelled

based on a single target). In contrast to QSAR, proteochemometrics incorporates both the ligand and target features in the activity model [Doddareddy and others, 2009].

Thus cell-based assays have become popular in elucidation of chemical similarity studies. “Activity *in vitro* does not necessarily mean that the compounds will be active *in vivo*”, at least it is generally agreed that one of the requirements for in vivo activity is that the compounds “must” be able to enter cells and induce cellular responses. So far, CAPE data from many studies have huge variations in the functional effects based on concentration. In this connection it is a bit difficult to establish the minimum inhibitory concentration, since it ranges from 1µM to 100µM. Similarly cell line specificity in relation to dose dependence of CAPE is widely varied, however, this could be due to the assumption that many factors can contribute to the differences in cell type specificity of a compound, including cellular uptake and metabolism or “off target”(compound target is unknown) interactions with other cell components. This necessitates the need to establish the *in vitro* and *in vivo* maximal inhibitory concentration (IC_{50}) values so as to have a comparative reference point.

The fundamental assumption in QSAR is that similar chemicals have sufficiently common mechanistic elements so as to share a common rate-determining step and similar energy requirements for activity. Only ligand features are considered and the activity is modelled, based on a single target. In contrast to QSAR, proteochemometrics incorporates both the ligand and target features in the activity model [Doddareddy and others, 2009]. For instance the NF-κB subunits have been known to be subject to many modifications and regulatory interactions that define their transcriptional activity and target gene specificity [Hayden and others, 2006, Perkins, 2007]. These interactions form a crucial interface with other signalling pathways, thus cell culture based assays can quickly give physiological hints on organic compounds based on structural and molecular similarity studies. It is further assumed that differences in reaction rates will give rise to observed differences in activity or quantitative potency. The key is to identify aspects of structure pertaining to the rate-determining, molecular-triggering event in the mechanism of action for the chemical and biological actions of interest. Hence, the mechanism of action is a guiding concept in determining both the groupings of chemicals suitable for study and the molecular descriptors potentially most relevant to activity. Ultimately, it is the linkage of SAR to mechanism that enables a scientific rationale to be constructed to account for activity variations in existing chemicals. This, in turn, provides the sound scientific basis for predicting the activity of new and untested chemicals.

2.6.1 Structural features

Two independent experiments have shown functional diversity of flavonoids in the inhibition of pro-inflammatory NF-κB transcription factor and other related inflammatory mediators and signalling pathways modelled under conditions of inflammatory bowel disease [Ruiz and others, 2007, Ruiz and Haller, 2006]. The preference of these studies was to a certain extent dependent on the available literature about anti-inflammatory effects where they show that, polyphenols indeed, have structural complexities [Ruiz and Haller, 2006], which can completely result in a loss or gain of function when metabolized [Ruiz and others, 2007]. However, it can also happen that structurally diverse compounds have similar activity [Pukkila-Worley and others, 2009, Ruiz and others, 2007, Weber and others, 2000, Xia and others, 2004]. In line with these views, determining the structure activity relationship of such compounds can be an exciting challenge with the overriding question "Can small changes in molecular structure necessarily be associated with small changes in activity". Such studies can generate more information and contribute to a better understanding of functional group diversity (functional group modification or substitution) and/or stereochemical diversity [exploring stereoisomerism-to create different binding patterns [Kaiser and others, 2008]]. This makes CAPE and the NF-κB signalling pathway a good study model, given that information on how it modifies or interacts with NF-κB subunits, and transcription coactivators and repressors is largely missing. Furthermore, many signalling pathways apart from utilizing the p65/RelA subunit can also use the other NF-κB [p105(p50), p100(p52), c-Rel and RelB] subunits to control the NF-κB pathway functions, probably due to promoter specificity [Perkins, 2007]. Because of the appreciation of the role of NF-κB in human diseases many drugs and natural compounds are used to target different points along the pathway (Figure 1). However, to effectively exploit CAPE it is necessary to fully understand the mechanisms of target selectivity now that it specifically targets the NF-κB but not the AP1, Oct-1 and TFIID transcription factors during cooperative DNA binding in the promoter regions [Natarajan and others, 1996].

2.6.2 Molecular similarity

The activity landscape has been compared to continuous SAR where small changes in molecular structure are associated with small changes in activity. Such SARs are attractive for molecular similarity analysis that is based on similarity –property principle. A discontinuous SAR or rugged activity landscape, however, is populated with molecules with small changes

in structure but large changes in activity. It can also happen that structurally diverse compounds have similar activity, which is the basis of scaffold hopping. Additionally, active regions with wide variations in chemical structure but small variations in biological activity may suggest different binding modes or sites or may reveal the effect of additional mechanisms such as the interaction with membranes that are not typically considered in modelling approaches. A 3D molecular representation is more important compared to 2D because of the occurrence of stereoisomers. Most computed molecular similarity data is in relation to the potency differences in a pair-wise structure activity relationship [Doddareddy and others, 2009, Rice-Evans and others, 1996]. Pair-wise comparisons are computed using Tanimoto coefficients in different platforms.

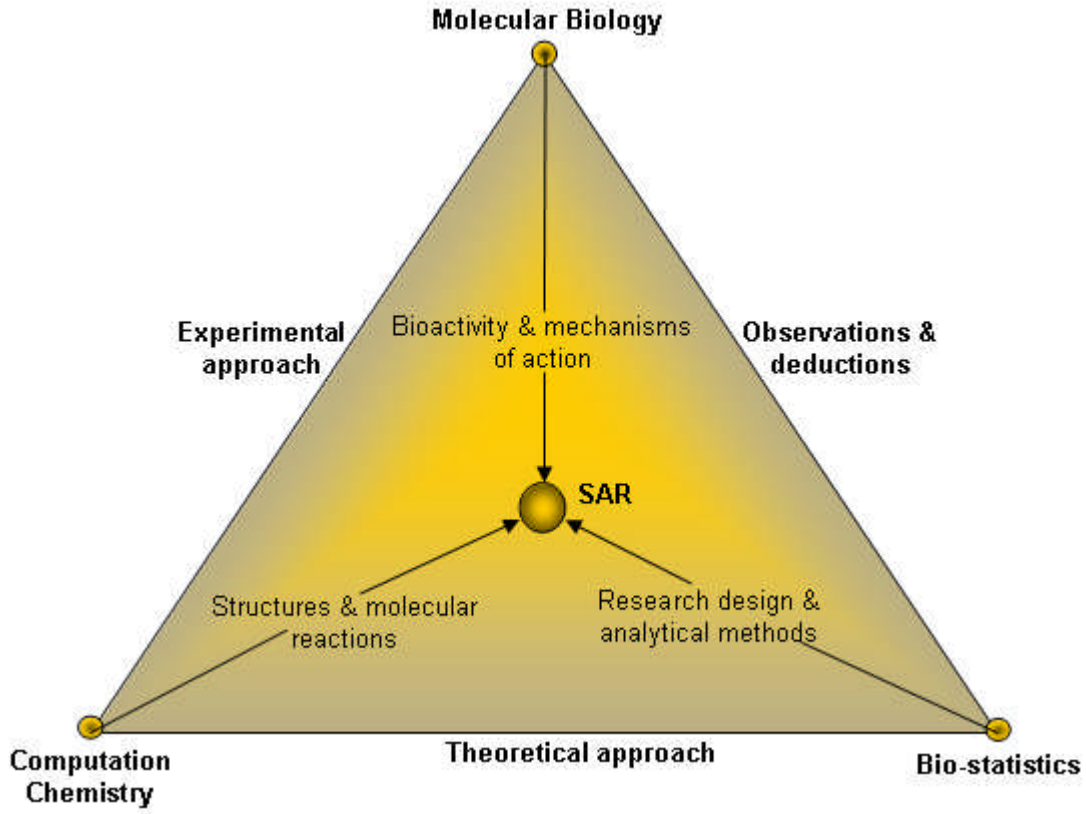

Figure 3. Modelling structure-activity relationship

2.7 Influence of CAPE on cell signalling

2.7.1 CAPE and the NF-κB signalling

The NF-κB family of proteins regulates many physiological processes, including embryonic development, cell proliferation, cellular stress response, apoptosis (programmed cell death), as well as innate and adaptive immune responses that altogether lead to regulation of proinflammatory signals. However, this transcription processes are cell type- and stimuli-dependent. Hence, the NF-κB dependent genes expressed by epithelial cells in response to

LPS are different from those induced in response to TNF [Gilmore and others, 2002, Gilmore, 1999, Gilmore, 2008, Hayden and others, 2006, Hoffmann and others, 2003, Ting and Endy, 2002]. This diversity in responses could indeed, be due to complexity of the signal transduction molecules involved in the NF-κB signalling, especially the resultant downstream effects [Gilmore, 1997, Hayden and others, 2006]. Furthermore, new regulators of NF-κB in inflammation have recently been identified downstream of the NF-κB signalling in the nucleus [Ghosh and Hayden, 2008]. In a 1996 landmark paper [Natarajan and others, 1996], it was reported that CAPE interfered with the DNA binding ability of NF-κB in the nucleus but did not inhibit IκB phosphorylation or its subsequent ubiquitination in human myeloid leukemia cells (U937) upon exposure to various stimuli that included TNF, H_2O_2, okadaic acid, ceramide and Phorbol 12-myristate 13-acetate (PMA). Subsequent studies involving use of CAPE as a therapeutic have therefore been modelled around this major finding [Coban and others, 2008, Ha and others, 2009, Onori and others, 2009, Orban and others, 2000, Wang and others, 2009], indicating that most of the activities modulated by CAPE require NF-κB proteins [Orban and others, 2000, Wang and others, 2009]. However, the molecular basis for the resultant multiple effects of CAPE, is not clearly defined with regard to different stimuli [Baeuerle and Henkel, 1994, Finco and Baldwin, 1995]. Furthermore, several steps are required in addition to IκB degradation for complete activation of NF-κB dependent gene expressions. As a result the ability to effectively target NF-κB for therapeutic intervention requires an understanding of how CAPE selectively regulates this transcriptional process.

The transcriptional activity of NF-κB is regulated in a stepwise manner in the signalling pathways leading to activation of the cellular machinery of inflammation. Regulation of NF-κB is mediated by the Inhibitor of κB (IκB) regulatory proteins within the cytoplasm. In un-stimulated cells, NF-κB dimers are inactive due to their association with the IκB proteins that mask their nuclear localization sequence (NLS), thus retaining them in the cytoplasm, and as a consequence prevent DNA binding in the nucleus. However, in stimulated cells IκB is rapidly phosphorylated by activated IκB kinase (IKK) complex followed by ubiquitination and degradation (Fig. 2). The IKK complex is comprised of IKKα and IKKβ catalytic subunits plus a third regulatory subunit, IKKγ better known as NF-κB essential modifier (NEMO) with newly discovered functions apart from activating NF-κB [Gilmore, 2008, Hayden and Ghosh, 2008]. The critical process of NF-κB activation and IκB phosphorylation has been comprehensively reviewed [Baldwin, 1996, 2001, Ghosh and Hayden, 2008, Gilmore, 1999, Gilmore, 2008, Hayden and Ghosh, 2008, Hayden and others, 2006,

Hoffmann and others, 2003, Karin and Ben-Neriah, 2000]. Typically, NF-κB binds to DNA as homodimers or heterodimers of five possible subunits which include p65 (RelA), c-Rel, RelB, p100/p52, and p105/p50; where p52 and p50 are truncated forms of p100 and p105 respectively. They are characterized by an N-terminal Rel homology domain (RHD), that mediates DNA binding and heterodimerization, with additional sequences important for nuclear localization and IκB inhibitor binding [Baldwin, 1996, Ghosh and Hayden, 2008, Gilmore, 1997, 1999, Hayden and others, 2006, Karin and Ben-Neriah, 2000]. RelA, c-Rel and RelB have a C-terminal transactivation domain (TAD) that is necessary for positive gene regulation, whereas p105/p50 and p100/p52 have a long C-terminal domain with multiple copies of ankyrin repeats that inhibit their activation [Hoffmann and others, 2006]. However, p105/p50 and p100/p52 can be activated when they associate with any TAD containing NF-κB subunit or other transcription coactivator proteins [Hayden and Ghosh, 2004, 2008, Hayden and others, 2006, Hoffmann and others, 2006].

Interestingly, the NF-κB signalling cascade is known to have more than one distinct pathway [Du and others, 2007, Hayden and others, 2006, Perkins, 2007]. These pathways are classified into atypical, canonical or non-canonical depending on the stimuli, proximal adaptor proteins and downstream cascading proteins involved. The most studied and well understood, is the canonical pathway that is triggered through TRAF and RIP complexes in conjunction with TAK1 that proceeds downstream via IKK complex signalling (Figure 1) with about six potential target points for NF-κB inhibition. Therefore, to determine the actual point of CAPE interference in NF-κB signalling given that there are multiple therapeutic targets in the pathway it is worthwhile to follow through the path and determine the functional consequences of CAPE at each point or better still determine the specific mechanisms of action involved. In addition, other mechanisms could be involved in the regulation process altogether, especially posttranslational modifications that affect the ability of NF-κB dimmers to interact with other transcriptional regulatory proteins. These includes the coactivators and repressors that are typically recruited to the promoters and enhancers by interacting with DNA bound transcription factors [Gerritsen and others, 1997, Ghosh and Hayden, 2008, Zhong and others, 2002].

Cell stimulation for instance, by inflammatory cytokines such as TNF and IL-1β, T–cell and B–cell activation signals, microbial and viral products, growth factors and stress inducers results in IκB phosphorylation, polyubiquitination (by the E3 ubiquitin ligase complex) of nearby lysine residues that culminates in degradation by the 26S proteasome. Degradation of

IκB results in the release of phosphorylated NF-κB that rapidly translocates to the nucleus. Once in the nucleus it binds to target DNA (κB sites) elements within the promoters and enhancers inducing expression of NF-κB responsive target genes through recruitment of coactivators and repressors. These include cytokines, chemokines, adhesion molecules, acute phase proteins and anti –microbial peptides as well as genes involved in regulation of apoptosis and cell growth control [Hayden and Ghosh, 2008]. Apart from NF-κB deregulation, information on how CAPE interacts with other elements of the transcription machinery is lacking. Regulation of CBP/p300 can trigger crosstalk between NF-κB and other transcription factors. For instance inactivation of GSK3β results in reduced CBP/ NF-κB binding activity [Martin and others, 2005]. NF-κB translocation into the nucleus also exhibits a feedback mechanism that leads to rapid resynthesis of IκBα proteins that sequester the NF-κB from the nucleus and shuttle back into the cytoplasm bringing the transcription process to an end. However, it has been revealed that IκB proteins are no longer just simple inhibitors of NF-κB, activity. They can also promote formation of otherwise unstable RelB dimmers, thereby influencing the range of NF-κB dimmers within a given cell [Hoffmann and others, 2006] and stabilization of the nuclear and DNA bound dimmers [Ghosh and Hayden, 2008]. In addition, nucleosomal acetylation of RelA, probably via CBP/p300 and other associated histone acetyltransferases has been linked to increased transcription [Chen and Greene, 2004]. Taken together this additional information provides grounds for further investigations that could reveal effect of CAPE on this NF-κB transcriptional binding partners.

2.7.2 Anti-inflammatory effects of CAPE

Studies show that NF-κB is a key activator of both immune and inflammatory responses through its ability to induce expression of various genes [Gilmore, 2008]. In addition to this function of activating inflammatory genes, NF-κB also prevents the destruction of tumour cells [Barkett and Gilmore, 1999]. This latter effect involves the inactivation of cell regulatory proteins that normally recognize cancer cells and marks them for destruction via apoptosis. Considerable progress has been made in understanding the details of the signalling pathways regulating NF-κB activity, specifically with response to the TNF and IL-1 proinflammatory cytokines. Activation of NF-κB occurs as a response to both acute and chronic inflammatory diseases in humans, such as septic shock, arthritis, and Inflammatory bowel disease due to its ability to regulate the promoter regions of a host of genes yielding several pro-inflammatory mediators [Neurath and others, 1996]. Some of the inflammatory immune responsive genes

induced by NF-κB includes the cytokines, IL-1, IL-2, IL-6, IL-12, IL-17, IL-23, and TNF; chemokines, IL-8, RANTES, MIP-2, IP-10, and MCP-1; inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 enzymes; and the adhesion molecules—ICAM1, VCAM1, and E-selectin [Baldwin, 1996, 2001, Ghosh and Hayden, 2008, Gilmore, 1999, Hayden and Ghosh, 2004, 2008, Hayden and others, 2006, Hoffmann and others, 2003, Wang and others, 2007, Yamamoto and Gaynor, 2001]. Whether this activation is the cause of the underlying inflammatory process or develops as a response to curb the processes has been a subject of research for a long time. Nonetheless, many scientific reports are of the view that NF-κB activation is a critical step in the inflammatory cascade and agents that can modulate NF-κB activity have the potential for *in vivo* therapeutic interventions [Atreya and others, 2008, Baldwin, 2001]. Results from some experiments indicate that CAPE significantly modulates some pro-inflammatory signals through inhibition of NF-κB activation [Coban and others, 2008, Ha and others, 2009, Kart and others, 2009, Lee and others, 2008, Natarajan and others, 1996, Wang and others, 2009, Wang and others, 2009].

Efforts are under way to produce drug like compounds capable of interacting with NF-κB to control this inflammatory pathway. For instance Neurath and co-workers [Neurath and others, 1996] reported that administration of the antisense phosphorothioate oligonucleotides to the p65/RelA subunit abrogates experimental colitis in mice. Of much interest, however, is the use of some less high-tech, natural compounds of plant origin, including green tea, herbs, spices, and red wine, which have been shown to modulate the immune system, both *in vitro* and *in vivo*[Holmes-McNary and Baldwin, 2000, Jin and others, 2008, Kunimasa and others, 2009, Lee and others, 2008, Natarajan and others, 1996, Orban and others, 2000, Pukkila-Worley and others, 2009, Ruiz and others, 2007, Ruiz and Haller, 2006, Wang and others, 2007, Xia and others, 2004]. There mechanistic effect appears to involve, at a minimum, the inhibition of NF-κB. The beneficial effect of these botanicals is attributed to the present polyphenolic compounds rather than to known nutrients and/or vitamins. Polyphenols exhibit a variety of biological effects (anti-inflammatory, anticancer and antioxidative). For example electrophoretic mobility shift assay of THP1 cells show that trans-resveratrol repressed TNF and LPS induced NF-κB activation [Holmes-McNary and Baldwin, 2000, Tsai and others, 1999]. It should be noted, however, that although TNF and LPS activate NF-κB through IKK complex activation, the responses to each stimuli are different [Cheong and others, 2008].

The NF-κB transcription factor is ubiquitously expressed in many cell types and its properties seem to be broadly exploited in immune cells, where it has been implicated in activation of a large number of genes in response to infection, inflammation, and other stress conditions that require rapid re-programming of gene expression [Baeuerle and Henkel, 1994]. Phosphorylation at serine^{276} of p65/RelA is required for the induction of most, but not all RelA regulated transcriptional responses. Uncontrolled activation of the NF-κB pathway constantly maintains the cells in a chronic state of inflammation; hence, therapeutic inhibition of excessive NF-κB activation may be a useful strategy for control of NF-κB depended inflammations. For a detailed review on therapeutic potential of NF-κB pathway inhibition in the treatment of inflammation and cancer see ref. [Yamamoto and Gaynor, 2001]. From this perspective the cellular genes and disease states associated with NF-κB activation can be controlled through an understanding of the mechanisms involving prolonged activation. The fact that NF-κB regulates TNF expression and is a key effector of this cytokine is consistent with development of therapies aimed at blocking TNF. For instance NF-κB was activated in the inflamed synovium of rheumatoid arthritis patients as well as animal models of the same disease [Marok and others, 1996, Miagkov and others, 1998]. Primary synovial fibroblasts experiments showed that NF-κB is a requirement for induction of multiple inflammatory cytokines, IL-1β and TNF, and direct suppression of NF-κB resulted in TNF and Fas receptors-mediated apoptosis[Marok and others, 1996, Miagkov and others, 1998]. Consistent with its essential role in inflammation, NF-κB activation is assumed to directly affect other inflammatory diseases such as inflammatory bowel disease [Neurath and others, 1996] and is known to be a target of anti-inflammatory compounds. In short, the strong dependence of disease causing gene expression responses on NF-κB has made it an important and potential therapeutic target. Hence, most of the inhibitors of NF-κB either increase activation of IκB or inhibit its phosphorylation and degradation, consequently preventing the release of NF-κB. However, direct interference of the NF-κB/DNA interaction – by interfering with the DNA binding region in the NF-κB subunits seems to be a more potent approach of designing specific inhibitors.

One such compound is CAPE, which has over time received a broad functional claim for inhibiting this pathway. These is against the background that many of the already established immune suppressive drugs like asprin, corticosteroids, sulfasalazine and anti- TNF compounds are known to mediate their anti-inflammatory effects (at least in part) through inhibition of the NF-κB pathway [Guidi and others, 2005, Thiele and others, 1999, Weber and

others, 2000]. Whereas glucocorticoids induce increased expression of IκB which in turn retains NF-κB in the cytoplasm [Thiele and others, 1999], CAPE is presumed to interfere with the NF-κB/DNA complex in the nucleus consequently affecting the process of transcription [Natarajan and others, 1996]. According to Natarayan and coworkers, CAPE completely blocked the activation of NF-κB induced by a variety of inflammatory agents, including TNF, PMA, ceramide, okadaic acid and H_2O_2 *in vitro*. This inhibitory effect was found to be specific for NF-κB binding to the DNA as other transcription factors (AP-1, Oct-1, and TFIID) were not affected [Natarajan and others, 1996]. Interestingly, there seems to be an emergent of contradictory information on the influence of CAPE on NF-κB dysregulation. In one study [Natarajan and others, 1996] it was shown that CAPE did not inhibit TNF – dependent phosphorylation and degradation of IκBα although it delayed its re-synthesis. The delayed re-synthesis could be due to a feedback regulation, since re-synthesis is dependent on NF-κB activation [Hoffmann and others, 2003, Hoffmann and others, 2002, Karin and Ben-Neriah, 2000]. However, contrary to this finding, Some studies [Toyoda and others, 2009, Wang and others, 2009] show that CAPE inhibits IκB phosphorylation and degradation in a dose dependent manner, whereas in another it decreases AP1 expression in gastric epithelial cells [Abdel-Latif and others, 2005]. Inasmuch as these findings could be cell type specific the question of dose dependence arises given that information on the inhibitory concentration 50 (IC_{50}) of CAPE was not provided. Besides, specific inhibitory mechanisms of CAPE on the NF-κB/DNA interface are not well known. Thus additional information on the functional consequences of the effects of CAPE at the chromatin is needed as it could be directly interacting with the NF-κB, masking the DNA (κB site) binding motif in the nucleus or simply interfering with the nuclear kinases activity.

A recent and interesting research finding involves a newly described protein called Akirin. These are evolutionarily conserved nuclear proteins that are ubiquitously expressed, and are said to function in parallel with the NF-κB transcription factor in regulating innate immune responses [Crosby and others, 2007, Goto and others, 2008], where the mammalian gene has been encoded- Akirin1 and Akirin2. According to Goto and colleagues [Goto and others, 2008], mice that lack Akirin1 are overtly normal, and cells from these mice show a mild increase in IL-6 secretion in response to IL-1β but not TNF stimulation. However, deletion of Akirin2 leads to early embryonic lethality, while secretion of IL-6 in mouse embryonic fibroblasts ($Akirin2^{-/-}$) is decreased in response to IL-1β, TNF, LPS, or MALP2 (macrophage activating lipoprotein 2; a TLR2 and TLR6 agonist).

They further show that although degradation and re-synthesis of IκBα is relatively normal, the expression of other genes like BCL-3, IP-10 and RANTES is greatly reduced in the absence of Akirin2. This strongly implicates Akirin in chromatin remodelling of specific NF-κB responsive genes. What seems to be unclear is the mechanism of action that is thought to be independent of nuclear localization and binding to IκB sites [Ghosh and Hayden, 2008]. Taken together, this study reveals that Akirin is a previously unknown nuclear factor that, together with or downstream of NF-κB, can regulate the immune deficiency pathway making it an interesting subject of study given that similar inhibitory effects have been observed when CAPE is applied in different studies.

2.7.3 CAPE versus NF-κB in apoptosis

CAPE has been implicated in various inflammatory cell signalling mechanisms that includes the NF-κB, MAPK, PAK1 and apoptotic pathways [Demestre and others, 2009, Ha and others, 2009, Jin and others, 2008, Lee and others, 2009, Natarajan and others, 1996]. Apoptosis is a natural form of cell death that can be initiated either via the extrinsic or intrinsic pathway. Deregulation of the apoptotic pathway results in a wide range of pathologic disorders as seen in conditions of chronic inflammation and cancer. NF-κB subunits are subject to many modifications and regulatory interactions that define their transcriptional activity and target gene specificity, these interactions form an important interface with other signalling pathways in the cell. Phosphorylation of NF-κB subunits by nuclear kinases[Haller and others, 2002], and modification of the subunits by acetylases and phosphatases, can result in transcriptional activation and repression as well as promoter specific effects following cellular exposure to a distinct stimulus like CAPE [Ha and others, 2009, Kart and others, 2009, Orban and others, 2000, Ozyurt and others, 2007]. These regulatory mechanisms provide explanations for cell type and stimuli specific effects of NF-κB such as the ability to either inhibit or facilitate apoptosis as shown in Table 1 [Barkett and Gilmore, 1999, Kucharczak and others, 2003, Perkins, 2007]. In response to many physiological stimuli, NF-κB is activated and suppresses apoptosis via transcriptional activation of antiapoptotic genes. However, NF-κB has also been found to exhibit both pro-apoptotic and anti-apoptotic mechanisms [Barkett and Gilmore, 1999]. This relatively recent apoptotic observation was made earlier by Baltimore and colleagues, who showed that mice lacking p65/RelA component of the NF-κB experienced embryonic lethality and liver degeneration [Beg and others, 1995].

Apoptosis is a desirable process that is usually targeted in cancer therapies by reactivating tumour suppressor proteins like p53 through inhibition of the NF-κB pathway. For instance, p53 and RelA directly compete for CBP/p300 promoting their cross-repression [Webster and Perkins, 1999]. p53 is a transcription factor that is activated by genotoxic compounds and induces apoptosis; this gene is frequently mutated or functionally inactivated in cancer cells. When p53 is inactivated through NF-κB interference, uncontrollable tumour growth is triggered (at least in some tissues) promoting tumorigenesis. Anti-apoptotic genes directly activated by NF-κB include the inhibitors of apoptosis [IAP (c-IAP-1, c-IAP-2, and IXAP)], the TNF receptor–associated factors (TRAF1 and TRAF2- adaptor molecules that augment NF-κB activation), and some of the Bcl-2 family of proteins [Kucharczak and others, 2003]. The IAP functions by inhibiting the distal cell death proteases, caspase 3 and caspase 7 and in addition prevents activation of procaspase 9 [Roy and others, 1997]. CAPE exhibits apoptotic effects, especially in oncogenic cells where it has been shown to reduce tumour progression [Chen and others, 2005, Demestre and others, 2009, Jin and others, 2008, Orban and others, 2000].

NF-κB directly induces the transcription of the bcl2 homolog A1 in activated B- and T-cells. Expression of this pro-survival gene is required to prevent apoptosis that occurs during the engagement of the B-cell antigen receptor. Apoptotic cells are characterized by changes in cell morphology that include nuclear and chromosomal DNA fragmentation, chromatin condensation and cell shrinkage resulting in the formation of apoptotic bodies that are engulfed by phagocytic cells. Mechanisms that regulate apoptosis have been unravelled by tumour biologists, and the first protein to be characterized was Bcl-2, which naturally suppresses mitochondrial release of cytochrome C [Creagh and others, 2003] . CAPE has been shown to inhibit the anti-apoptotic effects of Bcl-2 by mediating release of cytochrome C into the cytoplasm consequently activating caspase 9 which in turn activates caspase 3. This mitochondrial mediated apoptosis [Jin and others, 2008] is seen in U937 cells, where CAPE treatment decreased cell viability in a dose and time dependent manner. In addition CAPE activity was accompanied by release of cytochrome C, a reduction in Bcl-2 and increase in Bax expression, and activation of both caspase 3 and PARP. On the contrary other immune reactions culminate in the caspace-dependent apoptosis, especially the cell death associated apoptosis that originates at the TNF and Fas receptors that could be NF-κB dependent [Kucharczak and others, 2003]. Thus it is obvious that prolonged cell survival- especially tumour cells- depends on the proper balance between pro- and anti- apoptotic signals that

could be dependent or independent of the NF-κB signalling [Kucharczak and others, 2003]. However, it is worthwhile to note that the distinct NF-κB subunits (not all subunits have the capacity to suppress apoptosis induced by death receptors [Kucharczak and others, 2003]) play different roles in these pro- and anti-apoptotic balancing in addition to cell type specificity, thus CAPE selectivity and or specificity should be carefully analysed to determine how it exhibits its apoptotic effects, apart from silencing the NF-κB activity.

Table 1. Summary of disease related proteins regulated by CAPE treatment

Protein*	Disease	Source
β-catenin	Tumorigenesis	[Lee and others, 2009]
MMP-2, MMP-9	Hepatocellular carcinoma	[Lee and others, 2008]
PAK1	Neurofibromatosis	[Demestre and others, 2009]
TGF-β1	Pneumonitis	[Chen and others, 2005]
AP-1	Gastric ulcers	[Abdel-Latif and others, 2005]
GGT, ALT/GPT, IL1A, IL6	Cholestatic liver injury	[Coban and others, 2008]
ND	Ischemia-reperfusion injury	[Ilhan and others, 1999]
TNF, IL-2, IL-6, IFN-γ, iNOS	Helicobacter pylori-induced gastritis	[Toyoda and others, 2009]
IL-12 p40, IL-12 p70, IL-10, IP-10	Asthma	[Wang and others, 2009]
ND	Systemic candidiasis	[Pukkila-Worley and others, 2009]
Paraoxonase	Intestinal inflammatory diseases	[Precourt and others, 2009]
PCNA	Cholangiocarcinoma	[Onori and others, 2009]
Catalase	Ovary ischemia/reperfusion injury	[Kart and others, 2009]

ND: Not determined

*NF-κB pathway suspected

See Ref. [Gilmore, 2008] for a comprehensive list of proteins that can interact with Rel/NF-κB, IκB, and IKK signalling proteins

2.7.4 Mitogen activated protein kinases (MAPK)

Mitogen-activated protein kinases (MAPK) are a highly conserved, ubiquitously expressed family of serine/threonine kinases that control many cellular processes including

cell growth and apoptosis, differentiation, proliferation, gene expression and resonses to environmental signals and stresses. Basically the MAPK respond to extra cellular stimuli (mitogens, osmotic stress, heat shock and inflammatory cytokines) by phosphorylating other protein kinases, phospholipases and various transcription factors depending on the cellular context, while their down-regulation occurs through dephosphorylation by serine/threonine and tyrosine phosphatases or dual-specificity phosphatases and through feedback inhibitory mechanisms that involve the phosphorylation of upstream kinases. This interplay between phosphorylation and de-phosphorylation allows the cell to rapidly adjust its requirement for the active or inactive MAPK state [Kefaloyianni and others, 2006, Thornton and others, 2008, Yao and others, 2007]. They are activated and regulated by a signalling cascade of three successive enzymes of the following general structure: MAPK-MAPKK (MKK, MEK or MAP2K)-MAPKKK (MKKK, MEKK or MAP3K). Several groups of MAPK have been characterized into distinct pathways: Extracellular signal-regulated kinases [(Erk) Erk1 and Erk2 (Erk1/2)], c-Jun N-terminal kinases or stress-activated protein kinases [(JNK/SAPK) JNK1, JNK2, and JNK3], and the p38 with several isoforms (α, β, γ, and δ). Of interest to this study is the p38 MAPK subfamily which has been shown to interact with key inflammatory mediating proteins including TNF, IL-1β, IL-6 and NF-κB. In addition, a accumulating data support the role of MAPK in signal-mediated *ho1* gene activation. Although many of the inducers of HO1 activate one or more of the three major MAPK pathways in multiple cell types they do not automatically lead to HO1 induction. Notably, treatment of rat hepatocytes with SB203580 (pharmacologic inhibitor of p38) does not alter HO1 mRNA accumulation in response to arsenite [Yao and others, 2007].

2.7.5 Interferon regulatory factor 1 (IRF1)

Interferon regulatory factor 1 (IRF1) regulates expression of target genes by binding to promoter regions of interferon stimulated response element (ISRE) via an N-terminal helix-turn-helix DNA binding domain that is highly conserved among all IRF proteins [Du and others, 2007]. Besides its transcription activity, IRF1 has been shown to trans-activate the tumour suppressor protein, p53 via recruitment of the p300 co-factor. The cooperation between IRFs and between IRFs and other transcription factors is thought to be an important mechanism by which IRFs control the specificity and magnitude of a transcriptional event. Members of the NF-κB and IRF families cooperatively control the transcription of several cytokine genes. For instance evidence shows that IRF1 is required for full NF-κB

transcriptional activity via formation of a functional complex at the long terminal repeats (LTR) promoter regions in κB sites on the IP-10 promoter [Sgarbanti and others, 2008], indicating that IRF-1/ NF-κB complexes in adjacent or overlapping sites of many genes synergistically activate transcription [Sgarbanti and others, 2008].

2.7.6 Inflammatory mediators implicated in IBD

Inflammation is an important adaptive response to disruption of cellular and/or tissue homeostasis with many deleterious physiological consequences including tissue remodelling and repair, host defence, and metabolic regulation. These complex responses require many functional programmes to be either controlled co-ordinately in some situations or independently as a function of the inflammatory stimuli. As a result the mechanisms behind inflammatory processes have been sub-divided into distinct categories, namely, cell-, signal- and gene-specific mechanisms. Just like the name suggests, cell-specific mechanisms are cell type dependent that dictates the regulation of their recruitment and activation processes. Signal-specific mechanisms are defined by the regulatory signalling pathways involved. Gene-specific mechanism is more of a transcriptional response operating at the level of individual genes. Although these inflammatory responses appear to be distinct and specific there is always an overlap or cross-talk in between the three categories [Atreya and others, 2008, Hisamatsu and others, 2003, Kaser and Blumberg, 2009, Ruiz and others, 2006, Singh and others, 2008].

Studies of NF-κB regulation and TLR signalling have defined many elements of the signalling pathways involved in microbe-host interaction, but still much remains to be explored. TLR signalling is negatively regulated by several mechanisms including receptor compartmentalization, degradation, deubiquitination, and competition [Barton and Kagan, 2009]. Some of these regulators have an established role in controlling intestinal inflammation, while others have yet to be explored. A20, originally identified as an important negative regulator of TNF-induced NF-κB activation, is also a regulator of TLR-induced NF-κB activity.

2.7.7 Interferon gamma inducible 10 kDa protein (IP-10)

The chemokine Interferon gamma inducible 10 kDa protein (IP-10) or otherwise known as CXCL10 is a chemoattrant molecule of activated T-cells to sites of inflammation. IP-10 binds to a G protein coupled receptor CXCR3 that is preferentially expressed on T-cells. CXCR3 is also expressed by many cell types including intestinal epithelial cells and it has

been shown to be involved in epithelial cell movement via p38 MAPK and PI3K dependent signalling pathways. Production of IP-10 has been shown to augment induction of pro-inflammatory signals, which promote colonic Th1 cell population recruitment. Treatment of mice with severe colitis with anti-IP-10 adjuncts decreased clinical and histological disease severity scores, mRNA expression as well as the accumulation of mononuclear cells in the colon. This IP-10 targeted treatment specifically decreased the rate of Th1 cells recruitment into mesenteric lymph nodes and colon of IL-10 deficient mice as shown by one report [Hyun and others, 2005]. This study and many others demonstrate that IP-10 has merit in the pathogenesis of chronic intestinal inflammation. Overall IP-10 seems to play a dual role in the development of colitis by enhancing Th1 cell generation in inductive sites and promoting effector cell recruitment to inflamed tissue sites. Thus a blockade of IP-10 may be a useful adjunct to remission-inducing therapies in inflammatory bowel disease (IBD) by impairing disease recurrence through selective inhibition of effector cell generation and trafficking in vivo [Dufour and others, 2002, Ruiz and Haller, 2006].

2.7.8 Oxidative/Electrophilic stress response

2.7.8.1 Keap1/Nrf2 signalling

The Nrf2 transcription factor is known to transactivate cytoprotective genes through the antioxidant response element (ARE), a common DNA regulatory element. Nrf2 targets many genes including drug-metabolising enzymes, antioxidant proteins, metal-binding proteins, transcription factors, proteases, and molecular chaperones. Several Nrf2-activating compounds have been identified, albeit with no structural similarities. These includes but is not limited to diphenols, Michael reaction acceptors, isothiocyanates, thiocarbamates, trivalent arsenicals, 1,2-dithiole-3-thiones, hydroperoxides, vicinal dimercaptans, divalent metal cations, and polyenes. Although studies are ongoing to unravel how cells detect these chemicals the only common property identified is reactivity with thiol-reactive cysteine residues in Keap1 [Dinkova-Kostova and others, 2007, Dinkova-Kostova and Wang, 2011].

Keap1 binds and targets Nrf2 for ubiquitination and proteasomal degradation by acting as an adaptor protein for the Cullin 3-based E3 ubiquitin ligase. Keap1 has several reactive cysteine residues that form covalent bonds with electrophiles in vitro. Of these residues, Cys-151, Cys-273, and Cys-288 have been demonstrated to be essential in Nrf2 regulation. Under basal conditions, Nrf2 is rapidly degraded by the proteasome and little induction of target genes is observed. For Nrf2 to be activated electrophilic compounds have to directly modify

sulfhydryl groups of Keap1 cysteines by oxidation, reduction, or alkylation resulting conformational changes in Keap1. Keap1 loses the ability to repress Nrf2, which stabilises and translocates to the nucleus where it induces Nrf2 target genes that stimulates an adaptive response to electrophilic/redox stress in cells [Dinkova-Kostova, 2002, Dinkova-Kostova and others, 2007, Dinkova-Kostova and Wang, 2011, Hayes and others, 2010, Itoh and others, 1997, Motohashi and Yamamoto, 2004]. Many of these oxidative/electrophilic inducers of Nrf2 especially the isothiocynates and Michael acceptors are present in edible food plants. Synthetic triterpenoids bearing Michael acceptor function potently induce phase 2 enzymes besides the anti-inflammatory effects. In addition, some phenolic Michael acceptors capable of directly scavenging free radicals are thought to have synergistic cytoprotective effects by diminishing hazardous oxidants (direct and instantaneous action) and inducing the phase 2 enzyme response (indirect and long-lasting action) [Dinkova-Kostova and others, 2001].

2.7.8.2 Effect of Heme oxygenase-1 (HO1) in inflammation

The biological significance of HO1 is highlighted by several studies which show that HO-1 has an important protective role in cell physiology. This was revealed in HO1 deficient mice that exhibited embryonic lethality and anaemia [Poss and Tonegawa, 1997, 1997] and very recently it was shown that HO1 inhibits the expression of adhesion molecules associated with endothelial cell activation via inhibition of NF-κB $p65^{Ser276}$ phosphorylation [Seldon and others, 2007]. The main signalling pathway involved in the oxidative stress response is the Keap1/Nrf2 pathway. HO1 induction via Nrf2 signalling is known to be a major cellular defence against oxidative and electrophilic stress. Nrf2 is a substrate of the ubiquitin-dependent proteasome process that is constantly targeted for degradation by Keap1 in the absence of oxidative stress and by so doing represses Nrf2 transactivation activity. HO1 is a good example of inducible cytoprotective enzymes besides NQO1 and GSH that has been shown to also posses' anti-inflammatory ability. HO1, a stress-inducible protein, is the rate-limiting enzyme in the degradation of heme into biliverdin, bilirubin, free iron and carbon monoxide (CO). HO1 is induced by oxidative and electrophilic agents, hence it plays a critical role in the maintenance of cellular homeostasis under stressful conditions [Farombi and Surh, 2006, Maines, 2004, Yao and others, 2009].

2.8 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is defined as an inflammatory disorder of the gastrointestinal tract of unknown aetiology that presents in distinct clinical manifestations

including Crohn's disease (CD) and ulcerative colitis (UC). Based on various factors, including genetic predispositions such as the recently identified polymorphisms in the NOD2 gene, marker antibodies, and environmental factors, it is becoming clear that the varying clinical forms of IBD mirror unique pathogenic processes in the gastrointestinal tract (GIT). Although details concerning the etiology of IBD remain ambiguous, IBD is specifically seen as a representation of abnormal immune responses to luminal bacteria in the GIT, especially in individuals with a genetic predisposition to develop persistent mucosal inflammation [Danese and others, 2004, Kaser and others, 2010]. CD is regarded as a segmental and transmural granulomatous (macrophage aggregation) inflammation that runs through the full length gastrointestinal tract, whereas UC is a continuous non- granulomatous inflammation that is restricted to the colon. Both forms of IBD are assumed to have distinct endoscopic and histological features. In CD any site of the GIT may be affected with severe presentation in the terminal ileum where early mucosal lesions appear over Peyer's patches. UC is exhibited by diffuse mucosal inflammation in the colon accompanied with infiltration of copious amounts leukocytes in the lamina propria and crypts forming micro-abscesses[Kaser and others, 2010, Shanahan and Bernstein, 2009].

Even though the incidence of IBD varies between different countries where it has been reported to occur, accumulating evidence now shows that it has become a major public health problem. Furthermore, there is no cure for IBD, however a number of interventional strategies have been adopted as a remedy to reduce intestinal inflammation, these includes but are not limited to immunosuppressive therapies that target the TNF signalling and the application of probiotic micro-organisms as an alternative in restoring the homeostatic imbalance of gut microbiota. Many factors contributing to the pathogenesis and inflammation in CD and UC have been identified leading to development of new therapeutic concepts. Among these contributing factors are cytokines and chemokines, as well as adhesion molecules, which are relevant for the movement of immune cells into the intestinal mucosa [Xavier and Podolsky, 2007, Yamamoto and Gaynor, 2001]. Mucosal and systemic concentrations of many of these pro-inflammatory cytokines are elevated in IBD. Inadequate or persistent activation of the intestinal immune system plays an important role in the pathophysiology of persistent mucosal inflammation. Homeostatic imbalance between pro-inflammatory and anti-inflammatory cytokines has been described in the inflamed mucosa of IBD patients. While most individuals can maintain gut homeostasis and health in the face of extreme immunological challenges, others cannot [Xavier and Podolsky, 2007].

2.8.1 Intestinal epithelium

The gut is divided into regions based on physiological functions. The upper GIT comprises of the pharynx, oesophagus and stomach, which are involved in food uptake and digestion, and the small intestine where absorption of digestive nutrients occurs. The lower intestine includes the colon and the rectum for water and nutrient absorption as well as faeces formation. The intestinal tract is layered with a single column of epithelial cells that form villi structures to maximize surface area for digestion and absorption [Sartor, 2008, Sartor and Muehlbauer, 2007]. The epithelial cells are joined by tight junctions that regulate passage of large molecules and organisms in addition to a layer of membrane bound glycoproteins and mucus. Because of this barrier effect, transportation of water and nutrients across the epithelium is maximized while minimizing that of large molecules and organisms [Xavier and Podolsky, 2007].

2.8.2 Epithelial cells

In addition to digestion and nutrient absorption functions Intestinal epithelial cells (IEC) that line the gastrointestinal tract (GIT) underneath the mucus coat provide and maintain essential barrier integrity that prevents entry of commensal and pathogenic bacteria into the underlying lamina propria. IEC are heterogeneous and include columnar, goblet, paneth, enteroendocrine, undifferentiated stem cells and M (microfold) cells. IEC provide diverse protective systems including the microvilli, lipid bilayer of plasma membrane, intracellular organelles containing degradative enzymes, and tight junctions between adjacent cells. Each cell type has unique morphological features with distinct physiological roles. For instance M cells which overlie the follicle associated epithelium of the Peyer's patches is enhances mucosal immunity by facilitating luminal sampling. M cells have no microvilli and exhibit reduced mucin secretion, however, they have modified apical and basolateral surfaces to promote uptake and transport of luminal contents to antigen-presenting cells (APC). Other effector functions of these protective systems are enhanced salt and water secretion, expression of antimicrobial proteins and mucin.

The intestinal epithelium is recognised increasingly as an active partner in the mucosal immune system. IEC produce and respond to a wide range of cytokines and express molecules able to interact with immune cells of the gut associated lymphoid tissue (GALT), lamina propia lmphocytes (LPL) and intraepithelial lymphocytes (IEL), especially in response to bacterial invasion [Sartor and Muehlbauer, 2007]. Since the IEC interacts with luminal

bacteria it acts as the first line of defence that signals the innate immune system to rapidly release chemokines in the presence of bacterial antigens. This signalling cascade in IEC is due to expression of pattern recognition receptors (PRR) including toll–like receptor (TLR) proteins that bind to and recognise certain classes of microbial molecules based on molecular patterns. The well described TLR4 binds bacterial lipopolysaccharides (LPS) resulting in activation of the MyD88 pathway that leads to downstream activation of the NF-κB signalling leading to production of pro-inflammatory mediators. Besides the immunologic tolerance to antigens and non-invasive bacteria, the GALT has been shown to initiate both regional and systemic responses to certain antigens and pathogens. However, the precise mechanisms involved in this intricate balance between tolerance and responsiveness is still a subject of study [Lohner and others, 2007, Sartor and Muehlbauer, 2007, Vidal and others, 1993, Wirtz and others, 2007].

2.8.3 Epithelial barrier function

The goblet cells, Paneth cells, M cells and enterocytes form an indispensable part of the intestinal epithelium mucosal barrier. The epithelium is under continuous and rapid replacement of dead cells in the crypts through apoptosis and exfoliation. This process occurs without disrupting the functional integrity of the tight junctions involved in the regulation of paracellular permeability important in immune system homeostasis that is shielded from microbes and food antigens on the luminal surface of the mucosal barrier. Some of these enteropathogenic bacteria including *Shigella flexneri*, *Clostridium difficile*, and *Salmonella typhimurium* alter barrier functions by secreting toxins that target tight junction associated proteins disrupting the intestinal barrier [Sartor, 2008, Sartor and Muehlbauer, 2007, Shanahan and Bernstein, 2009, Singh and others, 2008]. Inflammatory mediators modulate the epithelium rejuvenation process as a result they enhance host defence mechanisms. For instance IL-13 and IP-10 have been shown to accelerate epithelial cell turnover that in turn enhances the rate of intestinal parasites removal. Conditional knockout of IκB kinase-γ (NEMO) or both IKKα and IKKβ in IEC results in a loss of capacity for NF-κB activation and as a consequence the mice develop severe chronic intestinal inflammation [Sartor and Muehlbauer, 2007].

2.8.4 Experimental mouse models of IBD

Although no animal model exactly reproduces human IBD, experimental mouse models of IBD now give insights into the Pathophysiological mechanisms involved in intestinal

inflammation and the effect of emerging therapeutic strategies. In fact some models exhibiting many of the features of UC that appear to be bacteria-driven, have helped unravel the pathogenesis [Kaser and others, 2010, Neurath and others, 1997]. Most of these models involve some form of genetic manipulation, either insertion (transgenic) or selective deletion (knockout) of a gene. Mice resulting from genetic manipulation are collectively referred to as 'induced mutants' to distinguish them from mice with spontaneously occurring mutations. The induced mutants that develop IBD, usually in the absence of any further manipulation, represent a small subset of the total number of immune system genes that have been mutated. This argues that the mutations that have resulted in disease must represent genes involved in pathways critical to the maintenance of mucosal homeostasis. Some of these mouse models that have been developed with distinct features include; models of impaired T cell regulation, excessive effector cell function, spontaneous IBD, perturbations of the epithelium and models involving chemical or environmental stressors [Wirtz and others, 2007]. It is worthwhile to note that TNF plays an important role in many of these models where it acts as an initiating factor that orchestrates the inflammatory response leading to intestinal inflammation in the ileum.

2.8.4.1 Models of impaired T cell regulation

IL-10 knockout mice develop anaemia, growth retardation and chronic colonic inflammation with age. Under germfree conditions IL-10 mice do not develop colitis, a condition that is reversed when they are reconstituted with bacteria flora. The effector cell mediating colitis in IL-$10^{-/-}$ is the $CD4^+$ T cell [Hoermannsperger and others, 2009, Ruiz and others, 2006]. Thus transfer of $CD4^+$ or $CD4^+CD8^+$ T cells isolated from the lamina propria of IL-10 mice into RAG-$2^{-/-}$ results in colitis, whereas transfer of CD8+ cells does not. The RAG-$2^{-/-}$ lamina propria lymphocytes are $CD4^+$ or $CD4^+CD8^+$. The major role played by the CD4 Th1 subset has been confirmed by experiments showing that administration of anti- IFN-γ, anti-IL-12 and IL-10 attenuates colitis. Severe combined immunodefiecient (SCID) mice have a spontaneous mutation in a DNA dependent protein kinase catalytic subunit gene that results in a deficiency of both B cells and T cells; however, their immune system can be partially reconstituted by adoptive transfer of B cells and T cells. Transfer of $CD4^+$ T cells expressing high levels of the surface molecule CD45RB results in colitis. But transfer of whole $CD4^+$ T cell or $CD4^+$ subset expressing low levels of the CD45RB molecule does not result in disease. Treatment with anti-IFN-γ, anti TNF or IL-10 prevents disease development, indicating that the colitis is mediated by Th1 effector cells. Other related T cell regulatory

models include IL-2, T cell receptor α-chain, and TGF-β_1 deficient mice respectively [Williams and others, 2001, Wirtz and others, 2007].

2.8.4.2 Models of excessive effector cell function

TNF is involved in the regulation of inflammation at many levels; of particular interest for IBD is the role of TNF in the recruitment of circulating inflammatory cells to local tissue sites and granuloma formation. An important role for TNF as a pro-inflammatory cytokine in CD has emerged in recent years and this pivotal role is prominent in the $TNF^{\Delta ARE}$ (TNF 'knockin') mouse model of intestinal inflammation [Hoermannsperger and others, 2009, Kontoyiannis and others, 1999, La Ferla and others, 2004]. In this mouse model, deletion of Adenosine-Uracil Repeat Elements (ARE) in the 3' untranslated region of the *tnf* gene results in enhanced mRNA stability and increased TNF production. Both homozygous and heterozygous $TNF^{\Delta ARE}$ mice develop intestinal inflammation, although the pace of disease development and progression is accelerated in homozygous mice, which fail to thrive and die between 5 and 12 weeks of age. The disease is localized primarily to the terminal ileum, and less frequently, the proximal colon [Ruiz and others, 2007, Ruiz and Haller, 2006]. Initial lesions consist of villous blunting and broadening that is associated with mucosal and submucosal infiltration of chronic and acute inflammatory immune cells, including monocytes and neutrophiles. Severe intestinal inflammation is usually observed by 8 weeks of age in heterozygous $TNF^{\Delta ARE/+}$ mice, with an increased number of submucosal lyphoid aggregates and follicles. Transmural extension of inflammation is observed on disease progression that culminates in a complete loss of villous and poorly organised granulomas in old mice of about 4 months of age. Although the specific molecular and cellular mechanisms of the pathogenic action of TNF are not completely understood many of the TNF–induced inflammatory responses are mediated by the NF-κB, which regulates transcription of many other pro-inflammatory cytokines [Neurath and others, 1997]. Current evidence, though controversial, shows that the pathogenesis of IBD is not only mediated through the Th1 responses but also through Th17 (a newly discovered T lymphocyte subset). Th17 cells produce arrange of cytokines including IL-17, IL-21, and IL-22 which have been implicated in autoimmune diseases. Other models in this category include A20 deficient mice, STAT-4, IL7 and CD40 ligand transgenic mice.

Chemically induced models

The two best described models include Dextran sulphate sodium (DSS) induced colitis and Trinitrobenzene sulphonic acid (TNBS) induced colitis. Addition of 2-10% DSS (which is

believed to be directly toxic to gut epithelial cells) in drinking water induces colitis in mice that is manifested by bloody diarrhoea, weight loss, colon shortening, mucosal ulceration and neutrophil infiltration [Wirtz and others, 2007]. Thus this model is suitable for studies involving epithelial repair and the contribution of innate immune mechanisms of colitis respectively [Williams and others, 2001]. However, the main limitation of DSS colitis model is the non-specific injury that is independent of both T cells and B cells, hence; it is not well suited to address immunologic or therapeutic issues involving the acquired immune system. The TNBS model involves both chemical damage and T cell immune reactivity and colitis is only induced if the TNBS is administered in conjunction with ethanol which breaks the mucosal barrier. In TNBS-induced colitis the mucosal CD4+ T cells produce huge amounts of IFN-γ and IL-2, but not IL-4, consistent with a Th1 effector response and administration of anti-IL-12 reduces the colitis [Peluso and others, 2006]. In addition other agents that have been shown to prevent TNBS-induced colitis via inhibition of IL-12 production are IL-10 and an anti-sense oligonucleotide to NF-κB. Results that have been obtained from these models to date provide strong support for the immunological hypothesis that a dysregulated mucosal immune response, particularly a CD4+ T cell response, to antigens of enteric bacteria in a genetically susceptible host results in chronic intestinal inflammation. Taken together, these observations have led to the general agreement that T lymphocytes, in particular $CD4^+$ cells, play a key role in both normal and patho-physiological immune regulatory processes in the gut [Peluso and others, 2006].

2.8.5 Intestinal inflammation and homeostasis

Inflammation is a complex physiological response of cellular tissues to harmful stimuli including pathogens, damaged cells and environmental factors. These inflammatory responses are triggered to protect the organism from further injury as well as initiate the tissue healing process [Kaser and Blumberg, 2008, Neurath and others, 1996]. Intestinal mucosal surfaces are in direct contact with the external environment and hence are susceptible to microbial invasion and colonization. Thus, it must be tightly regulated to prevent abnormal responses to innocuous environmental factors and commensal bacteria that results in allergenic and or chronic inflammatory disorders. To fight these pathogenic invasions, elaborate mucosa-associated lymphoid tissues rich in innate and adaptive immune cells have been evolved in the mucosa. Intestinal inflammation is controlled by a complex interaction between innate and adaptive immune systems as well as non-immune process, however, disruption of this

homeostatic balance due to changes in gene expression or environmental factors can result in IBD [Neurath and others, 1997]. Many of the primary features of IBD remain unexplained despite the tremendous progress that has been made in understanding the biology of the disease. In fact, IBD is shrouded in mysteries concerning epidemiology and clinical courses where many questions are constantly being asked regarding its pathology and physiology. Some of the begging questions include: The biodiversity of IBD incidences; distinctions between UC and CD disorders; why UC is restricted to the mucosa while CD is transmural and the role of mesenteric fat in CD.

2.8.5.1 Biodiversity of IBD incidences

IBD is more common in the developed world than the developing world. It is generally agreed that the incidence of IBD has sharply risen in the last 50 years in the developed countries; with the increase occurring in the higher social classes in comparison to the lower classes, probably as a function of changing westernised lifestyle. However, the increase is too rapid to be accounted for by genetic changes (see IBD animal models) and strongly points to environmental factors, especially changes in diet and gut microbiota. Explaining such geographical restrictions of inflammation to the intestine is a major challenge given the broadness in disease-associated gene variation. Evidence shows that dietary changes and food preparation methods can influence intestinal microflora, however, the relationship between the pathogenic and commensal microbial communities in the gut and IBD is not well described.

2.8.5.2 Ulcerative colitis versus Crohn's disease

The distribution pattern of inflammation along the gut mucosa in UC and CD patients suggests that they could have different disease mechanisms. The tendency for UC to be confined to the colon in contrast to CD could be attributed to several reasons. The changes in immune responses may be so slight that the trigger required for inflammation can only be provided by the large bacterial load in the colon for UC, whereas a far less intense trigger may be required for CD development and so can occur anywhere in the gut. UC originates from the rectum as this region may be most conducive to the induction of cytokine milieu necessary for natural killer (NK) T cell development. Structural and physiological differences and variation in commensal bacteria between the colon and the small intestine may perhaps account for localized differences in UC and CD. Variation in expression of pathogen associated molecular patterns e.g. Toll-like receptors between colon and small intestine could also be an important factor in preventing small intestinal inflammation. Although genetic

predisposition in UC may affect genes that are mainly expressed and functionally relevant in the colon, this sharp contrast of involved and uninvolved areas is an endoscopic observation that does not march histological measurements in all UC patients.

The discovery of susceptible genes has shown the importance of both barrier function, and innate and adaptive immune systems in IBD development. Genome wide associated studies for IBD markers have identified a number of genes contributing to CD as NOD2/CARD15 (IBD1), IBD5, IL23R and ATG16L1 and these has been repeated in different studies. However, alteration in the interaction between gut microbiota and the mucosa is thought to be the driving force behind intestinal inflammation in IBD, perhaps due to environmental or host-related factors that could vary depending on the genetic predisposition.

2.8.5.3 Why UC is restricted to the mucosa while CD is transmural

There is general consensus that CD inflammation frequently goes further into the mucosa than UC inflammation due to the fundamental differences in the aetiologies of the diseases. In one case UC has been suggested to be confined to the epithelial cell layer whereas CD is predominantly due to disruption of the mucosal barrier in a genetically susceptible host that results in activation of the immune system in deeper layers of the gut. It has also been suggested that intestinal phagocytes fail to kill or neutralize the invading pathogens causing a perpetual of bacteria into the bowel walls in CD but not UC. Thus CD is analogous to localised infection that progresses into the bowel wall until the infection is at least partially controlled by the mucosal immune system. Other factors implicated in the variations of these two disease states include lymphatic obstruction, defective antiprotease activity and different disease causing bacterial flora in UC and CD respectively. For instance animals lacking T lymphocytes and T-bet, a transcription factor that regulates immune cell differentiation and function, develop colonic inflammation that mimics UC. This is mediated by production of TNF and loss of intestinal barrier function [Bereswill and others, 2010]. Interestingly, transfer of microbial flora from such genetically susceptible mice to mice with normal immune system cause a similar colitis indicating that the host immune response can modify the intestinal microbiota to become colitogenic.

2.9 Therapeutic implications of CAPE

The link connecting NF-κB activation and inflammation and/or apoptosis has been shown in various experimental animal disease models, and to some extent in human diseases.

These association has been achieved through genetic (gene knockout) studies and use of inhibitors substances. In most cases cells of the infected tissues initiate the inflammatory reaction by triggering pro-inflammatory signals through NF-κB in response to the stimuli [Hayden and others, 2006]. Activation of NF-κB results in the up-regulation of cell adhesion molecules and cytokines by epithelium within the tissue leading to recruitment and activation of effector cells (neutrophils, macrophages and leukocytes). Another important function of NF-κB protein is in the production process of antimicrobial effector molecules and the survival of leukocytes in inflammatory environment. Thus, a better understanding of the regulation of the NF-κB pathway may provide opportunities for the development of new interventions to inhibit its prolonged activation. As shown in Figure 1, NF-κB signalling is an obvious target for new types of treatment to block the inflammatory responses in cases where this process becomes dysregulated or chronic. A range of widely used anti-inflammatory molecules inhibit the NF-κB pathway (at least in part) as one of their targets, however, one major concern about inhibiting several of these points of the NF-κB pathway is the specificity of such compounds. For example, the proteasome responsible for IκB degradation has many other important functions, hence, inhibition of this proteasomal activity may possibly cause severe side effects [Yamamoto and Gaynor, 2001]. It might also not be practical to block the NF-κB pathway for prolonged periods, given that NF-κB plays a key role in the maintenance of host defence responses. For example, extended expression of a degradation-resistant IκB super-repressor protein in the liver of transgenic mice indicate that inhibition of NF-κB activity can occur with no liver dysfunction, although the mice are more prone to bacterial infection. Nevertheless, short term treatment with specific inhibitors of IKK activity might reduce such potential side effects [Yamamoto and Gaynor, 2001]. IKKβ plays a significant role in NF-κB activation in response to most inducers, whereas IKKα is not required for the phosphorylation and degradation of IκB in response to proinflammatory stimuli.

Activation of the NF-κB pathway is involved in the pathogenesis of chronic inflammatory diseases, such as asthma, rheumatoid arthritis and inflammatory bowel disease [Tak and Firestein, 2001]. In addition, altered NF-κB regulation may be involved in other diseases such as atherosclerosis [Collins and Cybulsky, 2001] and Alzheimer's disease [Mattson and Camandola, 2001], in which the inflammatory response is at least partially involved. Increases in the production of pro-inflammatory cytokines by both lymphocytes and epithelial cells has also been implicated in the pathogenesis of inflammatory bowel diseases, including Crohn's disease and ulcerative colitis [Ruiz and others, 2007, Ruiz and Haller,

2006]. Hence a potential application of inhibition of the NF-κB pathway in using small molecules like CAPE offers more diversity in treatment of inflammation and cancer (Table 1). However, it is essential to determine the effect of CAPE persistence, distribution and elimination in immune regulatory cells and disease related pathogenesis with regard to its inhibitory capacity. Overall, CAPE offers the promise of enhancing the efficacy of cancer chemotherapy as well as reducing excessive cytokine production under inflammatory conditions (Table 1).

To know whether the level of neurofibromatosis (NF) tumours is dependent on p21 activated kinase 1 (PAK1) for their growth, Demestre and co-workers [Demestre and others, 2009] exploited the anti-PAK1 therapeutic potential of CAPE. Using water soluble CAPE-rich extract of New Zealand propolis "Bio 30", they demonstrated that "Bio 30" completely suppressed the growth of both NF1 and NF2 tumour xenografts in mice, suggesting the possibility that "Bio 30" and other CAPE-rich propolis might serve as effective and safe neurofibromatosis therapy. In another study CAPE decreased radiation-induced interstitial pneumonitis and TGF-β expression both *in vitro* and *in vivo* via NF-kB [Chen and others, 2005], however, the mechanisms by which CAPE decreased TGF-β were unclear. It is well known that apart from pathogenic micro-organisms such as viruses and bacteria other inflammatory triggers include environmental and dietary factors, carcinogens, smoking, as well as reactive oxygen species produced during cellular metabolism. Over-exposure to any of these sources can lead to excessive production of NF-kB protein where CAPE administration would serve as a potent anti-inflammatory mediator. For example, pre-treatment of human gastric epithelial cells (AGS) with CAPE, inhibited *Helicobacter pylori* induced proliferation and cytokine production in a dose dependent manner [Abdel-Latif and others, 2005]. The protective effect of CAPE has also been observed in ischemia reperfusion injury. An injury in which the tissue first suffers from hypoxia as a result of decreased, or arrested, blood flow, and restoration of circulation triggers inflammation , which exacerbates tissue damage due to induced oxidative stress [Coban and others, 2008, Ilhan and others, 1999, Kart and others, 2009, Ozyurt and others, 2007]. From these established connections between NF-κB signalling and diseases [Gilmore, 2008], it is obvious that the ability to develop effective and target specific inhibitors of the NF-κB pathway with a low risk of undesired side effects will offer better therapeutic alternatives aimed at different diseases.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Preparation of stock solutions

3.1.1 Test compounds

The test compounds (CAPE & its analogues, EGCG, Resveratrol, ED, and EL) used in the experiments were dissolved in Dimethyl sulfoxide (DMSO) and stored at -20 °C as a 200 mM stock solution. For working aliquots the stock solution was diluted to 1mM in appropriate cell culture media and used in the experiments at the indicated end concentrations. The end concentration of DMSO in the cell culture medium was between 0.005- 0.05 %.

3.1.2 Pharmacological inhibitors

The pharmacological inhibitors (MG132, Cycloheximide, SB203580, Bay11-7082, CORM-2, Actinomycin D, and Tunicamycin) were dissolved in DMSO and the aliquots stored at -20 °C. Working dilutions were prepared from the aliquots at the indicated concentrations.

3.1.3 Pharmacological inducers

The lyophilized recombinant mouse TNF and IFN-γ were reconstituted in bidest water at a stock concentration of 100µg/ml supplemented with 0.1 % Bovine serum albumin (BSA) and the aliquots stored at -20 °C. Further dilutions of the aliquots in cell culture medium were prepared to make the final working concentrations as indicated.

3.2 Cell culture and mouse embryonic fibroblasts (MEF)

Freezing, thawing, storage and passaging of cells was done using laid down standard procedures. For freezing, confluent cells from 75 cm^2 culture flasks were washed in PBS, trypsinized, centrifuged (1,000 rpm, 5 min, 21 °C) and re-suspended in 2 ml ice-cold freezing medium plus 5 % DMSO and divided into 1 ml cryovial aliquots that were frozen at -80°C for 24 h before moving to liquid nitrogen(-80 °C) for long term storage. For thawing, the cryovial was gently warmed to ambient temperature before re-suspending pre-warmed Dulbecco's modified eagle medium (DMEM) to dilute excess DMSO. This was followed by a centrifugation step (1,000 rpm, 5 min, 21 °C), after which the cells re-suspended in DMEM and transferred to 75 cm^2 flasks for incubation. Briefly, the small IEC line Mode-K (passage 13-25) [Vidal and others, 1993] was cultivated in high glucose DMEM medium (Invitrogen) containing 10 % (v/v) FBS, 1 % Antibiotic/Antimycotic (PAA) and 2 mM L-Glutamine (Invitrogen). Cells were grown at 37 °C in tissue culture plates (Cell Star, Greiner bio-one) in a humidified atmosphere containing 5% CO_2. They were split every third day and grown to 80

% confluency before stimulation. All incubations for additional cell lines including PTK 6, $TNF^{+/+}$ and $TNF^{\Delta ARE/+}$ MEF, $NRF2^{+/+}$ and $NRF2^{-/-}$ MEF, RAW 264.7, HEK 293, NIH 3T3 and BMDC were carried out in a humidified incubator at 37 °C, 5 % CO_2. For preparation of embryonic fibroblasts, C57BL/6 WT and $TNF^{\Delta ARE//+}$ mice were killed by cervical dislocation at day 13.5 of pregnancy. Embryos were taken out, placed in separate wells and killed by decapitation. Heart and liver were removed. Embryonic tissues were rinsed with pre-warmed PBS, transferred into 3 ml of 0.25 % Trypsin/EDTA (supplemented with 2 % chicken serum), and minced into small pieces. After incubation time of 15 min at 37 °C, 10 ml of MEF medium was added. The suspensions were transferred into 15 ml falcon tubes leaving cell debris to settle. Supernatants were transferred and centrifuged (430 x g, 5 min, RT). Pellets were re-suspended in 7 ml MEF media [DMEM medium supplemented with 10 % FBS, 1 % Antibiotic/Antimycotic (PAA), 2 mmol/L L-Glutamine, 100 µM non essential amino acids (PAA) and 1 mM sodium pyruvate (Sigma)]. Cells from each embryo were seeded separately in T25 flasks and incubated at 37 °C, 5 % CO_2. The medium was changed after 24 h and cells were incubated for additional 24 h. Following genotyping, MEF were pooled together (passage 1) in MEF freezing medium (70 % MEF medium supplemented with 20 % FBS and 10 % DMSO) and stored in cryo-vials at -180 °C until use. Stimulation experiments were done using MEF between passages 1-7. $Nrf2^{+/+}$ and $Nrf2^{-/-}$ MEF (a generous gift from Dr. Albena Dinkova-Kostova and Prof. Masayuki Yamamoto) were maintained in culture as described previously [Wakabayashi and others, 2004].

3.3 Cell counting using trypan blue stain (viability)

The principle behind Trypan blue staining is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged, hence viable cells exclude the dye while dead cells stain blue. Briefly, following cell splitting (trypsinization), approximately 100 µl of cell suspension was suspended in 4 % Trypan blue at a dilution ration of 1:10. The hemocytometer counting chambers were loaded with the diluted cell suspension using a micropipette (10 µl per side chamber). The number of total (viable) cells overlying the 4 x 1 mm^2 (the grid surface is 0.1 mm below the cover slip, hence volume of fluid over one of the 1 mm^2 areas of the grid is 0.1 mm^3 or 10^{-4} ml) areas of the counting chamber was determined by microscopic observation. The cell concentration was calculated as follows.

(**A**)Total cells in 4 mm^2	(**B**) A / 4 = cells/mm^2	(**C**) B x dilution (10)	(**D**) C x 10^4 = cells/ml
120	30	300	3.0 x 10^6 cells/ml

3.4 Viability and cytotoxicity assay (WST-8)

WST-8 [2-(2-methoxy-4-nitrophenyl)-3(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] allows very convenient assay by utilizing a highly water soluble tetrazolium salt that produces a water-soluble formazan dye (orange) upon reduction in the presence of an electron mediator (dehydrogenases in cells). The amount of the formazan dye generated is directly proportional to the number of viable cells. A 100 µl of cell suspension (2 x 10^4 cells/ well) were dispensed in 96-well plates and pre-incubated for 24 h. The test compounds were added and plates incubated for a further 24 0r 48 h. Afterwards 10 µl of the WST-8 solution was added to each well and the plate incubated for 1 h. The absorbance was measured at 450 nm (detection) and 650 nm (reference) using a Multiskan spectrophotometer (Thermo scientific).

3.5 Ex plant (tissue) samples

Ileal tissues obtained from 12-week-old WT and $TNF^{\Delta ARE/+}$ mice were flushed with PBS and cut open into 4-mm-long tissue pieces which were placed onto Netwell inserts in DMEM medium with the serosal side touching the transwell membrane (Corning Life Sciences). After incubation with or without CAPE for 24 h, supernatants were collected while the tissue were mashed in lysis buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1mM EGTA as well as complete Mini (protease) and PhosStop (phosphatase) inhibitors (Roche). Tissue homogenates were centrifuged (16 000 x g, 4 °C, 10 min) and protein-containing supernatants used for immunoblotting experiments.

3.6 Reporter (SEAP) gene assay for NF-κB transcriptional activity

Briefly, *pNiFty-SEAP* transfected Mode-K cells were pre-incubated with CAPE and its analogues for 1 h before TNF stimulation for 24 h. Subsequent assays will be carried out according to the manufacturer's recommendations (InvivoGen). Samples will be measured at 405 nm in a Multiskan spectrophotometer (Thermo scientific).

3.7 Immunofluorescence and confocal microscopy

Cells were grown on cover-slips in 6-well plates to 80 % confluency, treated with CAPE for 1 h followed by TNF for 20 min- 1 h and before fixation with 4 % formaldehyde in PBS (15 min, RT). The fixed cells were blocked (1 h) in 5 % normal goat serum supplemented with 0.5% Triton X-100. After overnight incubation at 4 °C in anti-RelA

primary antibody [1:100 dilution; sc-372, (Santa Cruz)], cover-slips were rinsed three times and Alexa-Fluor 488 goat-anti-rabbit secondary antibody (Invitrogen) added. After 2 h at room temperature in the dark, rinsed slides were covered in VectaShield HardSet mounting medium with DAPI (Vector Laboratories). Cellular localization of RelA was determined using a Leica SP2 confocal laser scanning microscope (Leica Microsystems).

3.8 Immunoprecipitation

Immunoprecipitation is used as a method to isolate and purify proteins of interest from a cell lysate as well as to determine protein-protein interactions. An antibody for the protein of interest is incubated with a cell lysate extract so that the antibody will bind the protein in solution. The resultant antibody-antigen complex is then precipitated from the sample using protein A/G-coupled agarose beads with high affinity to the Fc moiety of immunoglobulins. The physically isolated protein of interest is dissociated from beads and purified through several washing steps. The sample protein is then separated by SDS-PAGE.

3.9 Western blot analysis

Western blotting is usually used to determine the presence and /or relative abundance of a particular protein. Sodium Dodecyl Sulfate (SDS) detergent is used to denature and give the proteins a negative charge. The amount of bound SDS is relative to the size of the protein, and the proteins have a similar charge to mass ratio. Bands in different lanes separate based on the individual components sizes. Polyclonal or monoclonal antibodies specific to the protein of interest are used for detection in a complex mixture of proteins. Briefly cells were lyzed in SDS-PAGE sample buffer and 10 µg proteins resolved on a 10 or 15% SDS-polyacrylamide gel. Antibodies against phospho-RelA (Ser536), phospho-p38 MAPK, p38 MAPK, phospho-IκBα, IκBα, phospho-ERK, ERK, phospho-SAPK/JNK, SAPK/JNK, phospho-AMPK, AMPK (Cell Signalling), RelA (Santa Cruz), IP-10 (R&D systems), HO-1 (Stressgen) and β-actin (MP Biomedicals) were used and the protein bands detected with the Amersham ECL detection kit (GE Healthcare). Since the stripping process does not alter the membrane bound proteins, the membranes were stripped with a solution containing a 0.2 M glycine-HCl, pH 2.5, 0.05 % Tween 20. The Polyvinylidine fluoride (PVDF) membrane was pre-wetted in 100 % methanol before incubation in the stripping buffer at 80 °C in a sealed plastic container for one hour with agitation in a water-bath. The membrane was washed with TBST and re-blocked in 5 % non-fat skim milk in TBST.

3.10 Electrophoretic mobility shift assay (EMSA)

EMSA is used to study (DNA-binding properties of a protein) the interactions between transcription factors and nucleic acid motifs. The principle behind this technique is that the electrophoretic mobility of the higher molecular weight protein/DNA complex is less than that of free probe resulting in a band shift when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis. This is because the rate of DNA migration is shifted or retarded when bound to a protein.

3.10.1 Experimental procedure

Cells were harvested using PBS by scraping and transferred to micro-centrifuge tubes (1,000 g 1 min 4 °C). The cells were washed with ice cold PBS and centrifuged using the above conditions. The cells were re-suspended in homogenization buffer and left to stand on ice for 5 min followed by centrifugation. The resultant pellet was re-suspended in low salt buffer to swell the cells for 1 minute before high salt buffer (nuclear extract buffer) was gentle introduced to the bottom of the tube. The tube was vortexed and left to stand for 10 min on ice. The tube was then vortexed and centrifuged (10,000 g 10 min 4 °C). The supernatant containing nuclear extracts was aliquoted and kept at -80 °C before use. The protein concentration of the nuclear extracts was determined by Bradford assay.

3.10.2 Binding reaction and electrophoretic separation

All binding reactions were carried out on ice except the final incubation stage which was done at RT. 1 µl of cy5 fluorescent labelled oligonucleotide was added to a reaction mixture containing 2 µl of 5x binding buffer (Appendix vii), 1 µl poly-dIdC (1 µg/ µl in TE), 2 µl nuclear extract (4 µg protein) and 5 µl ddH_2O. For a competition assay 1 µl of cold or mutant probe was added before adding the cy5-labelled probe. All the components in the reaction micro-centrifuge were span down to the bottom of the tube and incubated for 30 min at RT. the sample were then loaded on a 4.5 % non-denaturing polyacrylamide (0.5x TBE) gel buffer. The EMSA samples were loaded into the wells of the gel. The gel was run for 3 hours in 005x TBE at 1 mA/cm. The voltage was about 120 V at the start and was increased during the course of electrophoresis to 200 V. The gel was visualized for band shift (protein/DNA complex) with a Variable mode imager (Typhoon TRIO+) at 630 nm. The sequence of the wild type competition probe is the same as the labelled NF-κB and ARE specific probe, while the "mutant" probe has a nucleotide exchange within the recognition region (NF-κB and ARE binding site with mutated site in bold) as shown.

Oct-1 and NF-κB oligonucleotide consensus sequence

Oct-1 F: 5'-GAA GAA TTC ATG CAA ATG AAT TCG AAG AAG-3'

NF-κB (wild type) F: 5'-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3'

NF-κB (mutant) F: 5'-TTGTTACAA**CTC**ACTTTCCGCTG**CTC**ACTTTCCAGGGAGGCGTGG-3'

ARE oligonucleotide consensus sequence

ARE (wild type) F: 5'-GAG TCA CAG TGA GTC GGC AAA ATT-3'

ARE (mutant) F: 5'-G**C**G TCA C**C**G TGA GTC **TT**C A**C**A ATT-3'

3.11 ELISA analysis

Cells were pre-incubated with test compounds (10 μM) for 1 h followed by stimulation with or without TNF, IFN-γ or LPS for additional 24 h. Secreted IP-10, TNF, IL-6 and MIP-2 proteins in the supernatants were measured using mouse-specific DuoSet ELISA kits, according to the manufacturer's instructions (R&D Systems).

3.12 Chromatin Immunoprecipitation (ChIP)

ChIP is used for probing protein-DNA interactions by identifying multiple proteins associated with a specific region of the genome, or many regions of the genome associated with a particular protein. For instance, the assay is used to analyze binding of transcription factors, transcription co-factors, DNA replication factors and DNA repair proteins. Basically, cells are fixed with formaldehyde (reversible protein-DNA cross-linking agent) to "preserve" the protein-DNA interactions occurring in the cell. Cells are lysed and the chromatin harvested and fragmented by sonication or enzymatic digestion. The chromatin is then immunoprecipitated with protein specific antibodies. Any DNA sequence associated with the protein of interest co-precipitates as part of the cross-linked chromatin complex and the relative amount of that DNA sequence is enriched by the immuno-selection process.

ChIP was performed using the ChIP kit (Cell Signalling; #9003) as described by the manufacturer. Briefly, cells were pre-treated for 30 min- 4 h with CAPE before addition of TNF. After 30 min, cells were fixed in 1 % formaldehyde (10 min, RT). The chromatin digest was normalized according to the purified DNA concentration and immunoprecipitated against anti-phospho-RelA^{ser536}, anti-acH3, anti-acetyl CBP/p300 and NF-κB p50 (D-17) antibodies. H3 and normal rabbit IgG antibodies were used as positive and negative controls, respectively. Immunoprecipitated DNA, together with purified DNA as input control (2% of the total chromatin extract) was used as template for PCR amplification using the following promoter-specific primers: IP-10, F-5'-aaggagcacaagagggg, R-5'-attggctgactttggag; and Simple ChIP mouse RPL30 (Cell Signalling).

PCR products were resolved by electrophoresis on 1-2 % agarose gels mixed with gel red dye and visualized using the UV-imager (Syngene-bio imaging).

3.13 RT- PCR

Total RNA was extracted from cells using the Isol-RNA-Lysis according to the manufacturer's instructions (5-Prime). Nucleotide concentrations were determined using the Nanodrop spectrophotometer (Peqlab). Complementary DNA (cDNA) was obtained from 1 µg RNA by reverse transcription using random primers (Invitrogen) and M-MLV reverse transcriptase (Invitrogen). Real-time qPCR was performed using the UPL master mix and primer sets designed by the Universal Probe Library (Roche) on the LightCycler 480 (Roche). Crossing point (Ct) values were obtained using the Second Derivative Maximum method. Coefficients of regulation between treated and control samples were calculated from triplicate samples according to the ΔΔCt method [Pfaffl, 2001]. The following primer pairs (Universal Probe Library set, mouse; Roche Diagnostics GmbH) were used: GAPDH, F-5'-tccactcatggcaaattcaa, R-5'-tttgatgttagtggggtctcg (probe no. 9); IP-10, F-5'-gctgccgtcattttctgc, R-5'-tctcactggcccgtcatc (probe no. 3); TNF, F-5'-tgcctatgtctcagcctcttc, R-5'-gaggccatttgggaacttct (probe no. 49); MIP-2, F-5'-cctggttcagaaaatcatcca-3', R-5'-cttccgttgagggacagc-3' (probe no. 63); NF-κB Rel-A, F-5'- cccagaccgcagtatcct -3', R-5'-ttcttcgctctggacctcg -3' (probe no. 47); NF-κB p105, F-5'- cactgctcaggtccactgtc -3', R-5'-acttgaggccctatcactgtc -3' (probe no. 69); NF-κB p100, F-5'- tggaacagccaaacagc -3', R-5'-tacctccaaacggtccac -3' (probe no. 76); NF-κB RelB, F-5'- gtgacctctcttccctgtcact -3', R-5'-aacctttagtagctgcttatgt -3' (probe no. 80).

3.14 Feeding experiment in $TNF^{\Delta ARE/+}$ mice

The animal use protocol was approved by the Bavarian Animal Care and Use Committee (No. 55.2-1-54-2531-88-09). Twelve-week-old heterozygous $TNF^{\Delta ARE/+}$ and wild type (WT) C57BL/6 mice (n = 5 per treatment/genotype group) were fed gelatin pellets (15 % w/v gelatin and 20 % sucrose) with or without CAPE (10 mg/kg body weight) three times a week for six weeks in addition to their usual diet [Ruiz and others, 2007]. Mice were killed by cervical dislocation at the age of 18 weeks and samples prepared for histological scoring and intestinal epithelial cell isolation according to the described method [Ruiz and others, 2007].

3.14.1 Histology

Six weeks after CAPE feeding, mice were killed and the distal ileum fixed in a 4 % formaldehyde solution for 24 h and then transferred to a 70 % ethanol solution. The samples were then dehydrated using the Leica TP1020 tissue processor (Leica microsystems) and

embedded in paraffin. Fixed tissues were sectioned at 5 µm then transferred to a glass-slide and stained with hematoxylin and eosin (HE) using the Multistainer Leica ST5020 (Leica microsystems), before examination using a light microscope. The inflammatory state of each ileal section was characterised and scored for leukocyte infiltration in the Mucosa, Sub-mucosa and Muscularis and Epithelial damage (crypt loss) on a scale of 0- 3 (0 =.no inflammation and 3= severe inflammation). The overall histological score was the sum total of all the four sections (0, not inflamed, to 12, highly inflamed).

3.14.2 IEC isolation

After killing the animals the intestine was washed in PBS and kept in a petri dish containing DMEM. The colon and caecum was cut longitudinally and the gut contents removed using an inoculation loop. The colon was then washed to remove debris in PBS and kept in DMEM. The same procedure was done for the ileum. The colon and caecum was cut into 0.5 cm pieces and put in a 50 ml tube with 20 ml DMEM. 20µl of 1M DTT was added to each 50ml tube with DMEM and vortexed for 1 min followed by incubation for 15min at 37°C in a thermo shaker (200 rpm). The tube was vortexed for 1 min before sieving the tissues. The filtrate was centrifuged at 300 g (4° C) for 7min. The pellet was resuspended in 5 ml DMEM and kept on ice. A percoll gradient was prepared by addition of 3ml of 20 % Percoll solution to a 50 ml tube before another 3 ml of 40 % Percoll solution was introduced gently under the 20 % Percoll solution using a Pasteur pipette to form a gradient (a clear visible line between the two). The cell suspension was carefully pipette on top of the Percoll-gradient and centrifuged at 600 g (4°C) for 30 min. The resultant distinct band of IEC between the Percoll – gradient was sucked out using a Pasteur pipette and centrifuged again (~2min, 1,000 g, 4°C). The pellet was divided into two portions and kept at -80°C for subsequent RNA isolation and protein analysis.

3.15 Statistical analysis

All experiments were performed at least six times in triplicate unless noted otherwise in the figure legend. Values were expressed as mean ± SD of triplicate measurements representative of at least two independent experiments. Statistical analyses were performed using SigmaPlot 11 (Systat Software Inc.). Mean values were compared by ANOVA (data were tested for normal distribution and equality of variances). The Holm-Sidak test was used for pairwise comparisons. For all tests, the bilateral alpha risk was $\alpha = 0.05$.

CHAPTER FOUR

RESULTS

4.1 Function versus structure-activity of CAPE

4.1.1 Model pattern of CAPE research

The PUBMED library was screened for publications containing “Caffeic Acid Phenethyl Ester” in their title. The pace of research involving CAPE has accelerated over the last decade. To illustrate the steady growth of this area the number of annual publications containing ‘Caffeic Acid Phenethyl Ester’ in the title as indexed by PubMed is shown in Figure 4. The number of researchers working with CAPE was small between 1990 and 2000, however there was a dramatic increase in the subsequent years, suggesting that CAPE was efficacious and had more than one molecular target. The fundamental observation that we can derive from this search is that CAPE has some merit as a potential bioactive molecule to trigger research interests from various fields of science.

4.1.2 Evaluation of the effect of CAPE structural modification

About 15 CAPE analogues (Figure 5) were screened for biological activity to determine the most active and less active molecule using a CHOP (C/EBP homologous protein) gene reporter assay. CHOP is an endoplasmic reticulum stress-inducible protein that plays a critical role in the regulation of apoptosis. All of the analogues were synthesized in the laboratory with the exception of P3MC and PDC which were bought commercially. The synthesis involved methoxylation and amidation of certain bonds as well as bond hydrolysis and hydroxylation. The rationale behind analogue synthesis was based on a principle which states that structurally similar compounds are more likely to exhibit similar properties. The CHOP gene reporter assay in HEK 293 revealed that CAPE dose-dependently induced CHOP expression and it was the most active when compared to the analogues (Figure 7). Interestingly, CAPE effectively induced CHOP expression in low doses (50 µM) in comparison to known cellular stress response inducers including butyrate, thapsigargin and tunicamycin. (Figure 7B) Low molar concentration ranges of 1-5 µM did not induce the CHOP gene (Figure 6A); however, increasing concentration 10-50 µM induced the gene (Figure 7). Compounds 8 and 13 were found to be less active in comparison to CAPE, CAPEA and 6, probably due to transformation to the phenyl propionic acid functional group (Figure 6B). Compound 15 was also found to be inactive probably due to multiple substitution effect (methoxylation, amidation and hydroxylation).

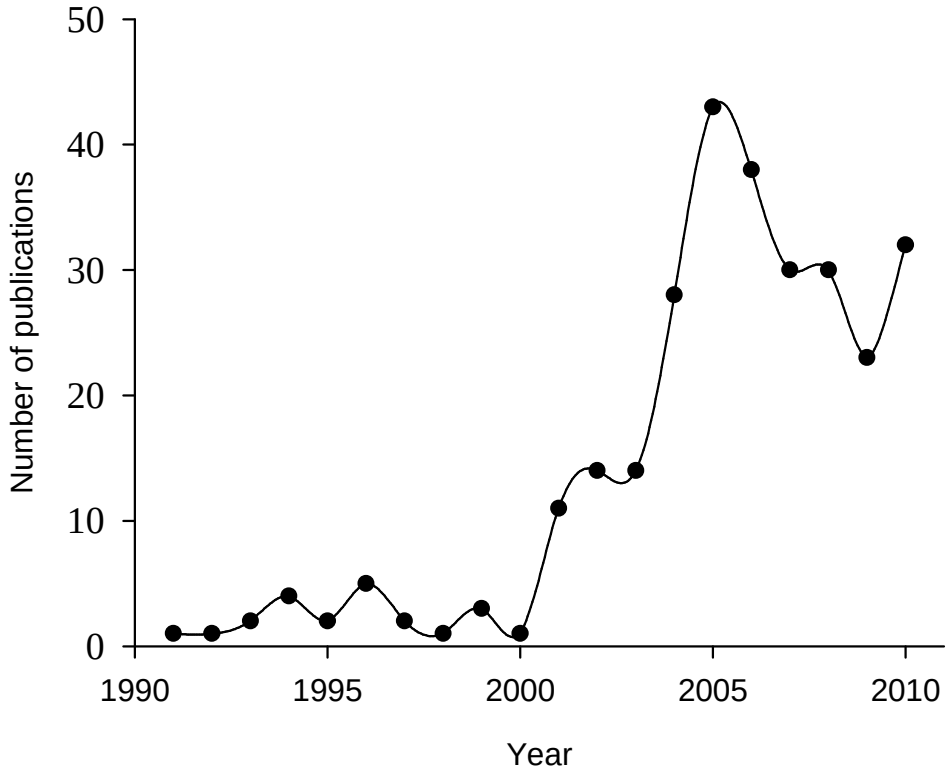

Figure 4. Annual publications containing ‘Caffeic Acid Phenethyl Ester’ in the title

This molecular similarity concept derived from structural cohesiveness (Figure 5) led to selection of the five compounds that exhibited high, medium to low biological activity (Figure 8A). Substitution of OH at C3 or C4 with CH_3 reduces activity while methoxylation of both hydroxyl groups on C3 and C4 of the catechol ring in CAPE results in reduced activity and loss of biological function (Figure 8B). Substitution of the ester bond with an amide group at O is tolerable (there is retention of activity), although the esters (CAPE) were found to be much more active than amides (CAPEA). Additional modifications on the phenol ring (CAT) were shown to result in reduced activity.

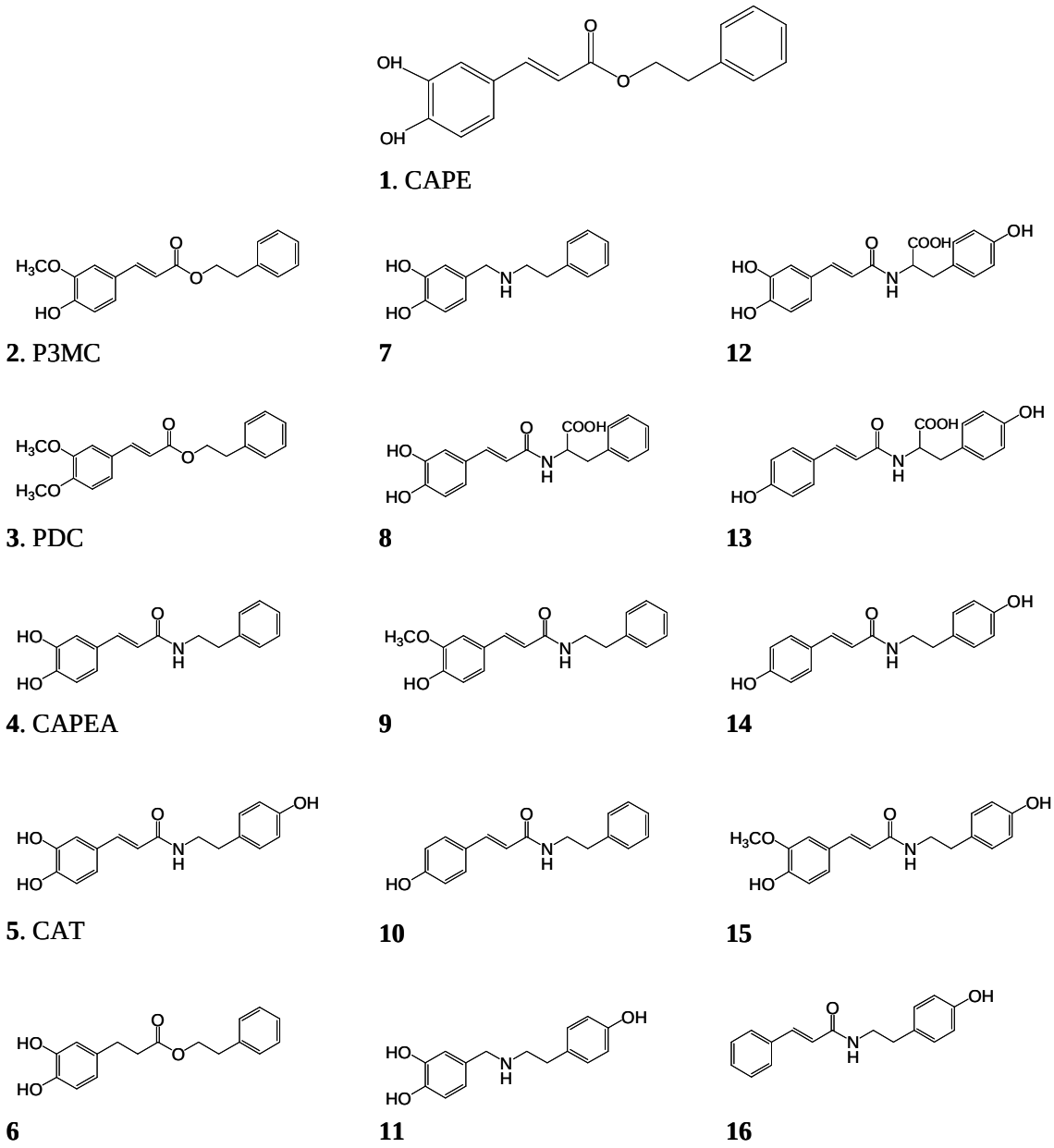

Figure 5. Structural analogs of Caffeic Acid Phenethyl Ester

CAPE, P3MCand PDC were commercially obtained while the rest of the analogues (compound 4-16) were synthesized in the laboratory with reference to CAPE as the parent molecule.

(**1**) 3-(3,4-Dihydroxy-phenyl)-acrylic acid phenethyl ester
(**2**) 3-(4-Hydroxy-3-methoxy-phenyl)-acrylic acid phenethyl ester
(**3**) 3-(3,4-Dimethoxy-phenyl)-acrylic acid phenethyl ester
(**4**) 3-(3,4-Dihydroxy-phenyl)-N-phenethyl-acrylamide
(**5**) 3-(3,4-Dihydroxy-phenyl)-N-[2-(4-hydroxy-phenyl)-ethyl]-acrylamide
(**6**) 3-(3,4-Dihydroxy-phenyl)-propionic acid phenethyl ester
(**7**) 4-(Phenethylamino-methyl)-benzene-1,2-diol
(**8**) 2-[3-(3,4-Dihydroxy-phenyl)-acryloylamino]-3-phenyl-propionic acid
(**9**) 3-(4-Hydroxy-3-methoxy-phenyl)-N-phenethyl-acrylamide
(**10**) 3-(4-Hydroxy-phenyl)-N-phenethyl-acrylamide
(**11**) 4-[2-(4-Hydroxy-phenyl)-ethylamino]-methyl-benzene-1,2-diol

(**12**) 2-[3-(3,4-Dihydroxy-phenyl)-acryloylamino]-3-(4-hydroxy-phenyl)-propionic acid
(**13**) 3-(4-Hydroxy-phenyl)-2-[3-(4-hydroxy-phenyl)-acryloylamino]-propionic acid
(**14**) 3-(4-Hydroxy-phenyl)-N-[2-(4-hydroxy-phenyl)-ethyl]-acrylamide
(**15**) 3-(4-Hydroxy-3-methoxy-phenyl)-N-[2-(4-hydroxy-phenyl)-ethyl]-acrylamide
(**16**) N-[2-(4-Hydroxy-phenyl)-ethyl]-3-phenyl-acrylamide

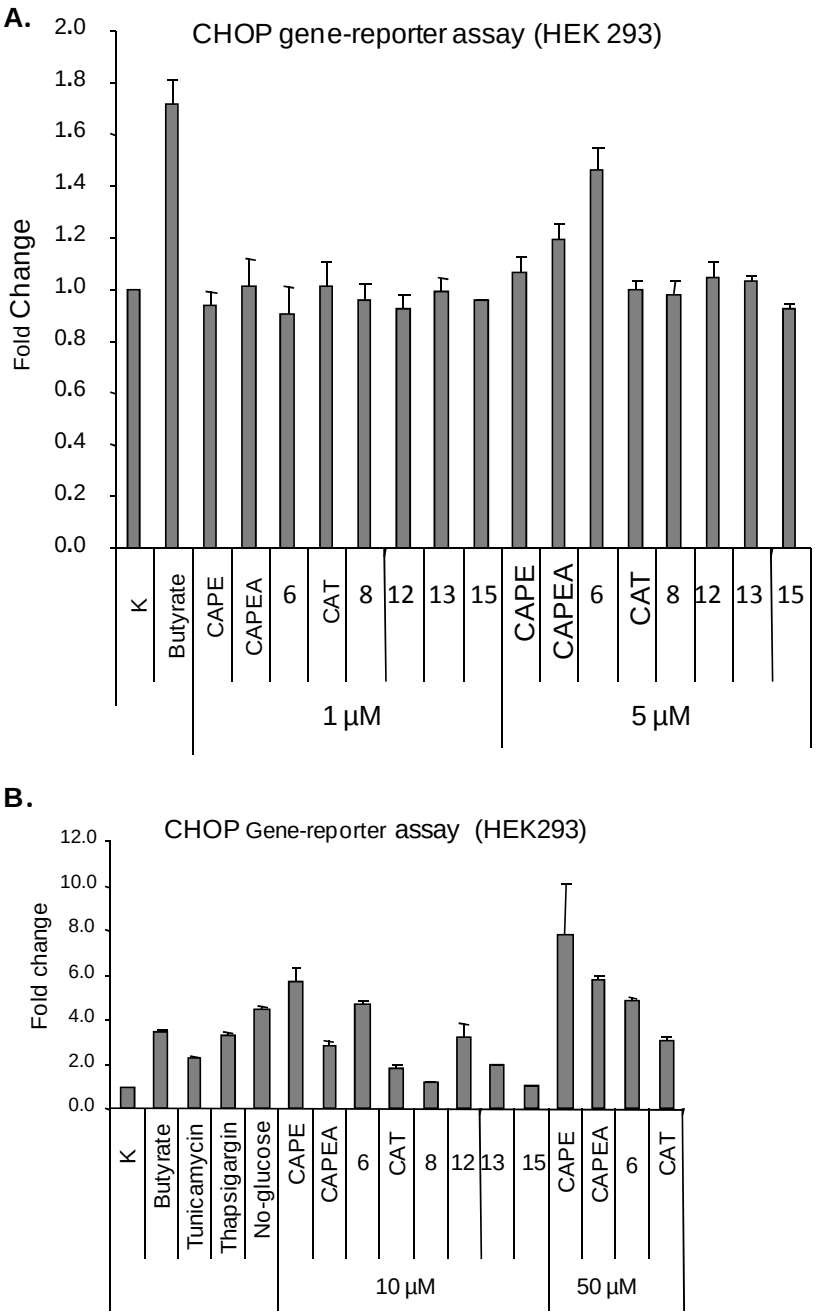

Figure 6. Effect of test compounds on CHOP gene reporter in HEK 293 cells
The potential of test compounds to induce gene expressions was tested in HEK 293 stably transfected with a CHOP (C/EBP homologous protein) gene. Cells were treated with the increasing concentrations of test compounds for 24 h (**A** & **B**) followed by fluorescence scan.

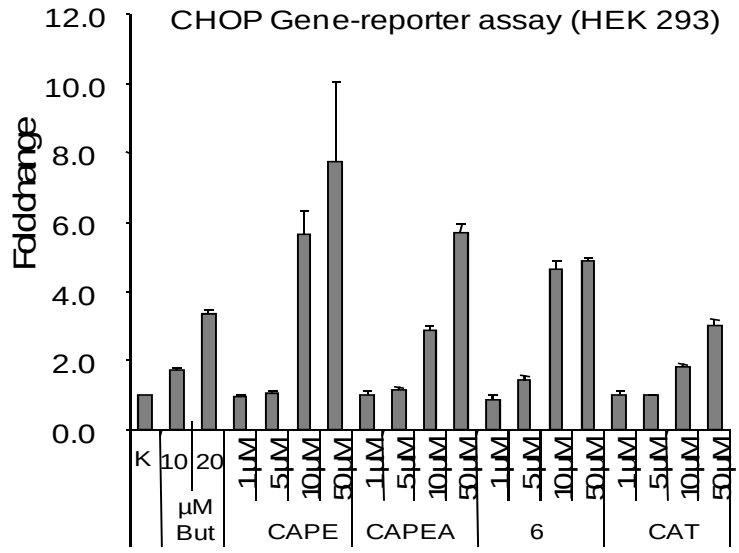

Figure 7. CAPE is more bioactive than the modified analogue (CAPEA)
CAPE induced over-expression of CHOP six-fold as opposed to CAPEA (four-fold) and CAT (two-fold) at 10 µM, and it was more potent than Butyrate, a known CHOP inducer.

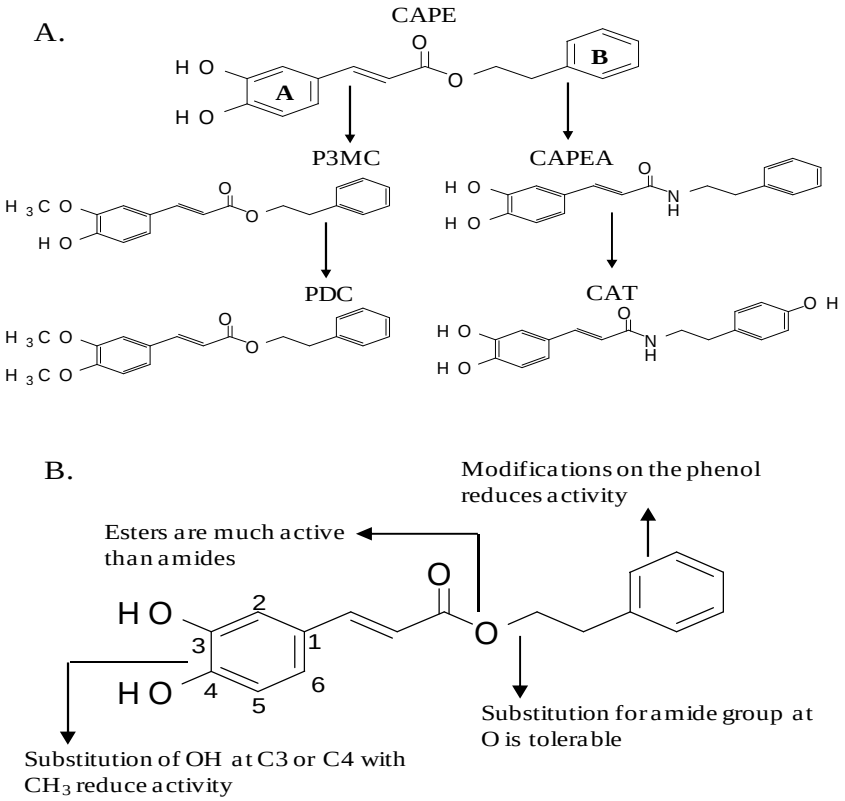

Figure 8. Substitution and modification of the CAPE parent molecule
(**A**) Chemical structures of CAPE and analogues. Phenethyl 3-methyl caffeate (P3MC) was substituted with one methoxyl, while Phenethyl dimethyl caffeate (PDC) had two substitutions on the hydroxyl groups of the catechol ring (A). Caffeic acid phenethyl amine (CAPEA) was prepared by substituting the ester with an amide, while Caffeic acid tyramine (CAT) was prepared by adding one extra hydroxyl on the benzene ring (B) of CAPEA.
(B), Shows the functional effects as a result of CAPE modifications.

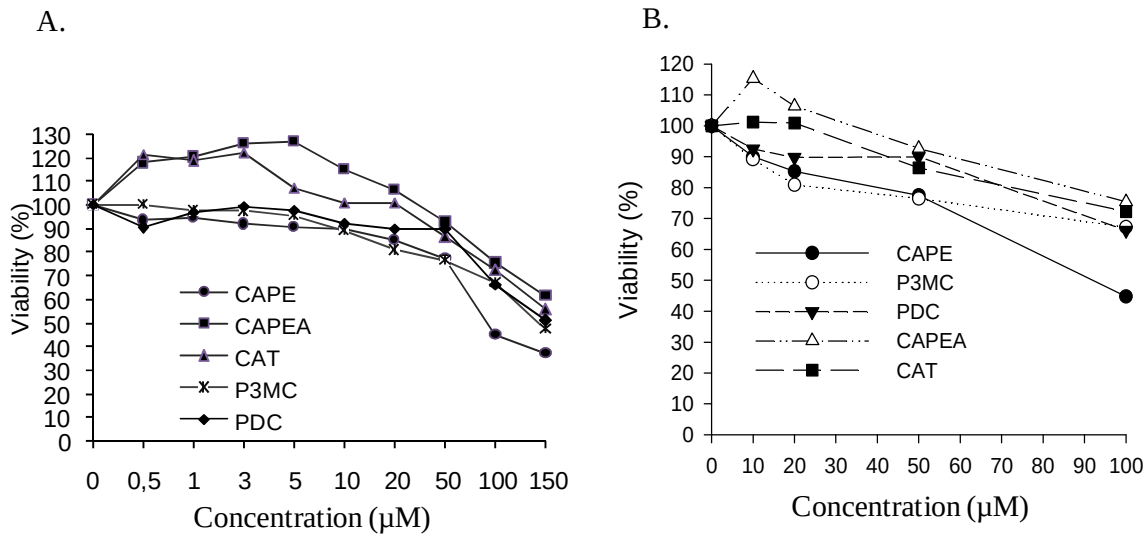

Figure 9. Cytotoxic effect of CAPE and its analogues in Mode K cells
Mode-K cells were incubated with the indicated compound concentrations for 24 h followed by WST-8 cell viability assay to determine the dose response

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4.1.3 Determination of the cytotoxic effect of CAPE on epithelial cells

To determine the effect on CAPE on cellular proliferation, a viability and cytotoxicity dose response test was carried out in Mode-K cells using WST-8 reagent. The tested compounds had no adverse proliferative effects in the sub-optimal molar concentration below 10 µM (Figure 9A). Most of the compounds exhibited cytotoxic capacities at high concentrations (above 50 µM). CAPE was potent than CAPEA at 100 µM (Figure 9), indicating that high concentrations of CAPEA can be tolerable to the cells while maintaining biological activity. CAPE dose dependently inhibits proliferation of both Mode-K (intestinal epithelial cells) and RAW 264.7 macrophages (immune cells); however the macrophages are more susceptible to the stimuli when compared to intestinal epithelial cells. 3 µM CAPE was sufficient to induce cytotoxity and was extremely cytotoxic above 5 µM in RAW 264.7 as opposed to Mode-K (Figure 10). These observations lead to the determination of the half maximal inhibitory concentrations 50 (IC_{50}) of all five compounds. The IC_{50} values were in the micromolar (cytotoxicity) and nanomolar (IP-10 inhibition) range and showed that CAPE was most potent (Table 2) suggesting that, even at low concentrations (< 1 µM) CAPE could inhibit IP-10 expression without compromising cell viability or prolifereation.

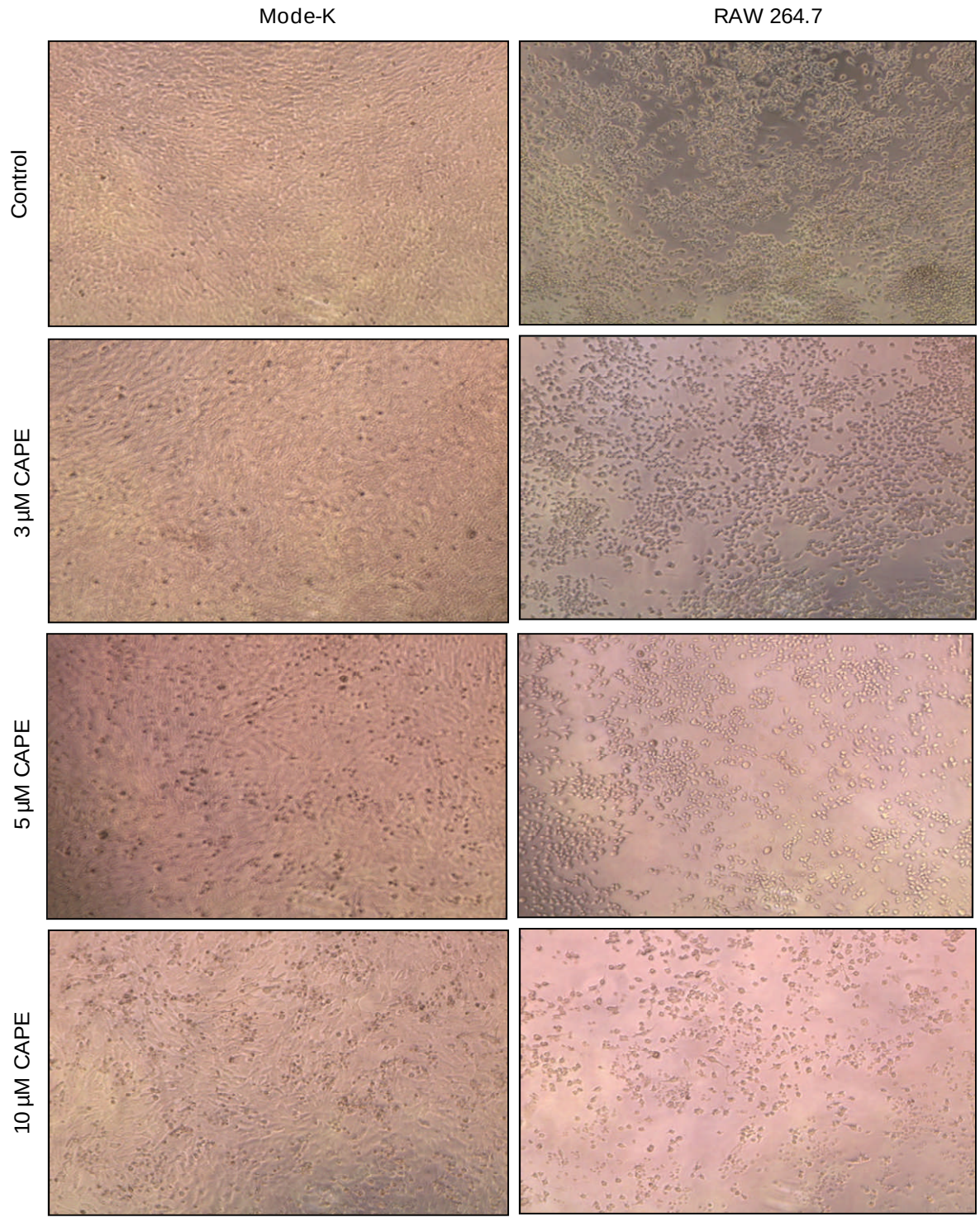

Figure 10. Effect of CAPE on epithelial (Mode-K), and immune (RAW 264.7) cells
A dose response effect of CAPE on Mode-K (left panel) and RAW 264.7 macrophages (right panel) proliferation after 48 hours of incubation was determined as indicated. The reddish background is due to the DMEM cell culture medium.

4.1.4 Effect of hydroxyl groups on the anti-inflammatory effects of CAPE

To investigate the structure-activity relationship of CAPE, a variety of analogues (Figure 8) were examined and their activity determined via measurement of cell viability and TNF-induced IP-10 expression in Mode K cells. CAPE inhibited IP-10 induction in a dose dependent manner, *i.e.*, both intracellular and secreted IP-10 protein levels were reduced in the presence of CAPE (Figure 11). Since a marked and reproducible inhibition was obtained using 10 µM and the test compounds were less cytotoxic at this concentration (80 % viability after 24 h) (Table 2), 10 µM was used as the concentration of reference in all subsequent experiments, unless otherwise stated. To determine how long CAPE sustained inhibition of TNF-induced IP-10 production, time-course experiments were performed. CAPE abolished IP-10 expression from 3 to 24 h of TNF stimulation, indicating that CAPE mediated inhibitory functions occurred at early time points and persisted over time (Figure 11).

To assess the extent of activity control by the chemical structure; CAPE analogues were tested whether they had similar inhibitory effects. Both ELISA and western blot analyses revealed that CAPE, P3MC and CAPEA significantly reduced IP-10 expression ($p < 0.05$), while PDC and CAT were significantly less active (Figure 13). The gradual decrease in cytotoxic IC_{50} CAPE> P3MC> PDC (Table 2) suggested that the catechol hydroxyl groups in ring A define functional effects, *i.e.*, the presence of one hydroxyl group retains activity while substitution of both hydroxyl groups in ring A leads to a loss of activity (Figure 8). Also, substitution of the ester functional group with an amide group (CAPEA) revealed only limited functional consequences, *i.e.*, led only to a slight decrease in IC_{50}. However, further modification of CAPEA to CAT by addition of a hydroxyl group to ring B markedly reduced the IC_{50} (Table 2 and Figure 13A). CAPE (Figure 12), as well as P3MC and CAPEA, did not affect the expression of another TNF-induced chemokine of the CXC family, namely macrophage inflammatory protein 2 (MIP-2), to the same extent as IP-10, i.e., return to basal levels as in the absence of TNF (Figure 13B).In addition CAPE, CAPEA and P3MC inhibited secretion of TNF-induced IL-6 but PDC and CAT did not (Figure 13D), indicating that slight structural modifications had great functional consequences with regard to cellular responses.

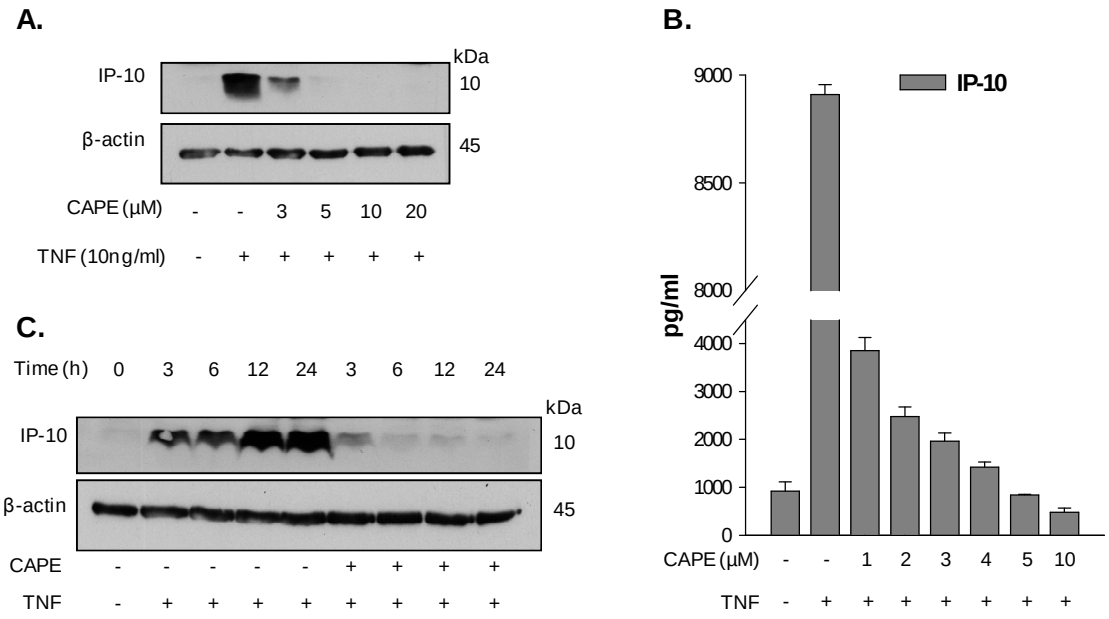

Figure 11. CAPE inhibits IP-10 expression in Mode-K cells
Mode-K cells were incubated with increasing CAPE concentration for 1 h followed by TNF for 16 h as indicated (**A**), Intracellular protein (**B**), Secretory protein. (**C**) Mode-K cells were pre-incubated with 10μM CAPE at the indicated time points.

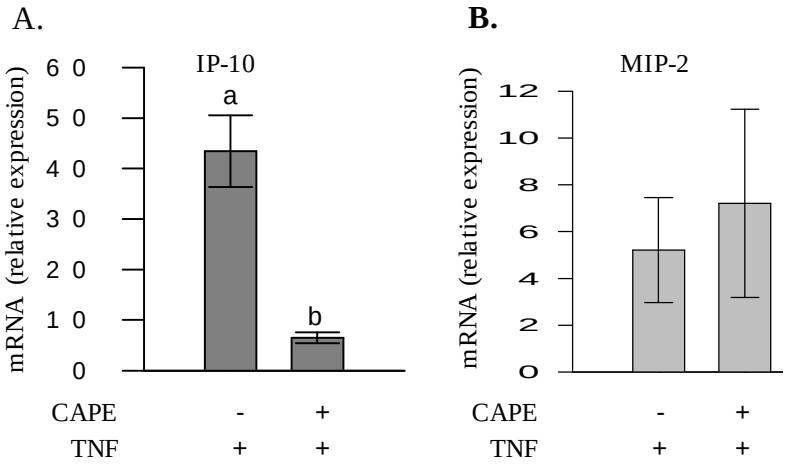

Figure 12. CAPE inhibits IP-10 but not MIP-2 mRNA expression
Mode-K cells were pre-treated with 10 μM CAPE for 1 h followed by 10 ng/ml of TNF for additional 4 h before RT-PCR analysis.

Table 2. The half maximal inhibitory concentration 50 (IC50) of CAPE and its analogues for cell cytotoxicity and IP-10 inhibition in Mode-K cells

Compound	Cytotoxicity (μM)	IP-10 inhibition (μM)
CAPE	61.0	0.85
P3MC	76.8	1.78
PDC	> 100.0	> 100.0
CAPEA	85.2	0.98
CAT	90.2	27.91

IC_{50} values were generated from three independent experiments (CAPE concentrations in the range of 0 to 200 μM) using the Four Parameter Logistic Equation in SigmaPlot 11 (Systat Software Inc.).

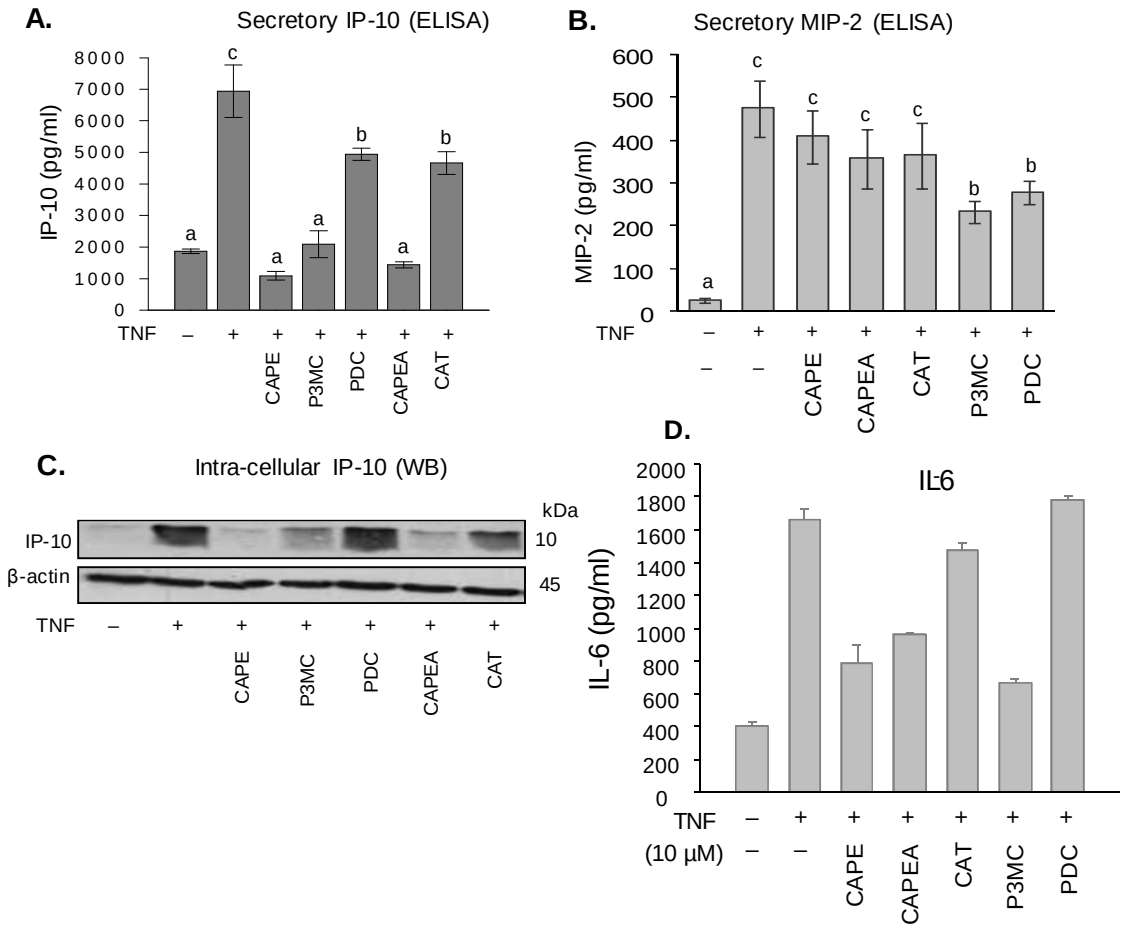

Figure 13. Effect of compound modification on IP-10, MIP-2 and IL-6 expression
Mode-K cells were pre-treated with 10 μM of test compounds for 1 h followed by TNF for additional 24 h before ELISA and WB analyses for (**A & C**) IP-10, (**B**) MIP-2 and (**D**) IL-6.

4.2 CAPE versus NF-κB signalling

4.2.1 Effect of CAPE on IκBα phosphorylation/degradation

The most important mechanism that prevents spontaneous NF-κB activation is the cytoplasmic retention via IκBα. IκBα phosphorylation, ubiquitination and subsequent proteasomal degradation is essential for full NF-κB activation. To further investigate molecular mechanisms of CAPE-mediated inhibition of the NF-κB pathway, TNF-induced IκBα degradation and RelA phosphorylation was determined. CAPE neither inhibited IκBα degradation nor RelA phosphorylation (Figure 14). Even at increasing concentrations, CAPE did not reverse the IκBα degradation process after 30 min of TNF stimulation (Figure 14A).

It has been shown that IκBα expression is induced by NF-κB providing a negative feedback that terminates NF-κB activation [Nelson and others, 2004]. However, NF-κB activation is known to be biphasic following
TNF stimulation (the initial phase lasts one hour followed by a persistent second phase that depends on the input stimuli concentration), and some genes require persistent TNF stimulation to be fully activated [Nelson and others, 2004]. To investigate the influence of CAPE on prolonged TNF stimulation, IκBα phosphorylation and degradation leading to NF-κB activation over eight hours was monitored. The results showed that CAPE neither altered the extent of TNF-induced RelA phosphorylation nor IκBα phosphorylation/degradation (Figure 14). The relA NF-κB subunit can be phosphorylated by kinases on serine 276 and 536 that consequently determines the lysine acetylation site that regulates discrete biological actions of the NF-κB complex [Chen and Greene, 2004, Chen and others, 2002]. Therefore the effect of CAPE on serine 276 and 536 was determined. After 16 h of treatment, CAPE did not inhibit Ser^{276} and Ser^{536} phosphorylation (Figure 14D). The effect of CAPE structural modification was also determined and none of the test compounds inhibited relA phosphorylation or I-κBα degradation (Figure 14E).

CAPE and BAY-11-7082 were compared with respect to their ability to inhibit NF-κB activity. BAY-11-7082 selectively inhibits NF-κB activation by blocking TNF-induced degradation of IκBα without affecting constitutive IκBα phosphorylation. The results confirmed that CAPE does not inhibit TNF induced IκBα phosphorylation or its subsequent degradation (Figure 15A). Surprisingly, CAPE enhanced accumulation of phosphorylated p38 MAPK in the nucleus in comparison to TNF Apart from inhibiting NF-κB activation, BAY-11-7082 also has the ability to inhibit nuclear translocation of p-p38 MAPK (Figure 15B). This is in accordance with [Miyamoto and others, 2010] who showed that BAY-11-7082

interfered with nuclear translocation of IRF7 and type I interferon production by plasmacytoid dendritic cells suggesting that CAPE most probably has target specificity.

4.2.2 Effcet of CAPE on relA nuclear translocation

Because the key molecular mechanism in NF-κB activation is translocation of relA to the nucleus following TNF stimulation it was assessed as to whether CAPE targets nuclear translocation of relA using immunoflourescent confocal microscopy experiments. In comparison to non-treated cells, CAPE treatment had no effect on relA translocation as demonstrated by immunofluorescence analysis (Figure 16). Indeed, relA was localized in the cytoplasm in non-treated cells and quickly shifted into the nucleus following TNF stimulation, even when cells were pre-incubated with CAPE.

These observations pointed at a process in which NF-κB activity is controlled at the level of transcription factor binding to the gene promoter and chromatin remodelling. To confirm that CAPE inhibits NF-κB recruitment to the promoter, Mode-K cells were stably transfected with pNiFty plasmid expressing the SEAP gene in the ELAM1 composite gene promoter. The effect of structural modifications on promoter activity using the SEAP reporter assay was also examined. Compared with control samples, SEAP expression was up-regulated after TNF administration. However, it was significantly reduced after treatment with CAPE and CAPEA, but not P3MC, PDC and CAT (Figure 17). Importantly, CAPE completely abolished NF-κB transactivation revealing that the catechol hydroxylic groups are essential for its bioactivity.

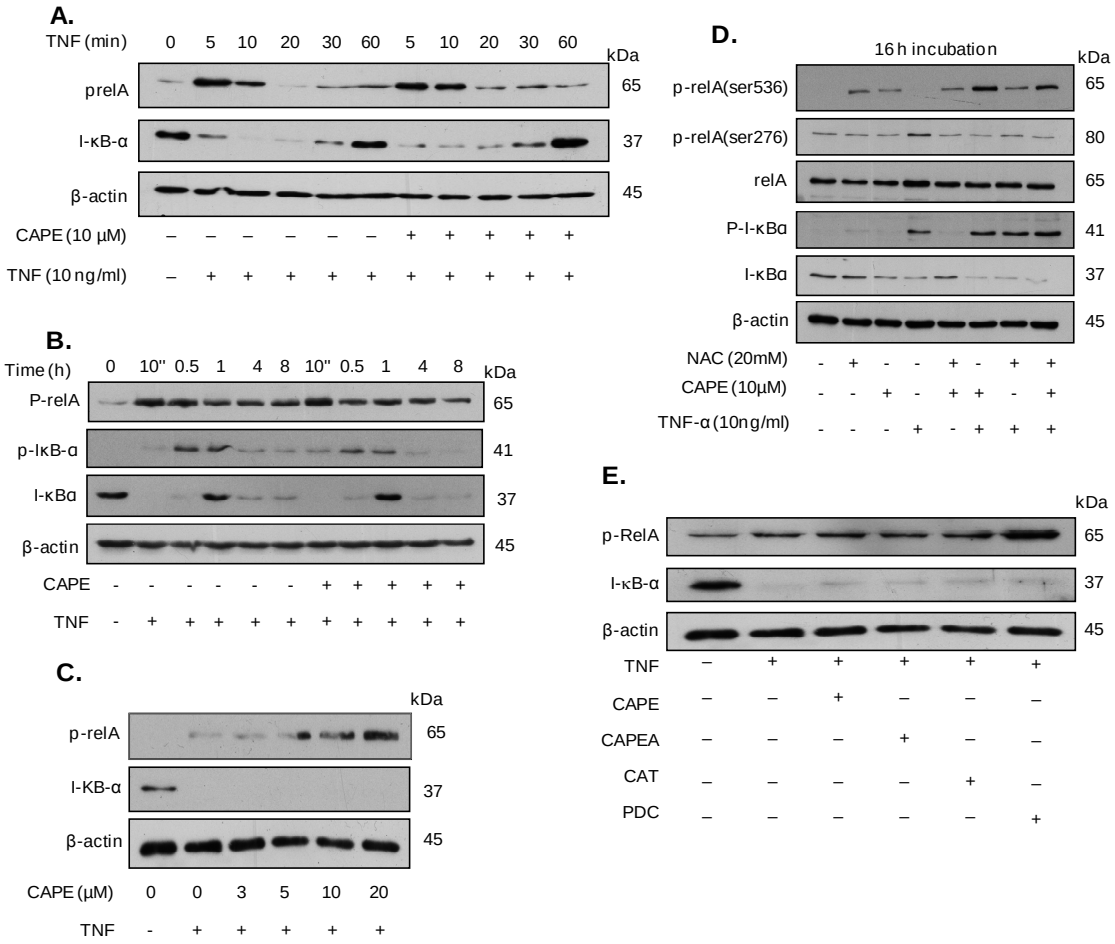

Figure 14. Effect of CAPE on RelA/p65 phosphorylation and I-κB degradation
CAPE neither inhibited TNF-induced IκBα degradation nor relA/p65 phosphorylation. (**A** & **B**) Time course for Mode-K cells treated with or without CAPE for 1 h followed by TNF induction. (**C**) Dose dependence for Mode-K cells treated with or without CAPE for 1 h followed by TNF for 30 min. (D) CAPE did not inhibit p-relA^{ser536} nor p-rel-A^{ser276} after 16h induction, and (E) Analogues had no influence on relA phosphorylation either.

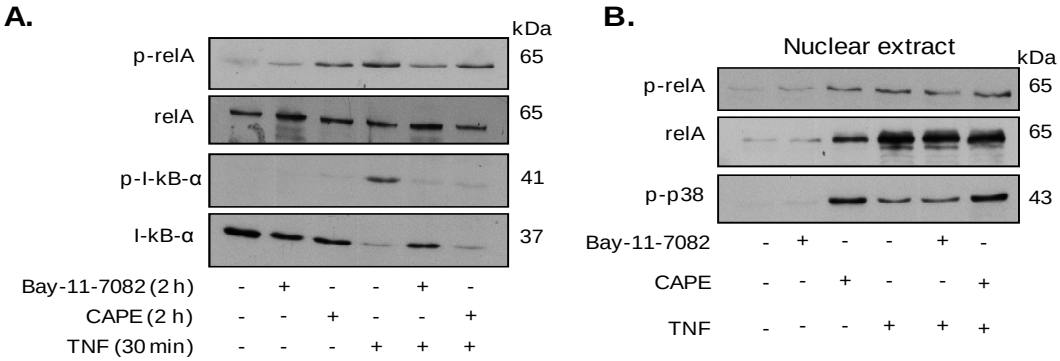

Figure 15. CAPE on I-κB-α degradation; and nuclear p-p38 MAPK accumulation
(A) Mode-K cells were pre-treated with Bay-11-7082 (an I-κB-α phosphorylation inhibitor) and CAPE followed by TNF at the indicated time. (B) The nuclear extract was immunoblotted against relA and p-p38 MAPK antibodies.

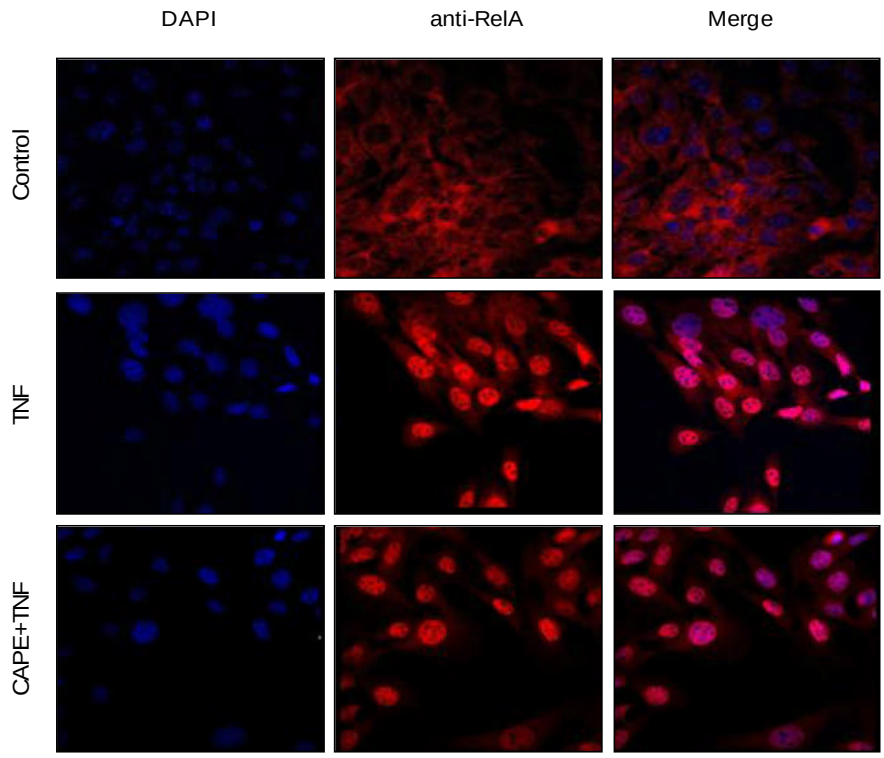

Figure 16. Effect of CAPE on p-relA/p65 nuclear translocation
Confocal immunofluorescence images (400 x) of cells labelled with anti-relA (red) showing cytoplasmic staining (control) and nuclear translocation (TNF-stimulated) and DAPI (blue) signals were generated from Mode K cells treated with or without CAPE for 1 h followed by TNF for 20 min.

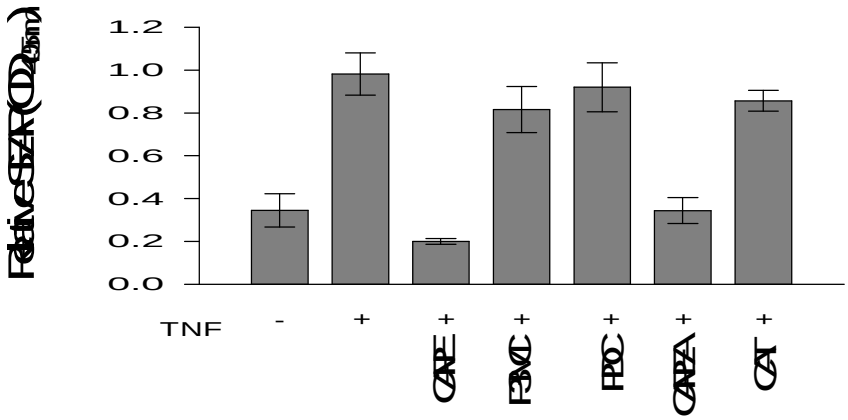

Figure 17. Effect of CAPE on NF-κB recruitment to its promoter sites.
CAPE targets the NF-κB site of the ELAM1 promoter. Mode K cells were stably transfected with a pNiFty plasmid expressing the SEAP gene under the control of an NF-κB-inducible ELAM1 composite promoter. Transfectants were pre-incubated for 1 h with 10 μM of indicated compounds before TNF stimulation for additional 24 h.

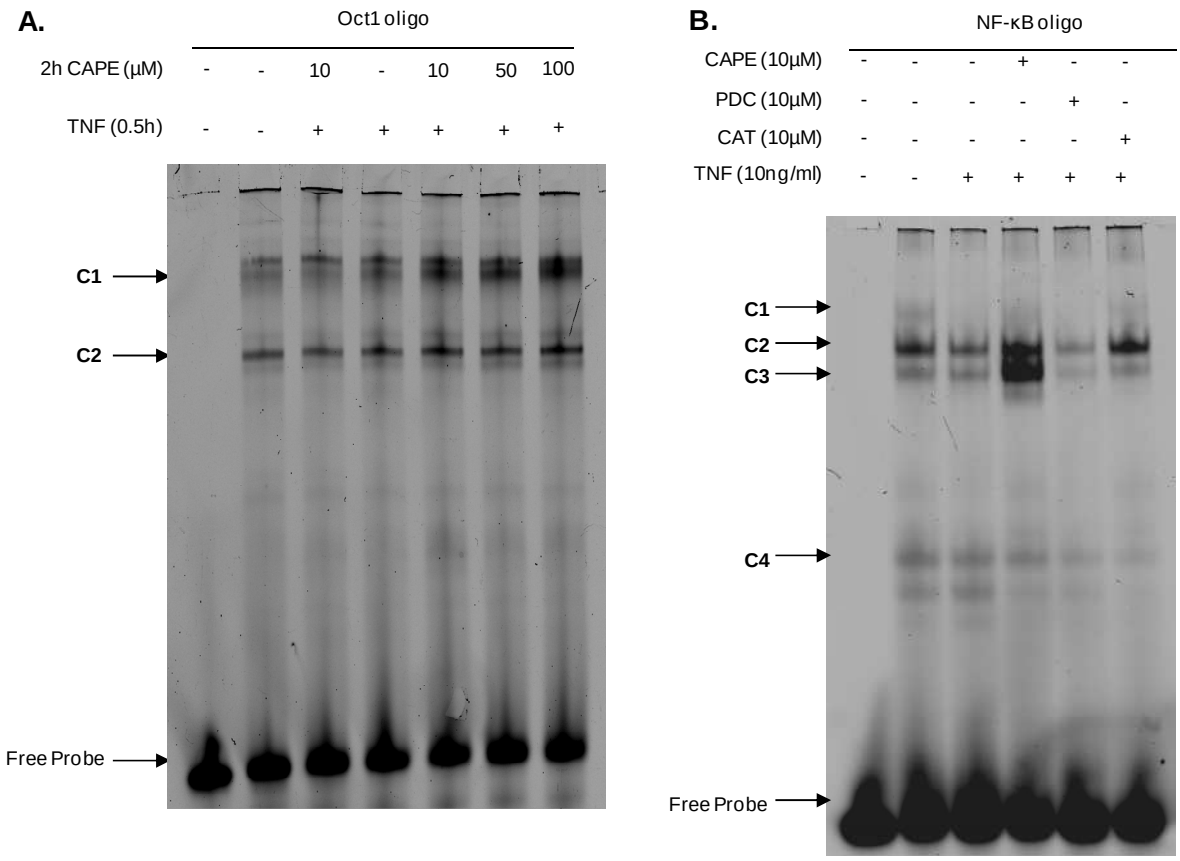

Figure 18. CAPE enhances accumulation of NF-κB binding proteins in the nucleus.
Mode-K cells were pre-treated with test compounds for 2 h followed by 30 min TNF. Nuclear extracts were prepared for EMSA and visualised using a fluorescent labelled dye (cy5). (**A**) Oct-1 oligonucleotide was used as the control [complex 1(C1) and 2 (C2)] because of relative abundance and stability in the nucleus. (**B**) CAPE enhances accumulation of NF-κB consensus sequence binding proteins as shown in lane 4, complex 2 (C2).

4.2.3 Effect of CAPE in accumulation of NF-κB consensus sequence binding proteins

The binding was detected using a Cy5-labeled oligonucleotide against Oct-1 gene sequence for experimental control because of its relative abundance and the NF-κB consensus gene sequence (Figure 18).Following the binding assay CAPE enhanced accumulation of complex 2 (C2) as opposed to the control sample, PDC and CAT (Figure 18B) suggesting that CAPE's structural features (the catechol ring) has some impact on the nuclear activity. To confirm the NF-κB specific binding a 100x cold oligonucleotide (unlabelled NF-κB) and 100x NF-κB mutant oligonucleotide were included as a specificity control (Figure 20). Nuclear extracts were subjected to cold competition to determine competitive binding from other proteins. Unlabelled excess (100x) NF-κB consensus sequence (cold competition) revealed that C1, C2, C3 & C4 (lanes 8- 13) were NF-κB- isomers. A mutant excess (100x) non- NF-κB consensus sequence, lanes 14- 19 revealed that C1and C3 proteins have a high affinity for the NF-κB consensus sequence. High concentration of CAPE (100 μM) enhanced the

accumulation of C2 and C3 as well as C4, in lane 6 (Figure 19A). C4 could probably be a degradative protein of the NF-κB family of proteins because of its low molecular weight. Following this observation Mode-K cells were incubated with CAPE for 4 h to determine if the effect of time. In deed there was persistence of C3 in CAPE treated cells, lane 6. Surprisingly, TNF stimulation did not have a robust effect on C3 accumulation when compared to CAPE; lanes 3 and 5 (Figure 19B). However, TNF alone induced accumulation of C4 more than CAPE, lane 4 (Figure 19A) and lane 5 (Figure 19C).To determine the composition of the NF-κB-oligonucleotide complexes, a western blot of the nuclear extract was performed. 100 µM CAPE (Figure 19B) as well as long incubation hours with 10 µM CAPE (Figure 19D) consistently enhanced the concentration of both p105 and its phosphorylated form. Although the effect on phosphor-p65 is inconclusive, the influence of CAPE on p65/relA was remarkable. CAPE drastically reduced the amount of TNF-induced p65/relA in the nucleus with concomitant p105 accumulation (Figure 19D) suggesting that had a direct effect on the p50 precursor, p105 subunit. It is worthwhile to note that no apparent effect was seen on the p50 subunit indicating that CAPE probably targeted specific proteins. Interestingly, increasing amounts of phosphorylated p38 MAPK were observed on the same nuclear extracts, however, the concentration of IRF1 (NF-κB binding partner) was diminished (Figure 19D).

The p50/relA heterodimer is the most common inducible complex that is synonymous to NF-κB, therefore its functional consequence following CAPE stimulation was determined by immunoprecipitation and immunoblotting experiments. The immunoprecipitate revealed that CAPE had no effect on the p50/relA heterodimeric complex association (Figure 21) suggesting that the CAPE-mediated NF-κB inhibition is probably downstream of the NF-κB signalling pathway at the chromatin. .

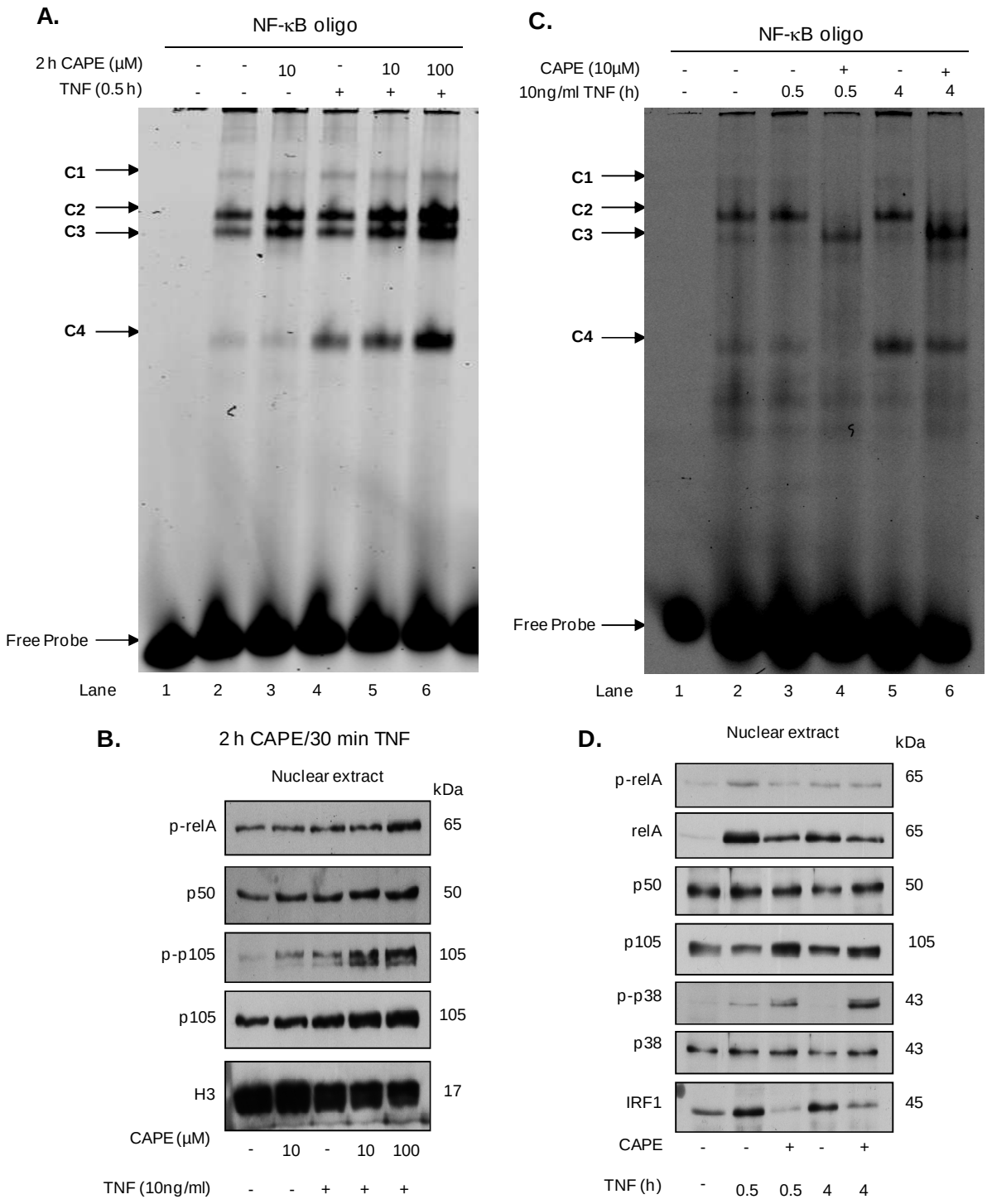

Figure 19. Effect of CAPE on p-p105, p-p38 MAPK and IRF1
(**A**) High concentration of CAPE enhanced C2 & 3 as well as a low molecular weight complex, C4 (lane 6). Western blots of the same nuclear extract (**B**) revealed high concentrations of p-p105 (NF-κB1). (**C**) CAPE enhanced processing of C2 to C3 [(low molecular weight) (lanes 4 & 6)]. (**D**) CAPE enhances accumulation of p-p38 and inhibition of IRF1 in the nucleus.

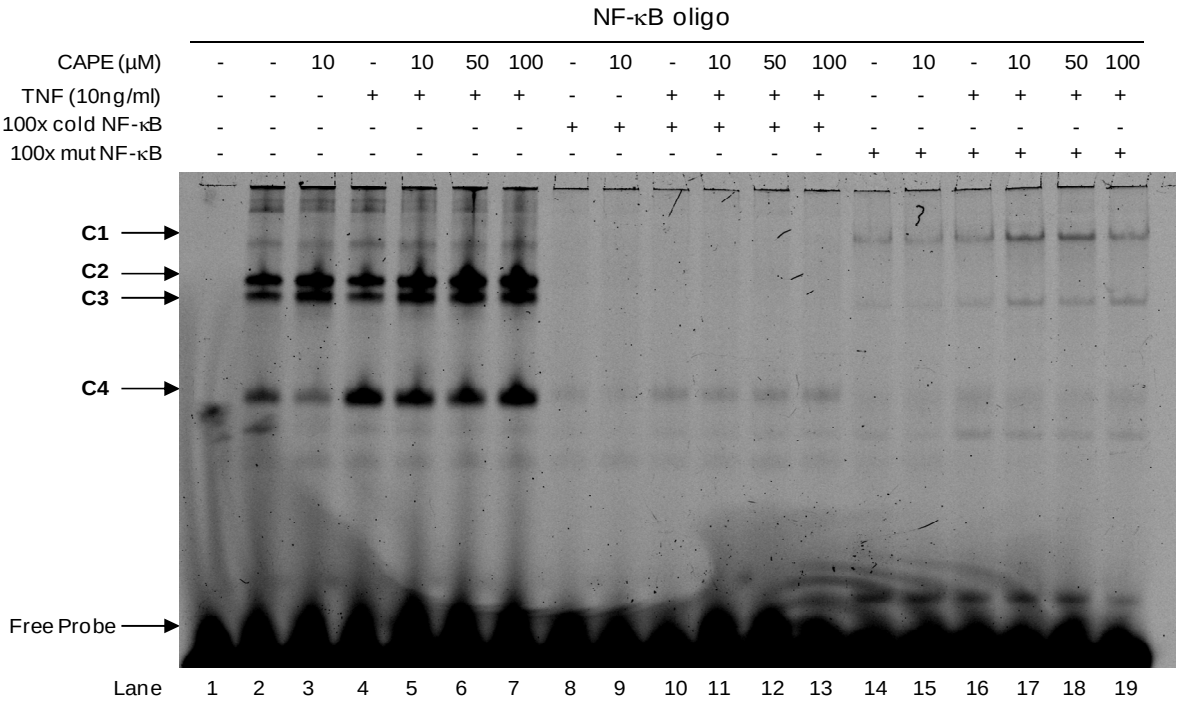

Figure 20. NF-κB consensus sequence cold competition effect

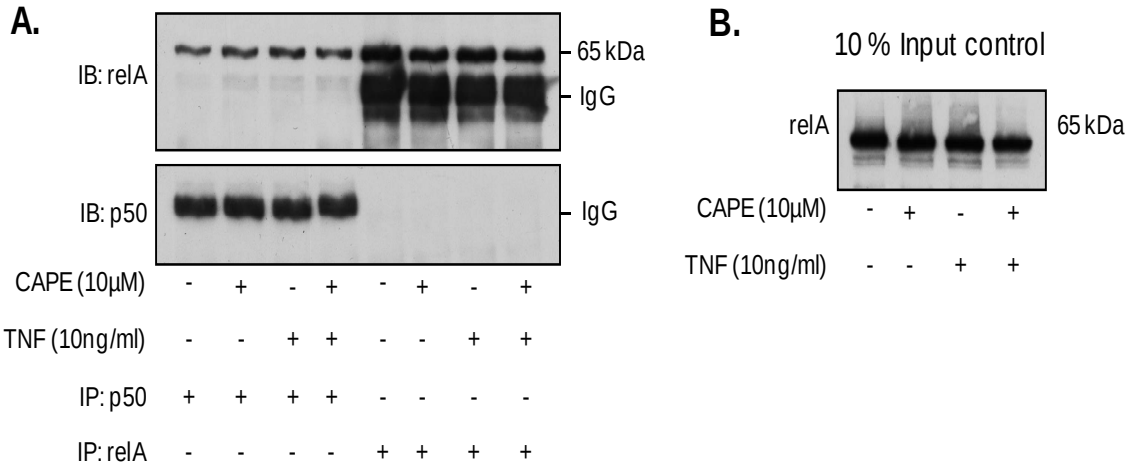

Figure 21. Influence of CAPE on p50/relA complex
Mode-K cells were pre-treated for 2 h with 10 μM CAPE followed by 30 min TNF, immunoprecipitated against relA and p50 NF-κB subunit antibodies followed by western blotting (**A**) and (**B**) input control.

In addition, the influence of CAPE on rel-B, p100, p105 and RelA mRNA expression was investigated. The results indicated that, in combination with TNF, CAPE induced the expression of p105; a p50 precursor (Figure 22C).CAPE time dependently induces phosphorylation of p105 (Figure 22E), however it does not induce p105 mRNA expression (Figure 22F).

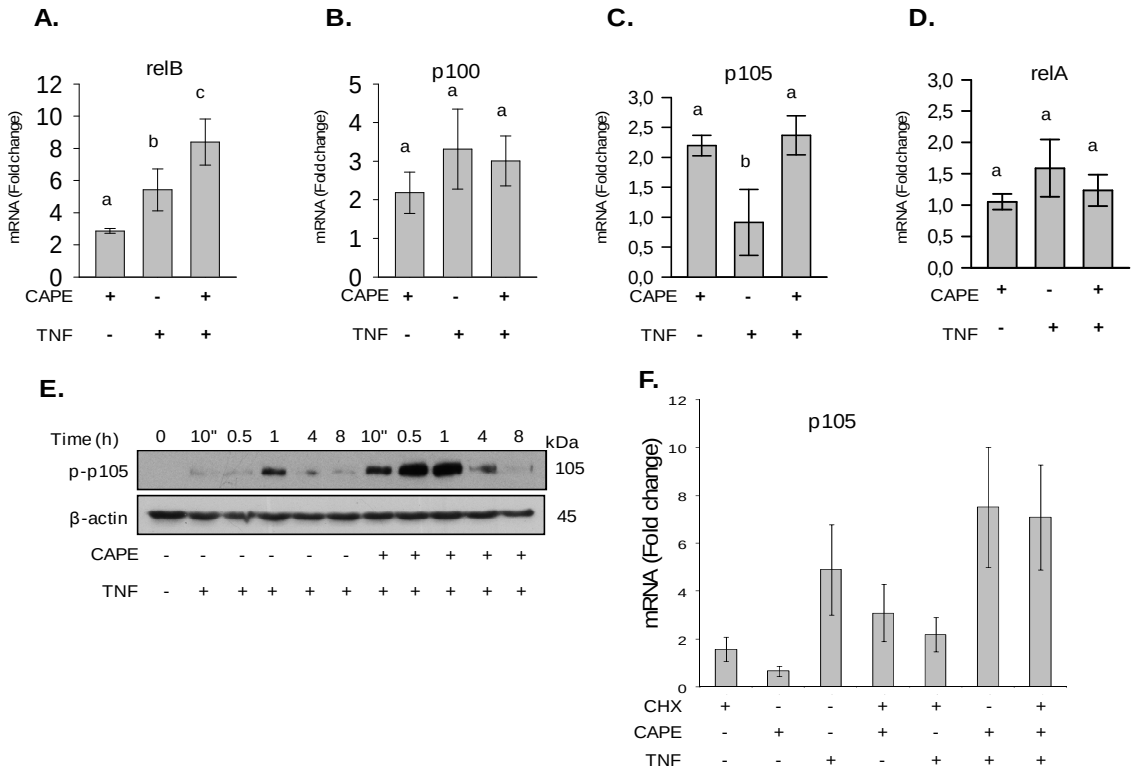

Figure 22. Influence of CAPE on NF-κB1/p105 processing and phosphorylation
Mode-K cells were treated with or without CAPE and TNF for 4 h and mRNA analysed by RT-PCR for (**A**) relB, (**B**) p100, (**C**) p105 and (**D**) relA. (**E**) CAPE induced phosphorylation p105 time-dependently. (**F**) Blockade of the protein synthesis machinery with cycloheximide (CHX) revealed that p105 phosphorylation is independent of new p105 protein synthesis.

4.2.4 Effect of CAPE on NF-κB recruitment to the gene promoter

To investigate the mechanisms by which CAPE regulates the NF-κB signalling pathway, IP-10 promoter regions for NF-κB binding using ChIP analysis, with emphasis on the functional specificity of the p50/RelA heterodimer were analysed. H3 and IgG immunoprecipitates were used as positive and negative experimental controls respectively. CAPE reduced TNF-induced IP-10 promoter occupancy of both p50 and phospho-relA NF-κB subunits (Figure 23B), suggesting that both subunits are essential for IP-10 expression. However, it had little effect on the MIP-2 promoter occupancy of relA as opposed to p50 (Figure 23C) suggesting that p50 was not essential for MIP-2 expression [Hoffmann and others, 2003]

4.3 Response of CAPE to inflammatory mediators

4.3.1 Effect of CAPE on IP-10 and TNF expression ex vivo in $TNF^{\Delta ARE/+}$ Mice

It is worthwhile to note that $TNF^{\Delta ARE/+}$ mice produce excessive amounts of TNF, a hallmark for chronic inflammation in the gastrointestinal tract.

Hence its quantification as a biomarker in a clinical setting enables some degree of prediction of disease progression and treatment. To validate the *in vitro* findings, the efficacy of CAPE was tested in the $TNF^{\Delta ARE/+}$ mouse model of ileitis. After six weeks of feeding, CAPE did not antagonize the development of severe Crohn's disease-like ileitis as no statistically significant differences were observed between control and CAPE-treated samples (Figure 24A). When compared with sham-fed control mice, $TNF^{\Delta ARE/+}$ mice were characterized by high levels of leukocyte infiltration in the mucosa and submucosa (Figure 24B). In agreement with the lack of protection of CAPE feeding at the level of tissue pathology, Western-blotting revealed that CAPE did not reduce IP-10 expression in the intestinal mucosa of $TNF^{\Delta ARE/+}$ (Figure 25C). To determine the expression of IP-10 in the ileum, *ex vivo* experiments using explant tissues from 12-week-old mice were performed. The results clearly showed that, although CAPE failed to reduce the development of ileitis in $TNF^{\Delta ARE/+}$ mice, it suppressed intracellular expression of IP-10 (Figure 25A/B). To further characterize the effect of CAPE in experimental ileitis, mouse embryonic fibroblasts were generated from $TNF^{\Delta ARE/+}$. Both transcriptional (Figure 26) and post- transcriptional (Figure 27) analysis confirmed that CAPE significantly inhibited IP-10 as well as TNF gene and protein expression indicating that it most likely targets the de novo synthesis of the mRNA and protein via the NF-κB pathway [Baldwin, 1996]. It also showed that CAPE not only targeted TNF signalling, but also the LPS-induced TLR4 signalling pathway.

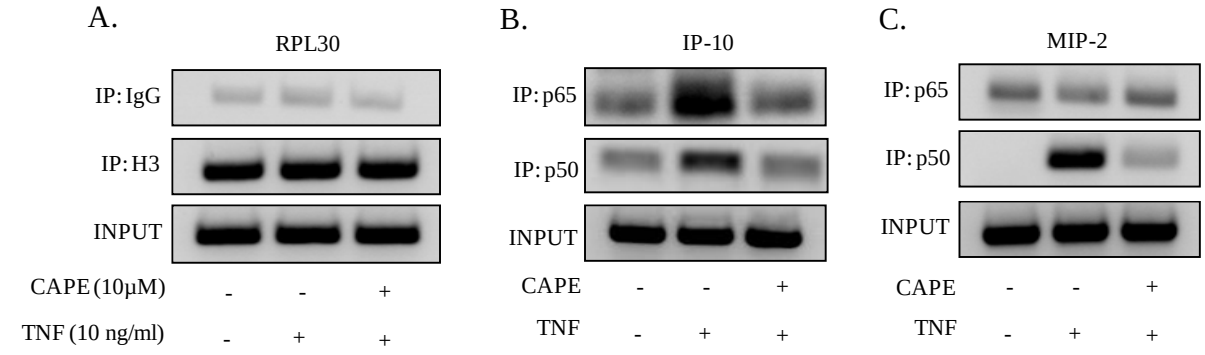

Figure 23. Effect of CAPE on relA and p50 recruitment to the IP-10 promoter
CAPE reduces recruitment of p50/relA heterodimer to the IP-10 promoter. Cells were pre-incubated with CAPE for 4 h before TNF stimulation for 30 min followed by relA, p50, H3 and IgG antibody immunoprecipitation of the enriched chromatin. (**A**) H3 and IgG were included as positive and negative controls against the ribosomal protein L30 (RPL30) gene. (**B**) Inhibition of both p50 and relA subunit recruitment to the IP-10 promoter. (**C**) Inhibition of p50 subunit recruitment to the MIP-2 promoter.

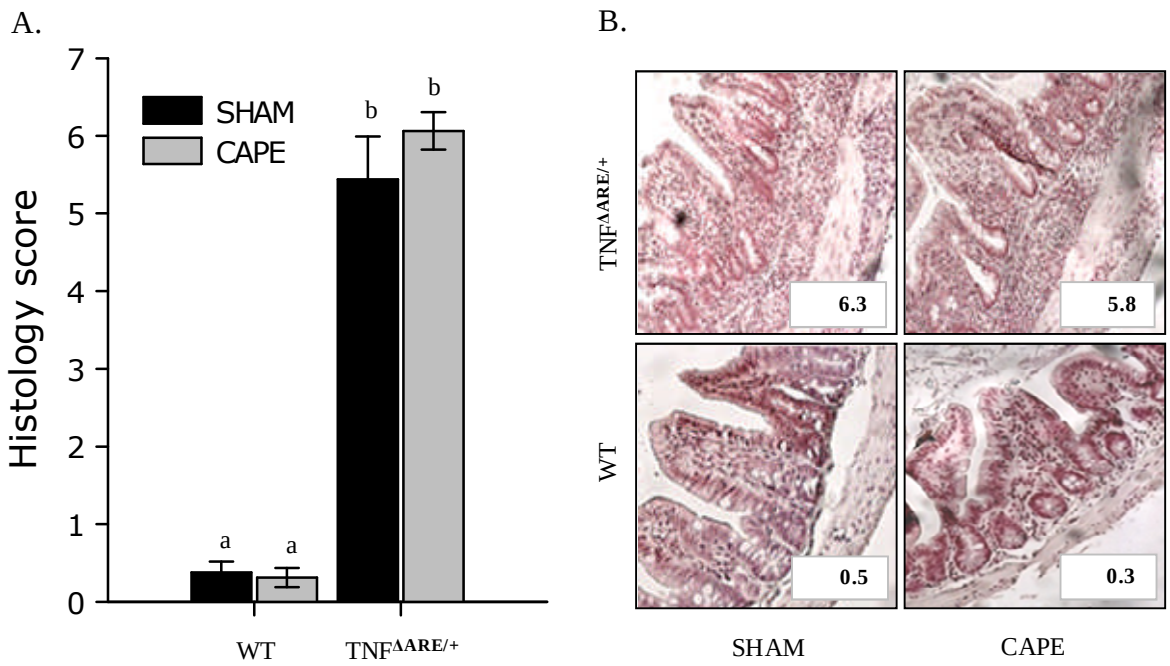

Figure 24. Effect of CAPE on experimental ileitis in $TNF^{\Delta ARE/+}$ mice
(**A**) Histology scores (0, not inflamed, to 12, highly inflamed) in the distal ileum of WT and $TNF^{\Delta ARE/+}$ mice (n = 5 mice per treatment/genotype group) treated with or without CAPE. (**B**) Cross-sections of HE stained distal ileum showing leukocyte infiltration for CAPE-treated and control WT and $TNF^{\Delta ARE/+}$ mice (200 x).

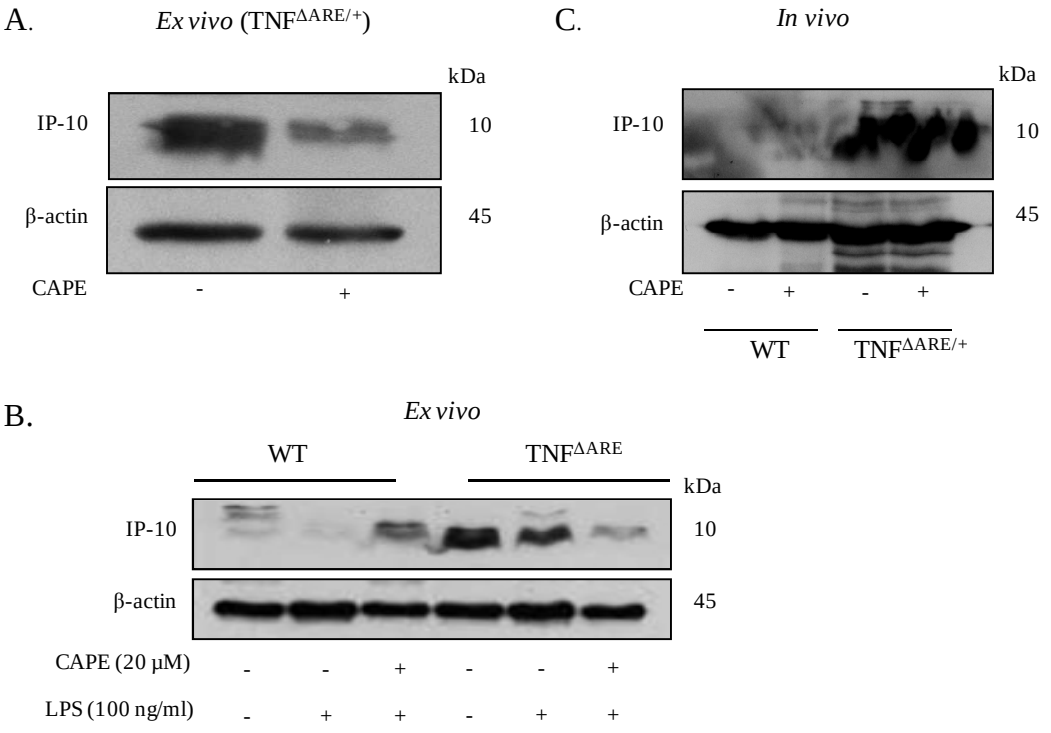

Figure 25. Effect of CAPE on IP-10 expression ex-vivo in $TNF^{\Delta ARE/+}$ ileal tissues
Ileal tissues were excised from whole ileum pieces after killing 12 wk old mice. The ex-plant was cultured for 24 h with or without CAPE (**A**) and LPS (**B**). The tissue was lysed and homogenised followed by western blot analysis. (C) Western blot of CAPE treated WT and $TNF^{\Delta ARE/+}$ mice.

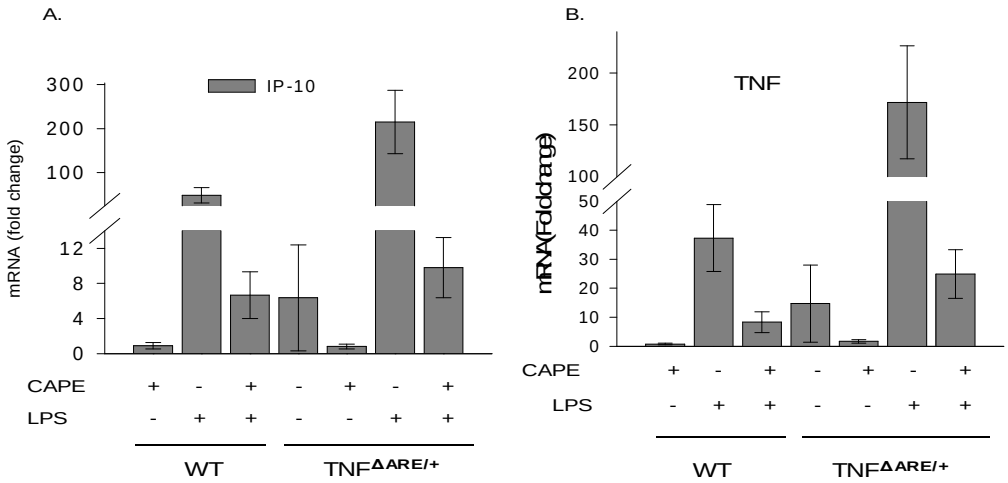

Figure 26. CAPE inhibits IP-10 and TNF mRNA expression in TNFΔARE/+ MEF
MEF were pre-treated with or without CAPE for 1 h followed by LPS for 4 h. mRNA was isolated and analysed by RT-PCR for (**A**) IP-10 and (**B**) TNF.

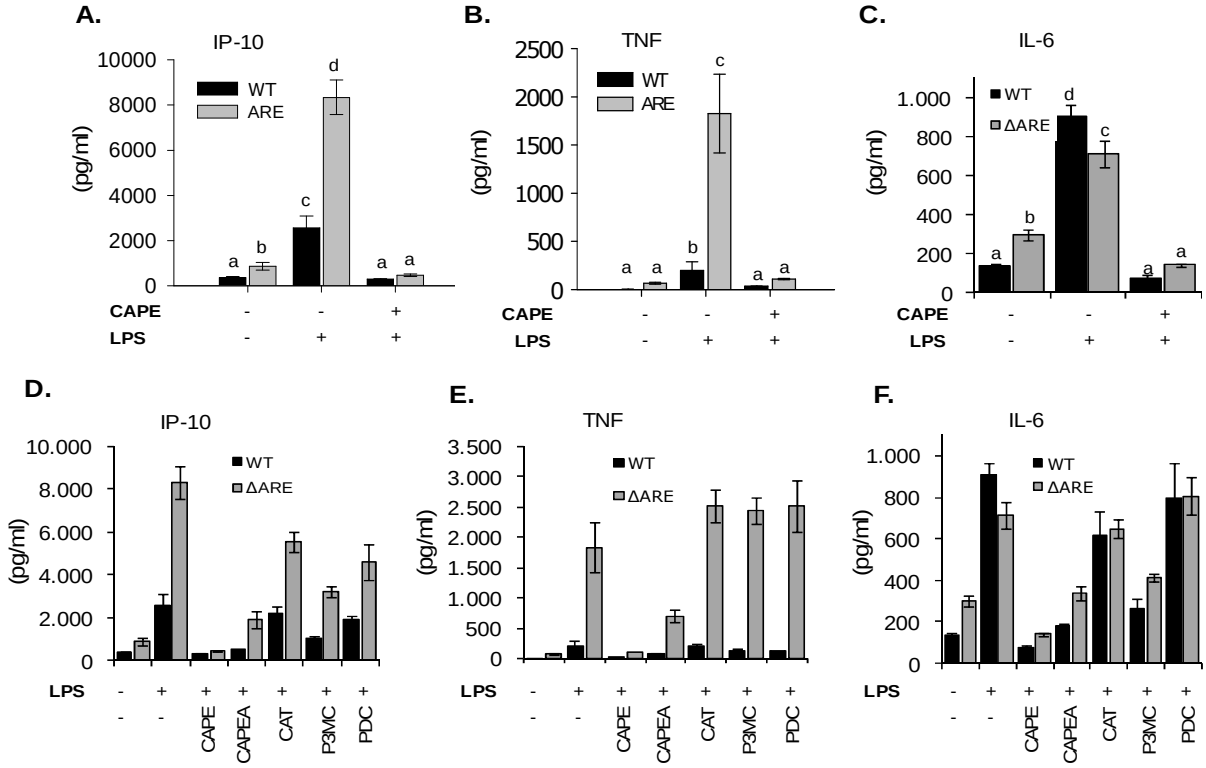

Figure 27. CAPE inhibits IP-10, TNF and IL-6 expression in TNF$^{\Delta ARE/+}$ MEF
Mouse embryonic fibroblasts (MEF) were prepared from WT and TNF$^{\Delta ARE/+}$ mice. The cells were cultured in with or without CAPE and LPS for 24 h and tested for (**A**) IP-10, (**B**) TNF and (**C**) IL-6 secretion using ELISA. The experiment was repeated using CAPE analogues for (**D**) IP-10, (**E**) TNF and (**F**) IL-6 secretion.

In view of the fact that NF-κB occupancy as assessed by ChIP and SEAP reporter do not indicate the functional activity of the promoter-bound heterodimer, the *in vitro* post-transcription levels of IP-10 expression in wild type and TNF$^{\Delta ARE/+}$ mouse embryonic

fibroblasts were measured. CAPE and CAPEA blocked IP-10, TNF and IL-6 protein as opposed to CAT and PDC (Figure 27D/E/FFigure 27)

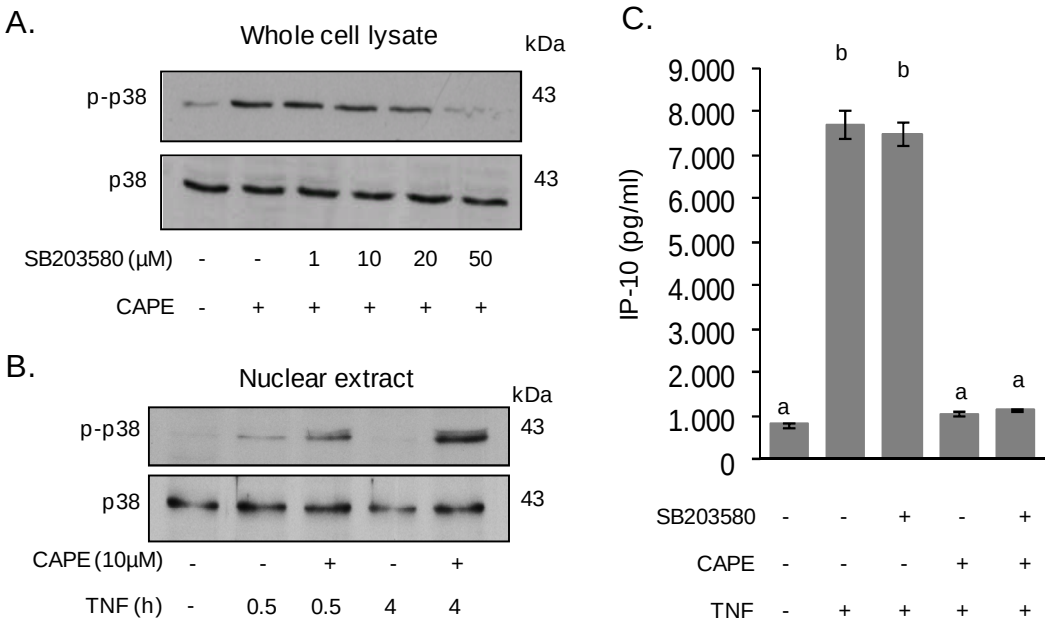

Figure 28. Effect of CAPE-mediated IP-10 inhibition on p-p38 MAPK accumulation
Mode-K cells were treated with or without SB203580 (inhibitor of p38 MAPK), CAPE and TNF for 24 h and analysed for p-p38 MAPK (**A**) whole cell lysate (**B**) nuclear extract and (**C**) IP-10 expression in culture supernatant.

4.3.2 Effect of Phosphorylated p38 MAPK in IP-10 inhibition

Transcriptional activation of NF-κB in the nucleus has been associated with p38 MAPK via MSK [Kefaloyianni and others, 2006] and many transcription factors are phosphorylated and activated by p38 MAPK in response to different stimuli. To gain further insights into the effect of p38 MAPK on CAPE-mediated IP-10 inhibition, Mode-K cells were pre-treated with SB203580, a cell permeable inhibitor of MAPK homologues. Inhibition of p38 MAPK phosphorylation (Figure 28A) had no significant effect on the CAPE-mediated inhibition of TNF-induced IP-10 secretion (Figure 28C). In addition data from nuclear extract western blot revealed that CAPE consistently increased the accumulation of the phosphorylated p38 MAPK in the nucleus in a time-dependent manner (Figure 28B). Taken together this finding indicates that accumulation of phosphorylated p38 MAPK in the nucleus had distinct and independent functional consequences in CAPE-induced cellular responses that needed further elucidation with regard to HO1 induction.

4.3.3 Effect of HO1 in CAPE-mediated IP-10 inhibition

Given the potential physiological importance of HO1 in mediating cellular homeostasis as an inducible stress protein response in addition to the anti-inflammatory effects, the role of HO1 in inflammation was delineated. Here the role of CAPE-mediated HO1 activation in the inhibition of IP-10 [a chemoattractant molecule of activated T-cells to sites of inflammation that has been implicated in the pathogenesis of chronic intestinal inflammation [Hoermannsperger and others, 2009]] was determined.

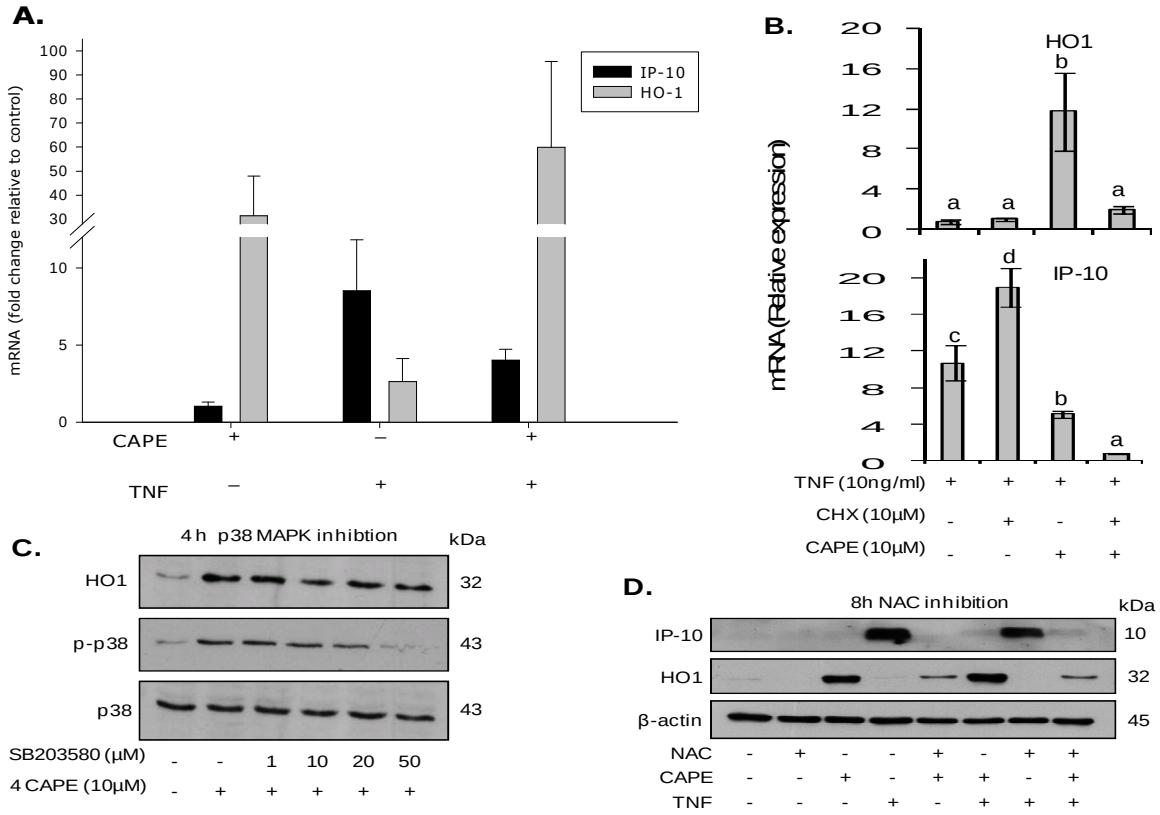

Figure 29. IP-10 inhibition, HO1 induction and p38 MAPK phosphorylation
IP-10 mRNA inhibition is independent of HO1 induction (**A** & **B**). HO1 induction is independent of p38 MAPK phosphorylation (**C**). IP-10 inhibition is independent of HO1 induction (**D**).

As expected there was no correlation between IP-10 mRNA inhibition and HO1 mRNA induction in CAPE/TNF treated cells (Figure 29A). These observations were further confirmed with CHX and NAC treatment whereby inhibition of HO1 expression did not reverse IP-10 mRNA and protein inhibition (Figure 29B/D & Figure 30). Inhibition of p38 MAPK phosphorylation had no influence on HO1 secretion (Figure 29C). Approximately 20 mM NAC was sufficient to completely diminish CAPE induced HO1 expression (Figure 31A) and the inhibition did not reverse the CAPE-mediated IP-10 inhibition (Figure 31B),

suggesting that IP-10 inhibition is independent of the oxidative stress response mechanism. Since carbon monoxide release during oxidative stress response has been shown to be a key mechanism for alleviating oxidative stress [Hegazi and others, 2005, Yao and others, 2009], cells were treated with a carbon monoxide releasing molecule 2 (CORM-2) to curb the induction of the oxidative stress responses as exemplified by HO1 expression.

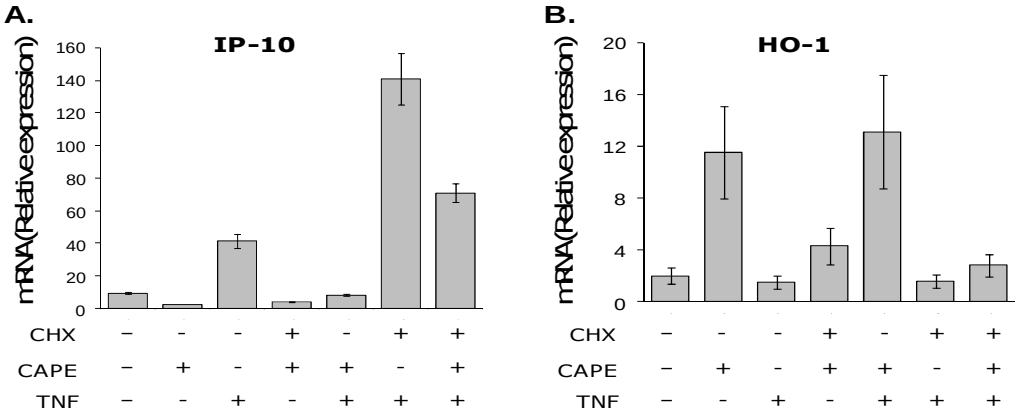

Figure 30. Relationship between IP-10 mRNA expression and HO1 mRNA induction.
Blockade of new protein synthesis by CHX revealed that (**A**) IP-10 mRNA synthesis was dependent on endogenous protein, while (**B**) HO1 mRNA synthesis required synthesis and post translational processing of Nrf2.

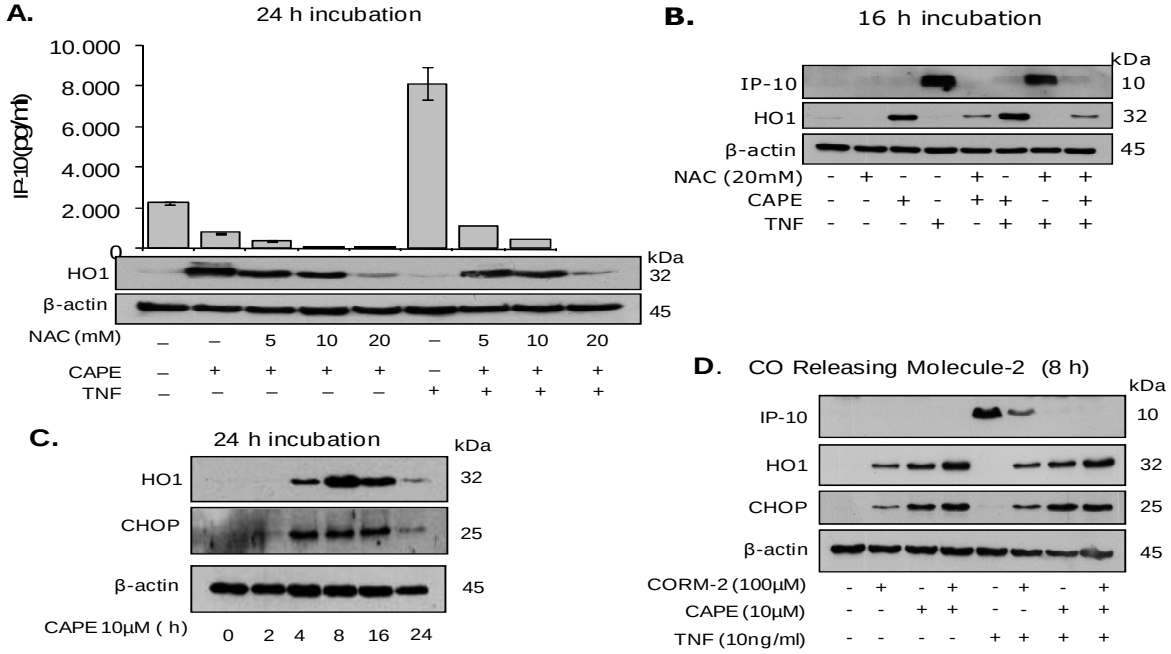

Figure 31. CAPE mediated IP-10 inhibition is independent of HO1 expression

Cells were treated with or without N-acetylcysteine [(NAC) oxidative stress inhibitor], CAPE and TNF for 24 h (**A**) and 16 h (**B**), and IP-10 inhibition is independent of HO1 expression. (**C**) HO1 and CHOP induction is time-dependent. (**D**) Pre-incubation of cells with Carbon monoxide releasing molecule-2 (CORM-2) does not abolish HO1 expression.

In contrast to the our expectations, CORM-2 also induced HO1 expression, however, the effect on IP-10 inhibition was not as robust like in CAPE treated cells (Figure 31D). From the initial experiments involving CHOP gene reporter assay CAPE induced the expression of CHOP (Figure 6 & Figure 7). Indeed as confirmed by the immunoblotting experiment, CAPE time-dependently induced CHOP protein expression (Figure 31C).

4.3.4 Influence of CAPE in Nrf2 deficient cells

Since the induction of HO1 has been shown to be dependent on Nrf2 signalling pathway [Calabrese and others, 2010, Dinkova-Kostova and Wang, 2011, Khor and others, 2006], Nrf2 deficient MEF were used to elucidated the physiological relevance of HO1 induction in CAPE-mediated IP-10 inhibition. Interestingly, CAPE inhibited expression of both Secretory (Figure 32A) and intracellular (Figure 32B) IP-10 in both wild types and Nrf2 deficient cells. Unexpectedly, CAPE induced expression of HO1 protein in Nrf2 deficient MEF cells (Figure 32C).

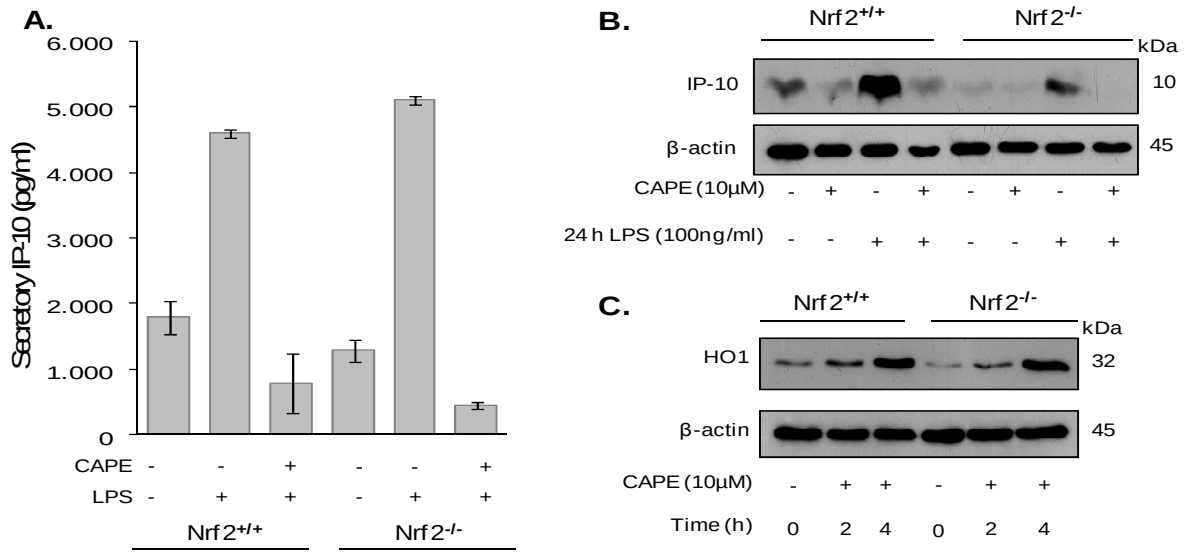

Figure 32. Effect of HO1 expression in IP-10 inhibition in Nrf2 deficient MEF
Nrf2 deficient MEF were treated with or without CAPE and LPS. Secretory (**A**) and intra-cellular (**B**) inhibition of IP-10 is independent of Nrf2 signalling. (**C**) HO1 expression in Nrf2 deficient MEF is via other compensatory mechanism.

Given the potent inhibition of IP-10 and robust induction of HO1 in Mode-K cells in response to CAPE, EGCG, which is structurally unrelated to CAPE with IP-10 inhibitory capacity was used to determine whether both compounds activate the *ho1* gene in the same manner in Nrf2$^{-/-}$ MEF. Interestingly, EGCG did not activate HO1 expression (Figure 33A), although traces of the basal HO1 were detectable. To delineate this mechanistic difference in

HO1 expression the regulation of activator protein 1 (AP-1), which is transcriptionally regulated by the action of SAPK/JNK (Figure 34) signalling cascades on the c-Jun transcription factor was determined. CAPE and not EGCG activated a protein (a higher molecular weight band) slightly above the predicted c-Jun 43 kDa band that was equally phosphorylated (Figure 33A). This additional mechanism offers a plausible explanation as to why $Nrf2^{-/-}$ MEF have upregulated levels of HO1 protein upon challenge with CAPE, probably via AP-1 signalling as a compensatory mechanism for Nrf2 deficiency, indicating that HO1 may be an endogenous protective factor. Incubation of $Nrf2^{-/-}$ MEF with a strong oxidative stress agent (H_2O_2) and CHX (protein biosynthesis inhibitor) revealed that indeed CAPE-mediated HO1 induction was dependent on excessive c-Jun expression and its concomitant phosphorylation as a compensatory mechanism for lack of the *nrf2* gene (Figure 33B). The c-Jun- binding partner (cFos) was completely stable and unaffected by CAPE treatment.

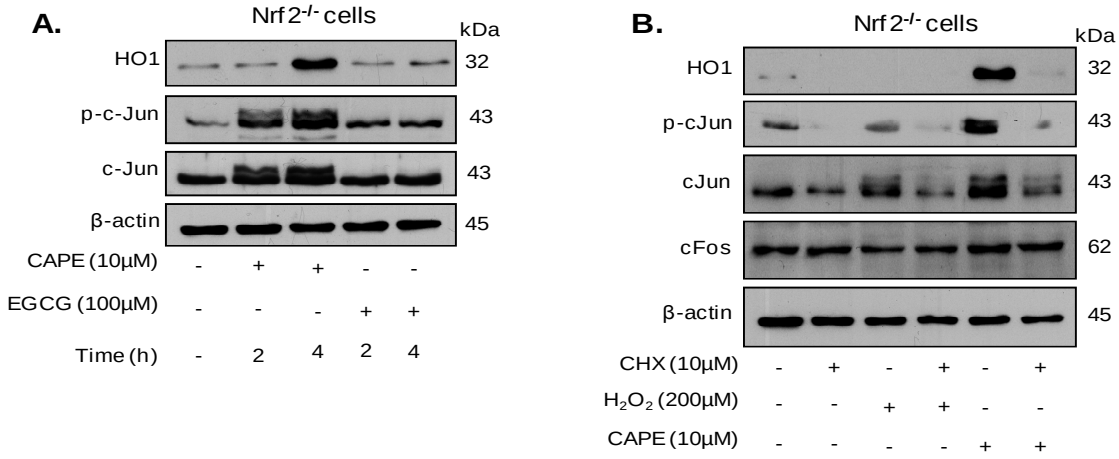

Figure 33. Impact of CAPE mediated HO1 expression in Nrf2 deficient MEF
(**A**) Nrf2 deficient cells were treated with or without CAPE and EGCG. CAPE enhanced Phosphorylation of c-Jun and HO1 expression but not EGCG after 4 h. (**B**) Cells were treated with or without CHX, H_2O_2 (strong oxidative stress agent). Blockade of HO1 expression using CHX revealed that c-Jun was the alternate compensatory pathway following Nrf2 deficiency.

4.3.5 Effect of CAPE on AMPK and SAPK/JNK phosphorylation

Phosphorylation of AMPK and SAPK/JNK were observed in Mode-K cells following CAPE treatment, but not ERK (Figure 34).Accordingly, CAPE triggers activation of p38 MAPK as previously shown (Figure 28 A/B) suggesting that phosphorylation of these kinases had some cellular functional effects following CAPE administration.

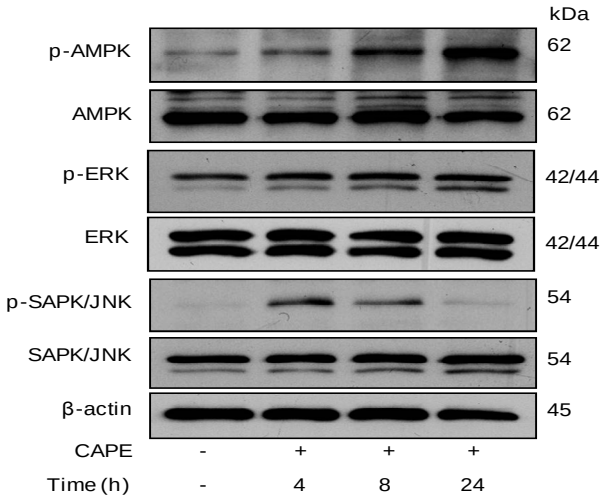

Figure 34. Effect of CAPE in phosphorylation of AMPK and SAPK/JNK
Mode-K cells were treated with 10 μM CAPE as indicated followed by Western-blotting. CAPE induced phosphorylation of AMPK and SAPK/JNK time-dependently; however, it did not activate ERK.

4.3.6 Effect of IRF1 in IP-10 regulation

IRF1 and NF-κB form a functional complex at the LTR kB sites that synergistically activate transcription. The IP-10 promoter has 2 kB sites in tandem with an IRF1 site in close proximity [Kollet and Petro, 2006, Sgarbanti and others, 2008]. To determine if IRF1 and NF-κB form a functional complex in IP-10 regulation Mode-K cells were stimulated with both TNF and IFN-γ. CAPE inhibited TNF-induced IRF1 but not IFN-γ.-induced IRF1. TNF-induced IP-10 inhibition directly correlated with IRF1 inhibition (Figure 35). Subsequent experiments confirmed that CAPE time-dependently abolished TNF-induced IRF1 protein expression (Figure 36A/B/C). However, expression of IRF1 protein following IFN-γ stimulation was weak in comparison to TNF (Figure 36D) suggesting that IFN-γ induced IP-10 expression was dependent on basal IRF1 protein expression [Schroder and others, 2004] and not necessarily the signal transduction pathway of the Janus Kinase (JAK) and signal transducers and activators of transcription (STAT) proteins [Du and others, 2007]. Low doses of IFN-γ (1 ng/ml) induced robust amounts of IP-10 (Figure 36E) as well as excessive IRF1 mRNA (40 fold) in comparison to TNF (Figure 36F) suggesting that this excess IRF1 mRNA was not wholesomely translated into the equivalent IRF1 protein (Figure 36D).

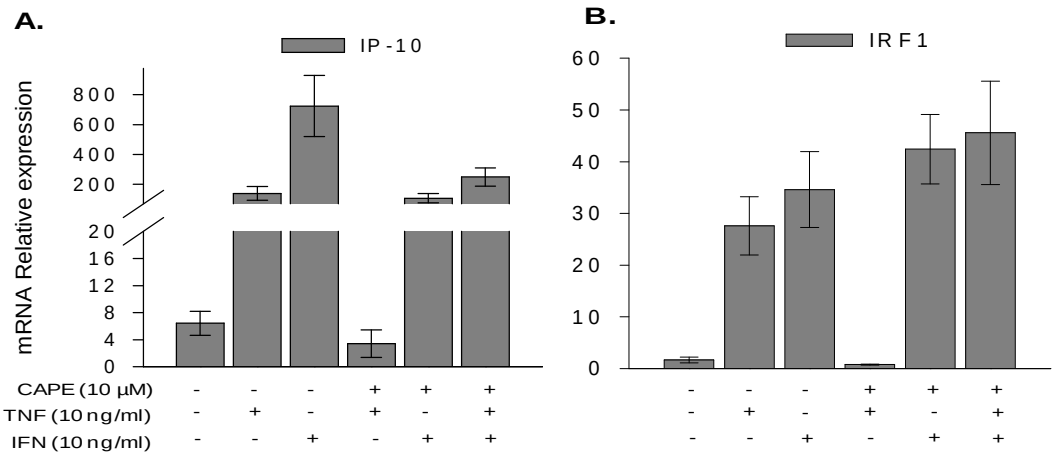

Figure 35. IP-10 regulation is tightly regulated by IRF1 expression
Mode-k cells were pre-treated with or without CAPE for 1 h followed by TNF and/or IFN-γ for 4 h. CAPE strongly inhibited TNF-induced and slightly IFN-γ induced IP-10 mRNA expression (**A**). Although, CAPE suppressed TNF-induced IRF1 mRNA it failed to suppress the IFN-γ induced IRF1 (**B**).

Whereas CAPE-mediated IP-10 inhibition was weak in Mode-K cells it was strong in NIH 3T3 cells, which expressed robust amounts following the synergistic effect of TNF/IFN-γ stimulation (Figure 37A/B/C). The physiological importance of IRF1 was confirmed in HEK 293 cells, which lack the IRF1 gene. Indeed IFN-γ stimulation failed to induce IP-10 expression as opposed to TNF, which induced approximately 2,500 pg/ml (Figure 37D) as opposed to 25,000 pg/ml in NIH 3T3 (Figure 37B). This finding suggesting that IRF1 and NF-κB interaction is essential for IP-10 expression.

4.4 CAPE versus oxidative/electrophilic stress response

4.4.1 Effect of CAPE in the de novo synthesis of Nrf2 and HO1 expression

The mechanisms of action leading to HO1 activation via the Nrf2/Keap1 signalling pathway were investigated. Inducible expression of HO1 by CAPE is time and dose-dependent manner (Figure 38). About 10 µM CAPE activates HO1 in the first 4 h and peaks at 8 h before diminishing within 24 h. HO1 induction correlates to Nrf2 depletion with no appreciable change in Keap1 (Figure 38A) and even at 1 µM CAPE, considerable amounts of HO1 are expressed (Figure 38C) suggesting that HO1 is dependent on Nrf2 protein expression levels. Whereas CAPE, CAPEA and P3MC induced HO1, the less structurally active compounds (CAT and PDC) did not (Figure 38D) lending more support to the previous observation in IP-10 inhibition which shows that the catechol ring plays a critical role in the molecules functionality (Figure 13).

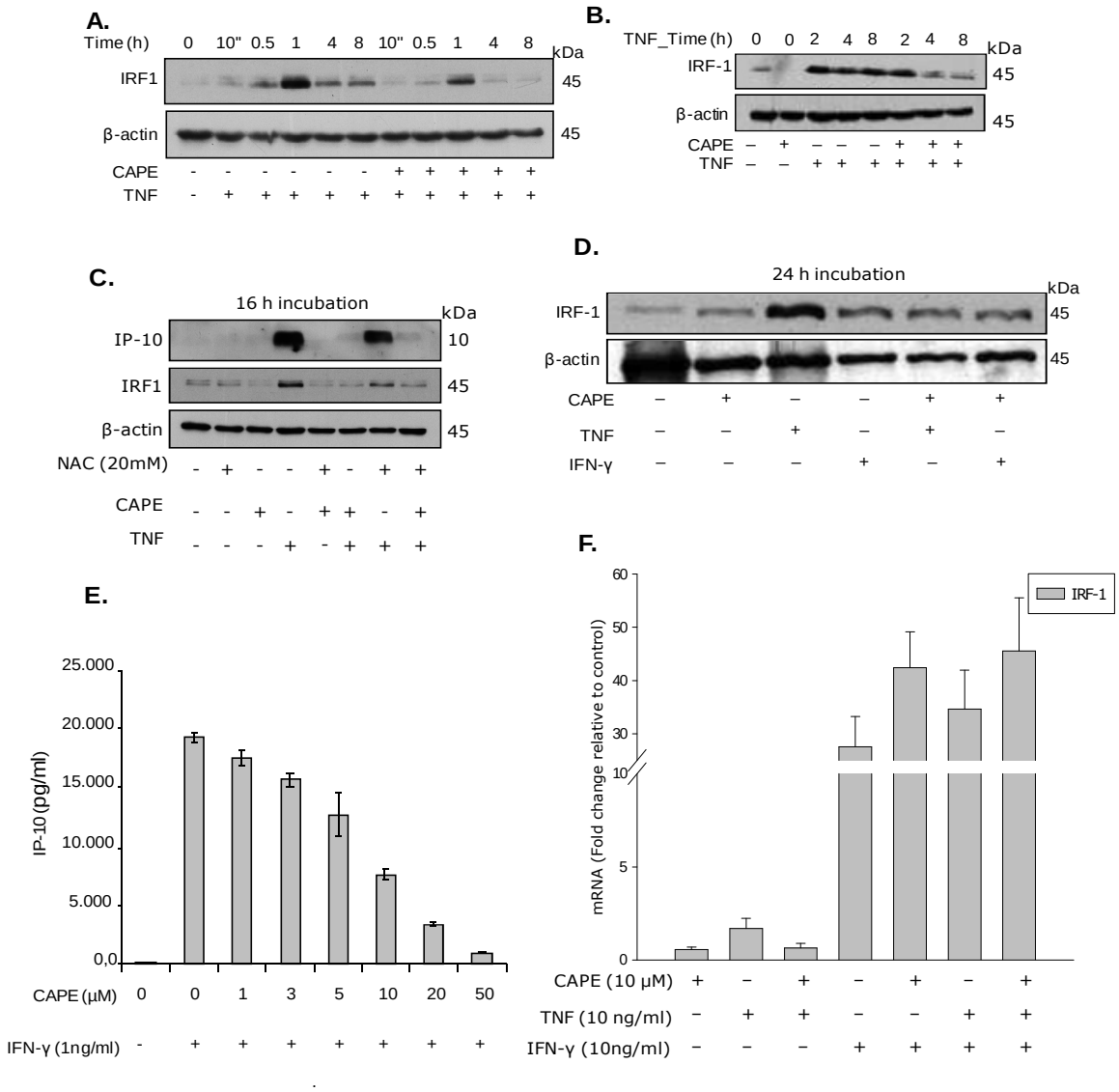

Figure 36. CAPE attenuates TNF induced IRF1 expression

CAPE time-dependently abolishes TNF-induced IRF1 protein expression (**A**, **B**, **C**). TNF sustains expression of IRF1 protein longer than IFN-γ (**D**). CAPE dose-dependently inhibits IFN-γ induced IP-10 (**E**). CAPE inhibits expression of TNF-induced IRF1 but not IFN-γ induced IRF1 mRNA (**F**).

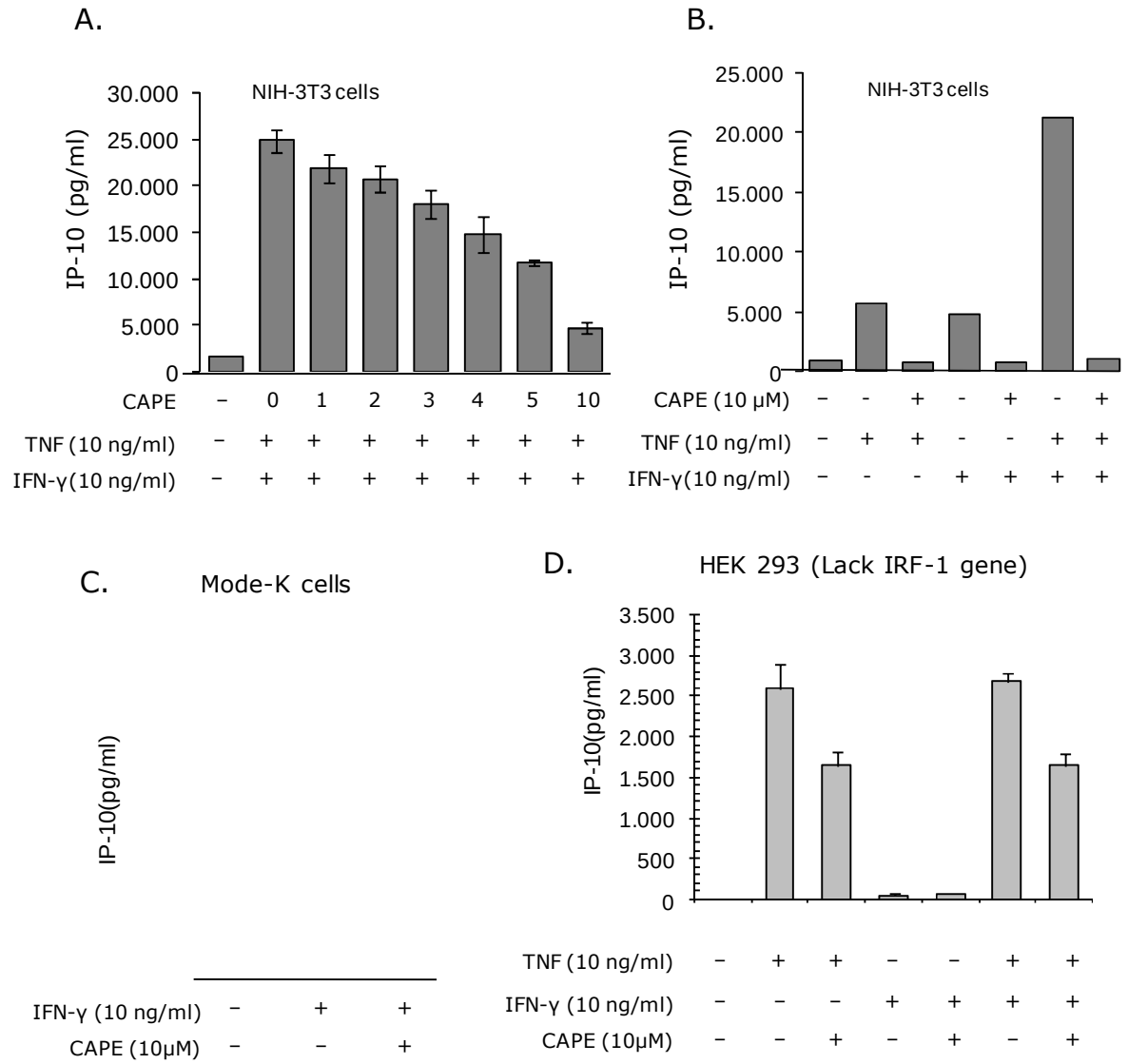

Figure 37. IRF1 tightly regulates IP-10 expression
CAPE dose dependently blocks over expression of synergistically (IFN-γ and TNF) induced IP-10 in NIH-3T3 cells (**A** & **B**). CAPE slightly inhibits IFN-γ induced IP-10 expression in Mode-K (**C**). HEK 293 cells which lack the IRF1-gene revealed that IP-10 expression is strongly dependent on IRF1 protein expression (**D**).

Blockade of protein synthesis by CHX revealed that Nrf2 protein was essential in HO1 induction (Figure 39A). In comparison to H_2O_2 (strong oxidant) CAPE activated robust amounts of HO1 (Figure 39B). To further determine the Nrf2 mechanism of action involved following CAPE administration, Mode-K cells were treated with actinomycin D [(ActD) inhibitor of transcription] and the nuclear extract subjected to EMSA. The band shift assay revealed that HO1 expression is dependent on newly synthesized and not endogenous Nrf2 protein (Figure 39C). This was confirmed by formation of complex 1 (C1) using the ARE

oligonucleotide consensus sequence, i.e. lane 4 and 5 have the same size of complex even after co-treating the cells with both ActD and CAPE, lane 5. To corroborate this observation Mode-K cells were co-treated with ActD, CHX and CAPE 4 h and the mRNA measured. ActD blocked synthesis of both Nrf2 and HO1 mRNA as expected, CHX significantly blocked HO1 but not Nrf2 (Figure 39D) indicating that HO1 expression was strongly dependent on de novo synthesis of Nrf2 protein.

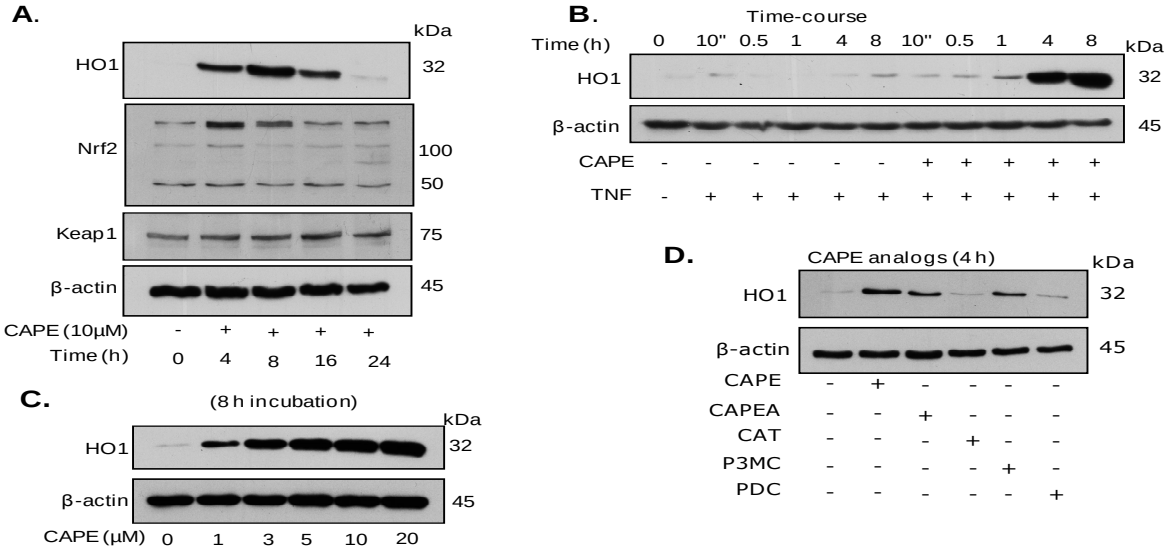

Figure 38. CAPE induces expression of HO1 in a dose-time dependent manner
CAPE induces HO1 in a time- (**A** & **B**) and dose- (**C**) dependent manner. Structural modification of CAPE results in reduced HO1 expression (**D**).

4.4.2 Influence of CAPE on proteasome activity

The hypothesis that proteasomal inhibition contributes to protein (Nrf2) accumulation, predicts that such responses should show increased ubiquitin conjugates. Therefore the levels of cellular ubiquitin in cells co-treated with CAPE and MG132 (proteasome inhibitor) were examined. Just like MG132, CAPE enhances the accumulation of 100 kDa Nrf2 proteins but not Keap1. Interestingly, the predicted form of Nrf2 at 50 kDa was the same in both the treatments (Figure 40A/C) indicating that the induction of ARE genes was most likely as a result of delayed proteasomal degradation of stabilized Nrf2 at 100 kDa.

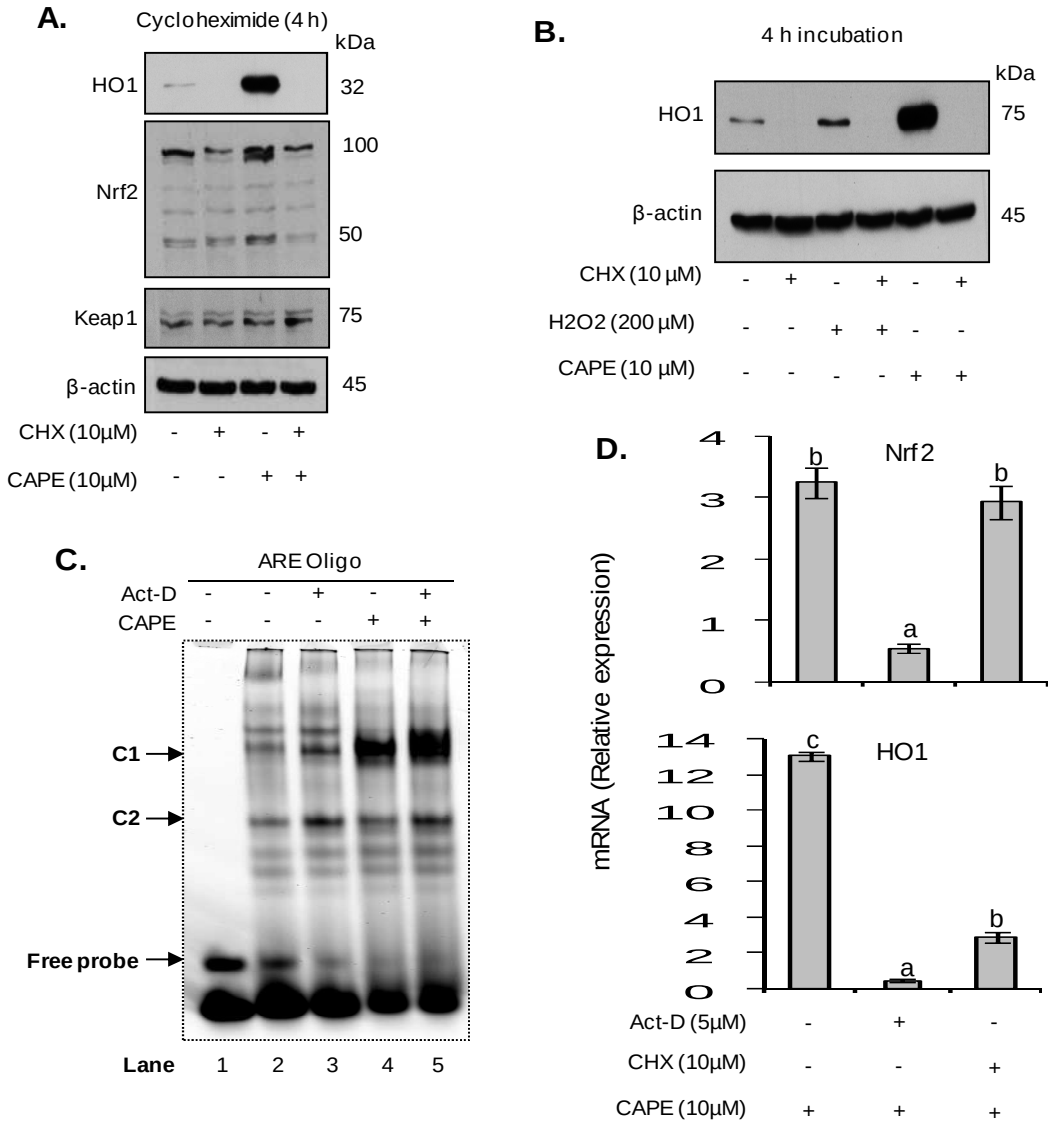

Figure 39. HO1 induction by CAPE is dependent on Nrf2 protein synthesis in Mode-K cells
Induction of HO1 in mode-K is dependent on Nrf2 synthesis (**A**). Cycloheximide blocks expression of HO1 (**B**). Binding of the ARE consensus sequence is independent of mRNA transcription (**C**). Blockade of the Nrf2 transcript expression by Actinomycin D reveals that HO1 expression is dependent on newly synthesized and not endogenous Nrf2 protein.

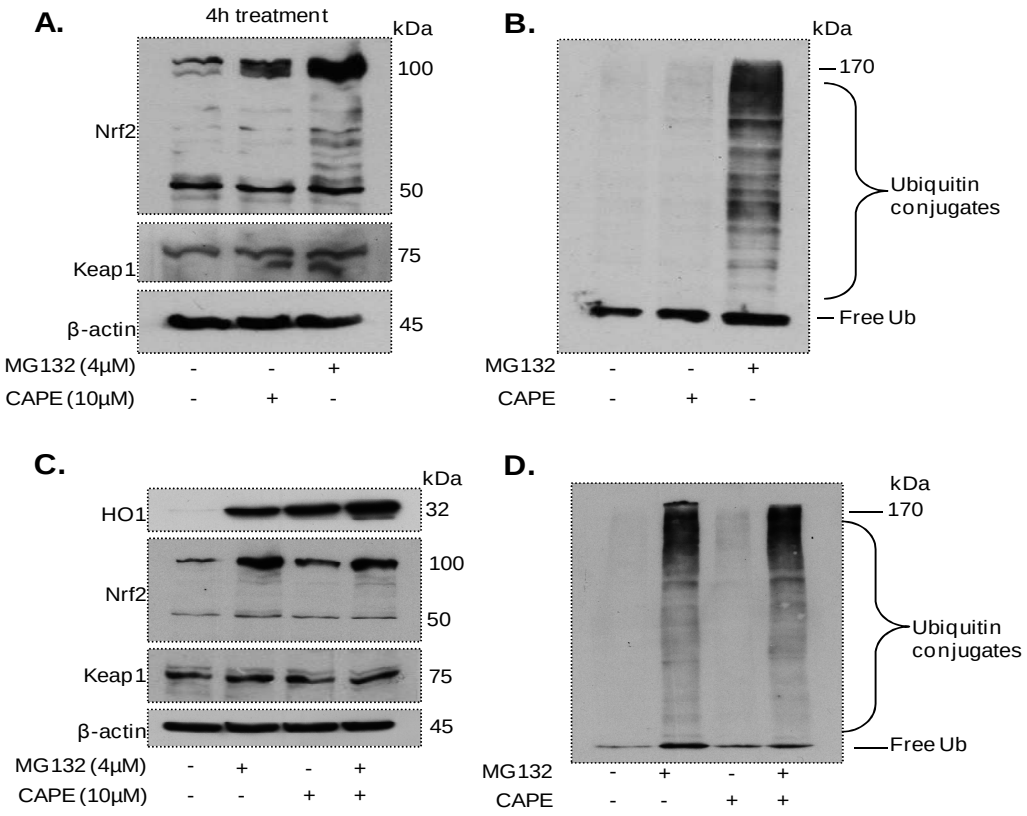

Figure 40. HO1 induction is independent of Nrf2 transcription factor proteasome activity
CAPE and MG132 enhances accumulation of the 100 kDa Nrf2 protein but not Keap1 (**A** & **C**). CAPE does not inhibit the proteasomal degradation machinery (**B** & **D**).

Accumulation of the 100 kDa protein band in the CAPE-treated cells in the same fashion like MG132, albeit mildly, encouraged us to investigate whether CAPE inhibited the ubiquitin-dependent proteasomal degradation machinery. The anti-ubiquitin antibody results revealed that CAPE did not influence the activities of the proteasome processing machinery. However, the immunoprecitation procedure performed to pull down Nrf2 from Mode-K cells lysate followed by western blot analysis with an anti-ubiquitin antibody failed to detect ubiquitin conjugates that only accumulate following inhibition of the proteasome with MG132 (Figure 40B/D). These data suggests that CAPE treatment does not result in proteasome loss of function and Nrf2 accumulation in the nucleus could be due to indirect, physiological effect of Nrf2 stabilization [Motohashi and Yamamoto, 2004, Nguyen and others, 2005].

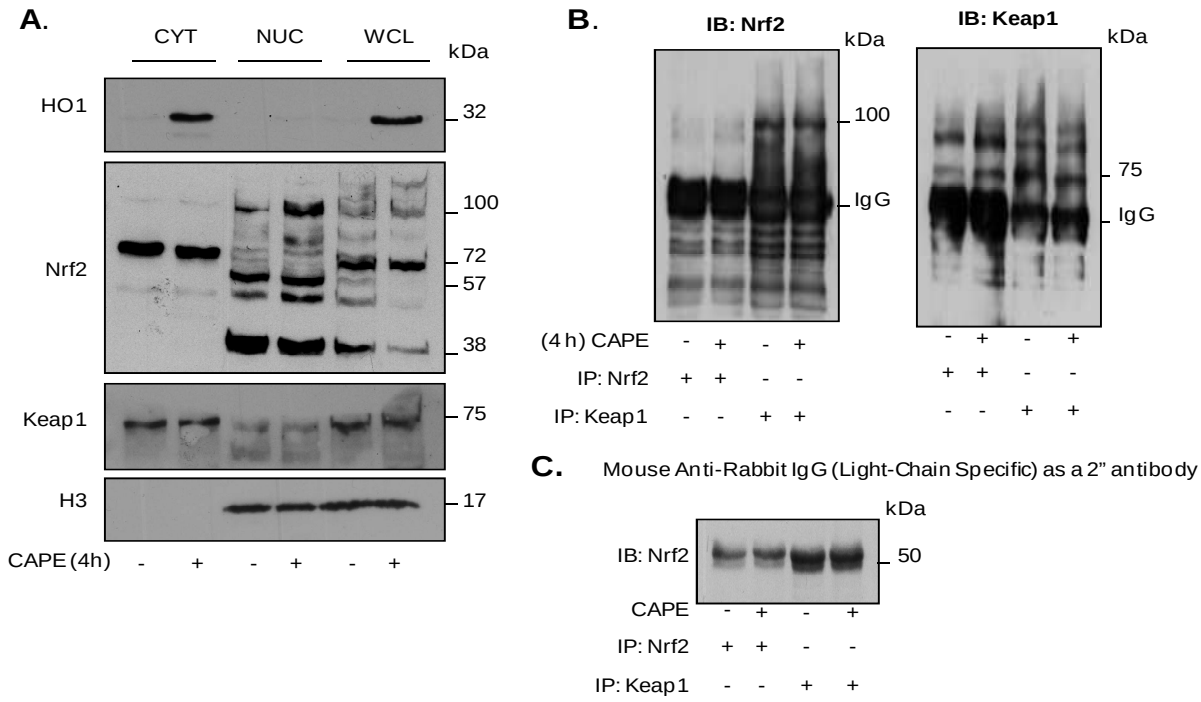

Figure 41. CAPE mediated HO1 induction in Nrf2 repression by Keap1
The nucleus has more Nrf2 conjugate proteins than the cytoplasm (**A**). Cells treated with or without CAPE were immunoprecipitated and immunoblotted with Nrf2 and Keap1 antibodies CAPE does not interfere with the Keap1/Nrf2 complex (**B** & **C**).

4.4.3 Effect of CAPE in the Keap1/Nrf2 complex dissociation

Given that Keap1 represses Nrf2 activity, it was investigated to determine whether CAPE led to the dissociation of the Keap1/Nrf2 complex. The whole cell, cytoplasm and nuclear extract western blots revealed a 72 kDa band in the cytoplasm, 100 kDa band in CAPE treated nuclear fraction and multiple degradative Nrf2 antibody binding proteins in the nucleus (Figure 41A). The Nrf2 and Keap1 pull-down assay showed that CAPE did not dissociate the Keap1/Nrf2 complex (Figure 41B). Since immunoglobulins migrate and bind in the 50kDa range creating a masking effect on the 50 kDa Nrf2 protein, the band was resolved using mouse anti -rabbit IgG (light chain specific) as secondary antibody. The subsequent western blot revealed that CAPE did not dissociate the Keap1/Nrf2 complex (Figure 41C).

4.4.4 Effcet of CAPE in the accumulation of ARE binding proteins in the nucleus

The binding was detected using a Cy5-labeled oligonucleotide against the ARE consensus gene sequence of the *nrf2* gene and other stress related response genes with sequence similarities. The EMSA revealed three reproducible complexes, unspecific complex, C1 and C2 Nrf2. Oct-1 was used as experimental control for sample preparation and loading (Figure 42A).

In the presence of CAPE excessive accumulation of C1, lane 4 was observed as opposed to ActD and CHX, however, the CHX sample had more of C1 than ActD (Figure 42B) C2 was probably degradation products following Nrf2 proteosomal processing. Surprisingly, MG132 enhanced accumulation of C1 just like CAPE. However, co-treatment of CHX and CAPE diminished the capacity to form C1, lane 6 (Figure 42C) indicating that indeed complex 2 was an ARE binding factor, which was probably Nrf2.

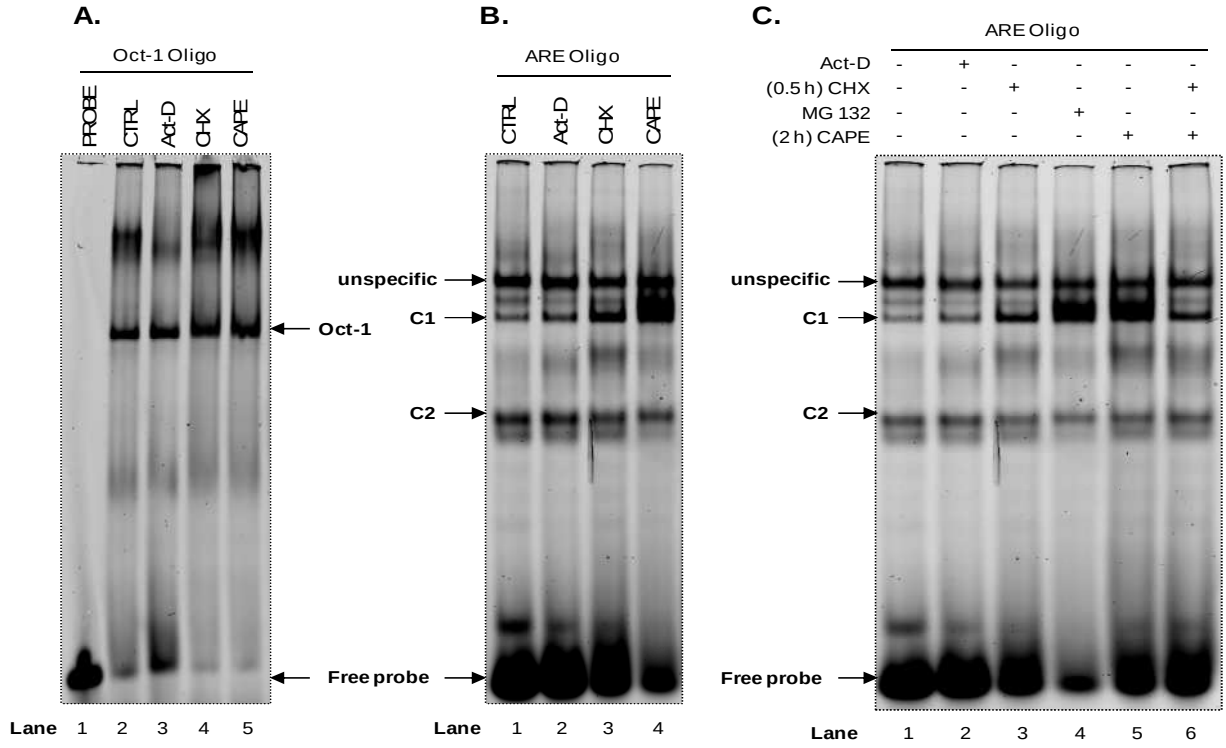

Figure 42. Effect of CAPE on Anti-oxidant Response Element binding proteins
Panels show gel shift assays and proteasome activity of 2 h CAPE-treated Mode-K cells. (**A**) Nuclear extracts probed against Oct-1 transcription factor binding site was used as a control for the EMSA to indicate equal sample loading. (**B**) EMSA of cy5- labelled ARE oligonucleotide consensus sequence revealed three complexes (unspecific, C1 and C2), of which C1 showed the highest binding affinity following CAPE treatment, lane 4. (**C**) Treatment with MG132 proteasome inhibitor revealed that CAPE partially increased accumulation of Nrf2 in the same manner as MG132 (lanes 4 & 5).

4.4.5 Effect of CAPE in NQO1 expression.

NQO1 is a highly-inducible enzyme under Keap1/Nrf2/ARE pathway regulation and its importance in combating oxidative stress has been elucidated [Dinkova-Kostova and Talalay, 2010]. The effect of CAPE on *nqo1* gene promoter activity was therefore determined in relation to the *ho1* gene using ChIP assay. The RPL30 gene was used as the experimental control for chromatin enrichment and loading (Figure 43A).

Whereas CAPE and H_2O_2 enhanced the Nrf2 promoter occupancy of the HO1 gene in equal measure (Figure 43B) this was not the case for NQO1 whereby only H_2O_2 enhanced the recruitment of Nrf2 to the NQO1 promoter (Figure 43C) suggesting that CAPE is probably more anti-oxidative than pro-oxidative.

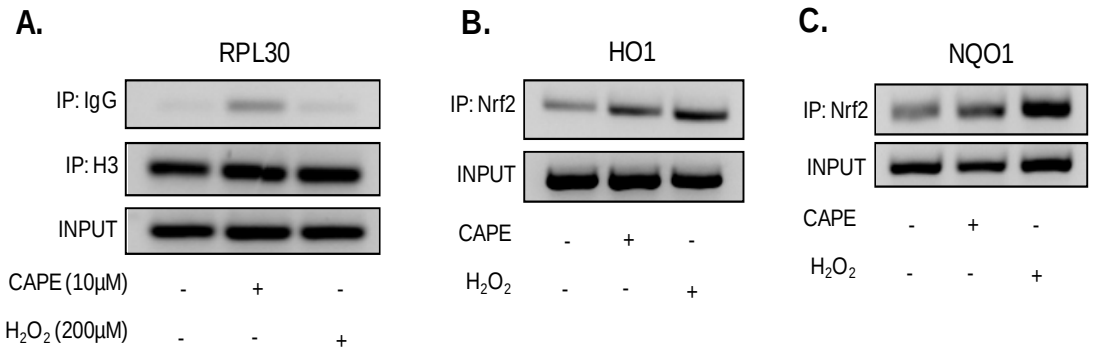

Figure 43. CAPE does not induce NAD(P)H Quinone Oxidoreductase 1(NQO1) expression
Mode-K cells were treated with or without 10 µM CAPE or 200 µM H_2O_2 for 2 h followed by IgG, H3 and Nrf2 chromatin immunoprecipitation. RT-PCR of the enriched chromatin was determined for (**A**) RPL30, (**B**) HO1 and (**C**) NQO1 genes.

4.4.6 Effct of CAPE in oxidative stress regulation

As a follow up the role of CAPE in oxidative stress response was determined. About 80 % of Mode-K cells were viable in the presence of 10 µM CAPE (Figure 44A) as opposed to 100 µM H_2O_2 (Figure 44B) after 24 h. of incubation. Hence H_2O_2 ranges above 100 µM were used in subsequent experiments to determine the level of protection on the 3, 5 and 10 µM CAPE ranges. After 48 h of incubation 10 µM CAPE (80 %) was more protective than 3 µM CAPE (70 %) and control (50 %) at 800 µM H_2O_2 (Figure 44).

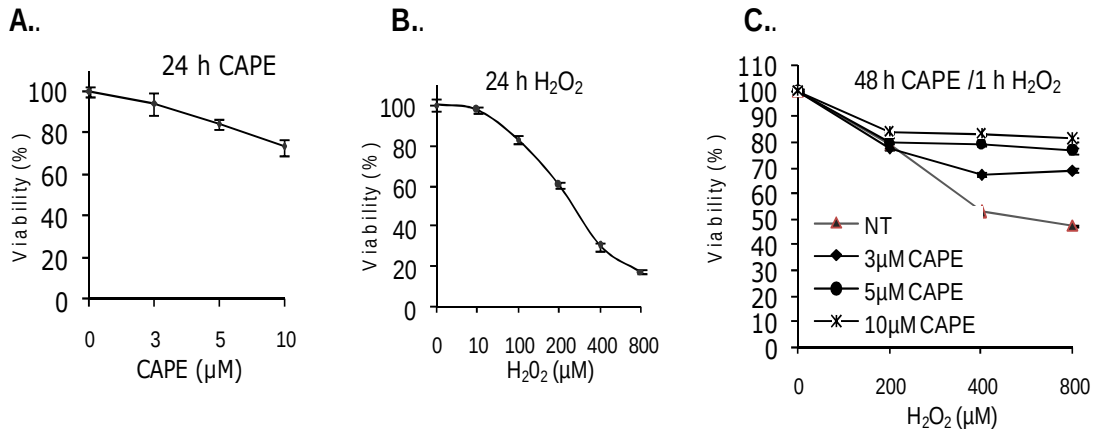

Figure 44. CAPE protects Mode-K cells from H_2O_2 induced oxidative stress
Mode-K cells were treated with or without CAPE or H_2O_2 as indicated. 80 % of cells were viable at 10 µM CAPE (**A**) and 100 µM H_2O_2 (**B**). 48 h incubation with 10 µM CAPE protected cells from H_2O_2 induced oxidative stress.

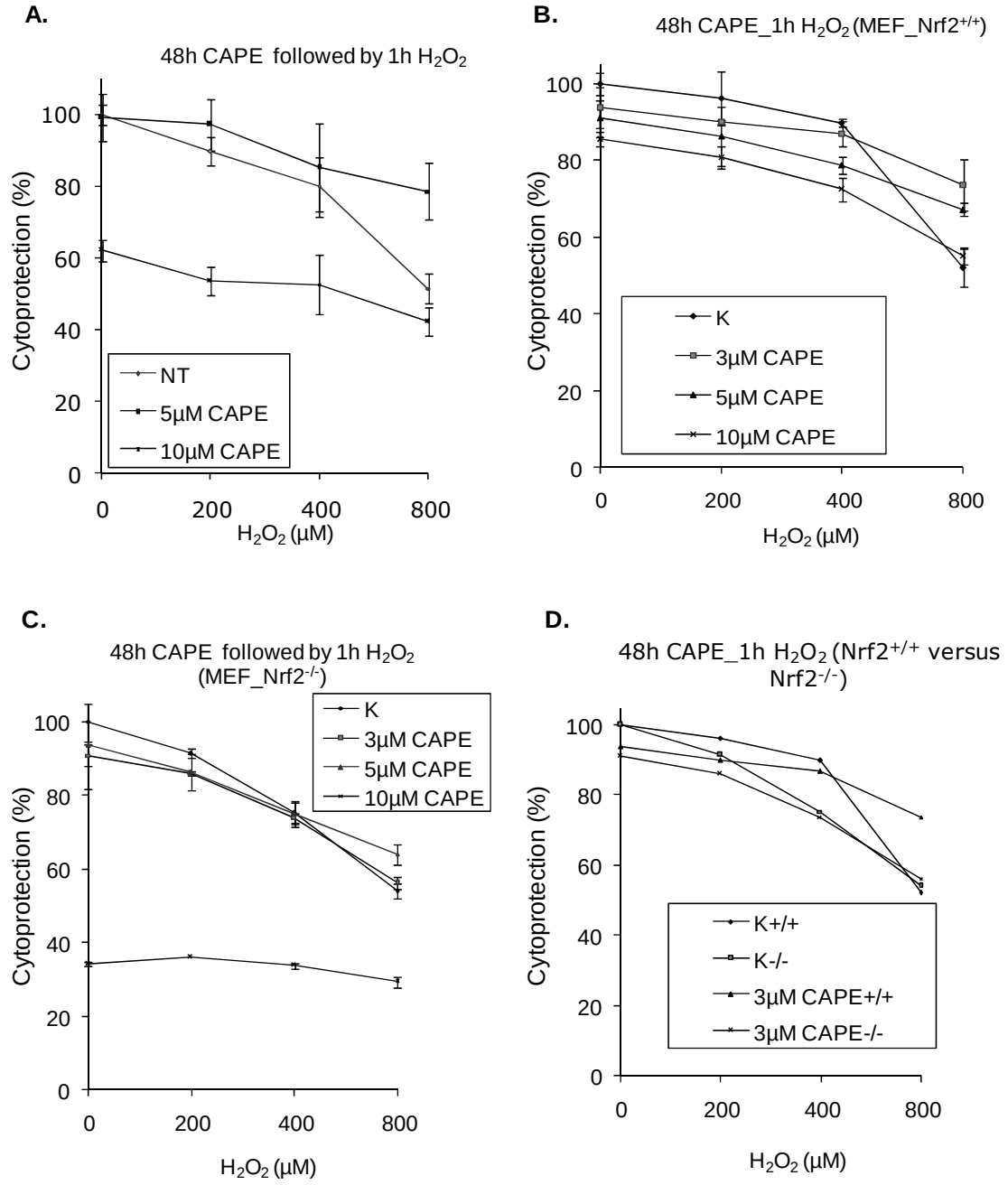

Figure 45. Nrf2 is required to protect cells from H_2O_2 induced oxidative stress
CAPE protects Mode-K (**A**), Nrf2$^{+/+}$ (**B**) but not Nrf2$^{-/-}$(**C**). Nrf2$^{-/-}$ cells are susceptible to H_2O_2 induced oxidative stress.

4.4.7 Effect of CAPE in the protection of Nrf2 deficient cells from oxidative stress

The rescue from toxicity by CAPE suggested that a critical effect of CAPE is to activate cellular levels of the Nrf2 transcription factor. Therefore cytoprotective effects of CAPE in Nrf2$^{+/+}$ versus Nrf2$^{-/-}$ cells treated by H_2O_2, a potent inducer of oxidative stress were examined. At least 5 μM CAPE was cytoprotective in Mode-K cells (Figure 45A).

Whereas Nrf2$^{+/+}$ MEF were protected (Figure 45B) by CAPE against H_2O_2-induced oxidative stress Nrf2$^{-/-}$ MEF were not (Figure 45C). The Nrf2$^{-/-}$ MEF were highly susceptible to H_2O_2 when compared to Nrf2$^{+/+}$ MEF at 3 µM CAPE (Figure 45D). A photographic representation of the same treatment effect shows necrotic Nrf2$^{-/-}$ MEF, left lower panel (Figure 46).This outcome is consistent with previous reports which show that mutants with reduced Nrf2 activity are sensitive to chemical inducers whereas elevated Nrf2 activity confers resistance in wild type cells [Chowdhry and others, 2010, Khor and others, 2006, Khor and others, 2008].

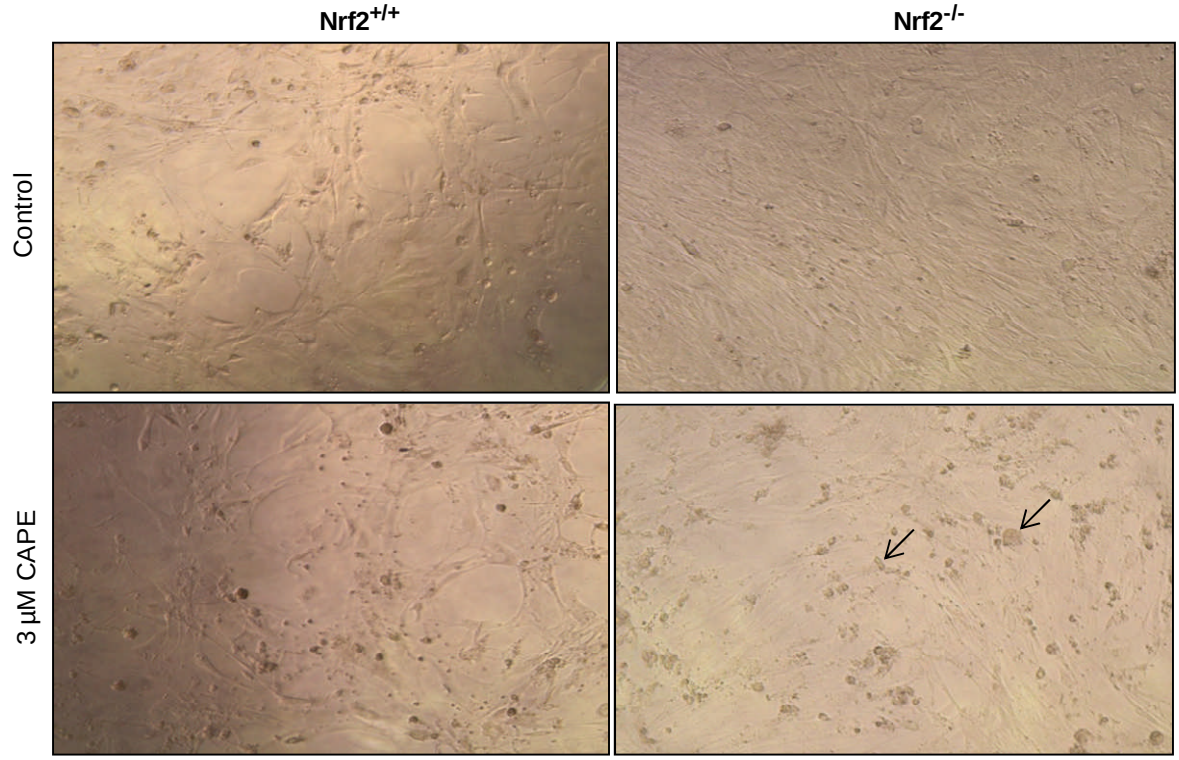

Figure 46. Nrf2 deficient MEF are susceptible to oxidative stress induced apoptosis
Nrf2$^{+/+}$ and Nrf2$^{-/-}$ MEF were treated with or without 3 µM CAPE for 48 h and visualized under the microscope. Nrf2 deficient MEF were susceptible to oxidative stress (left lower panel with arrows showing necrotic cells).

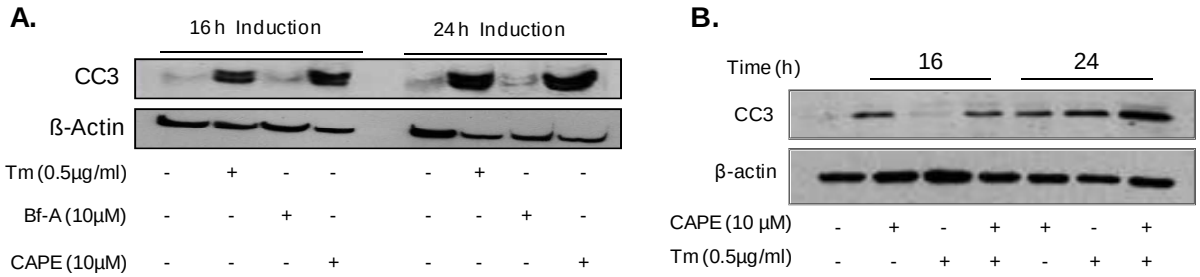

Figure 47. CAPE induces expression of cleaved Caspase 3 in Mode-K cells
CAPE induces Cleaved Caspase 3 (CC3) in equal measure to Tunicamycin (Tm) in a time-dependent manner (**A** & **B**).

4.5 Influence of CAPE in the expression of cleaved caspase 3

CAPE induced cleaved caspase 3, a known apoptosis biomarker in equal measure to tunicamycin in time dependent manner (Figure 47). This observation corroborates the induction of CHOP (Figure 31C/D) implicating CAPE in apoptotic related mechanisms [Jin and others, 2008, Onori and others, 2009, Orban and others, 2000].

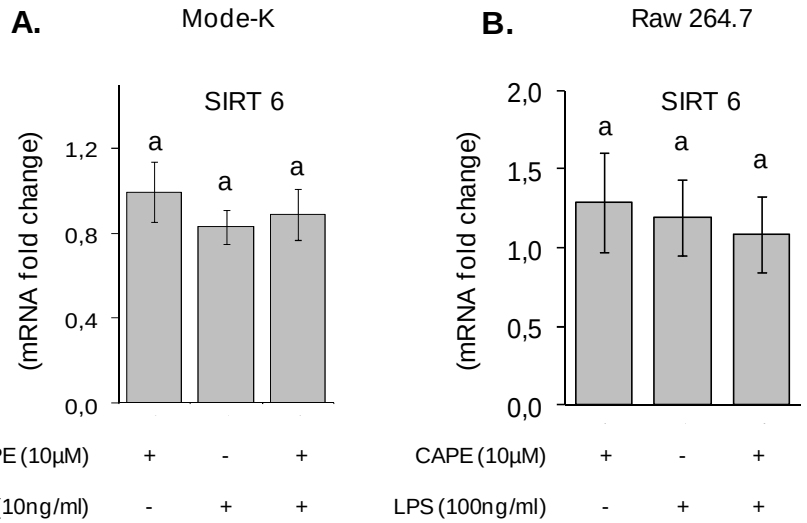

Figure 48. Influence CAPE on Sirt6 (NF-κB nuclear regulator) mRNA expression
CAPE does not induce SIRT 6 in Mode-K (**A**) and RAW 264.7 (**B**).

4.6 Effect of CAPE in Sirt6 mRNA expression

Sirtuin 6 (Sirt6) has been shown to attenuate NF-κB signalling at the chromatin whereby it has been shown to interact with promoters of relA target genes [Kawahara and others, 2009]. Sirt6 also deacetylates 'Lys-9' of histone H3 at NF-κB target promoters and may down-regulate the expression of a subset of NF-κB target genes. To determine the contribution of Sirt6 in NF-κB signalling both Mode-K and RAW 264.7 cells were assayed for SIRT6 mRNA expression. CAPE failed to induce Sirt6 mRNA expression in both cell lines (Figure 48) suggesting that probably CAPE had no direct effect on Sirt6 regulation at the transcript level. To this end it was not possible to determine whether Sirt6 protein depletion or activation affected NF-κB cellular functions.

4.7 Effect of CAPE in the production of cytokines and HO1 induction in dendritic cells

It was also determined as to whether CAPE could alter cytokine production and induce HO1 in Bone marrow derived dendritic cells following LPS stimulation. Indeed it inhibited IP-10 and TNF and robustly induced HO1 with 8 h (Figure 49).

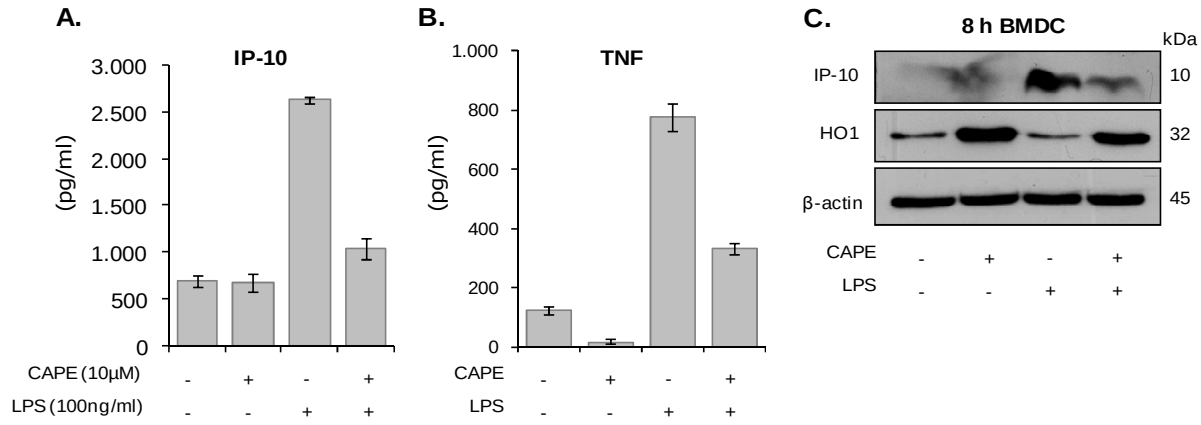

Figure 49. Effect of CAPE in Bone marrow derived dendritic cells
CAPE inhibits LPS induced IP-10 (A) and TNF (B) while inducing HO1 (**C**).

4.8 Influence of CAPE in RAW 264.7 macrophages

Following the effects of CAPE observed in the intestinal epithelial cell line, Mode-K, thee RAW 264.7 RAW immune cell line was investigated to determine if the cellular responses were of the same nature. CAPE induced HO1 and a blockade by CHX indicated that the synthesis was dependent on newly synthesized Nrf2 protein (Figure 50). CAPE was found to be a potent inducer of HO1 in Mode-K cells as well as RAW 264.7 macrophages, with Activation of HO1 occurring as early as 4 h in time-dose dependent manner (Figure 51), however, LPS also somewhat induced expression of HO1. As expected CAPE inhibited LPS-induced IP-10, TNF and IL-6 protein expression (Figure 52). Expression of mRNA in the immune cells also followed a similar pattern as observed in Mode-K cells (Figure 53). However, ATF4 [(activating transcription factor 4) implicated in the transcriptional activation of CHOP] was induced while TTP [(tristetraprolin) interferes with TNF biosynthesis [Mahtani and others, 2001]].was inhibited

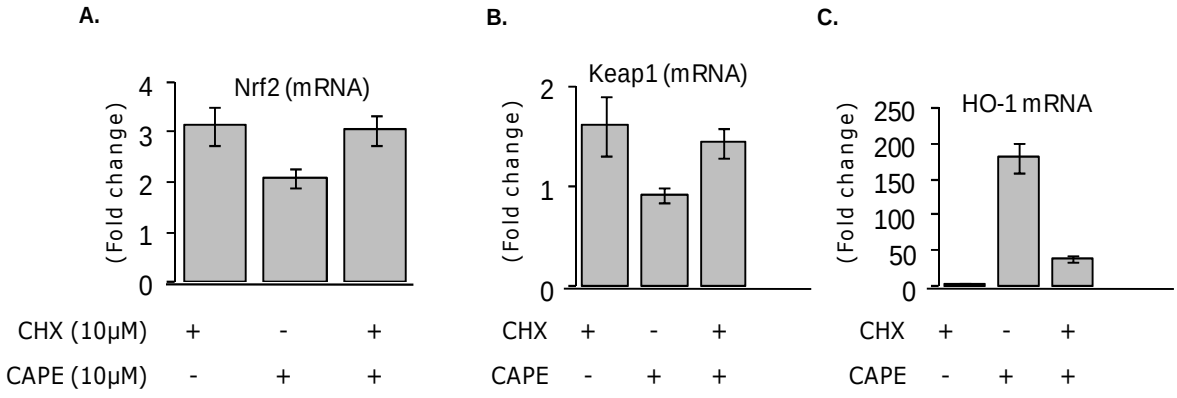

Figure 50. CAPE induced HO1 is dependent on newly synthesized Nrf2 protein
RAW 264.7 macrophages were treated with or without CHX and CAPE for 4 h and the mRNA analysed by RT-PCR for (**A**) Nrf2, (**B**) Keap1 and (**C**) HO1 gene expression

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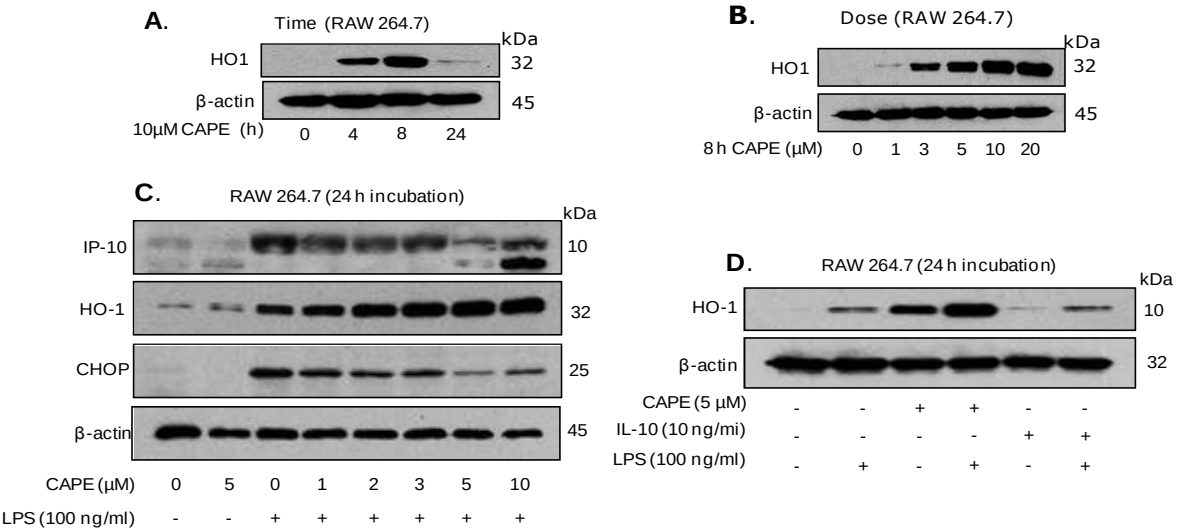

Figure 51. Effect of CAPE in HO1 induction in RAW 264.7 macrophages
CAPE induces HO1 in a time- (**A**) and dose- (**B**) dependent manner. CAPE inhibits LPS-induced IP-10 in a dose dependent manner (**C**). IL-10 did not inhibit LPS-induced HO1 expression (**D**).

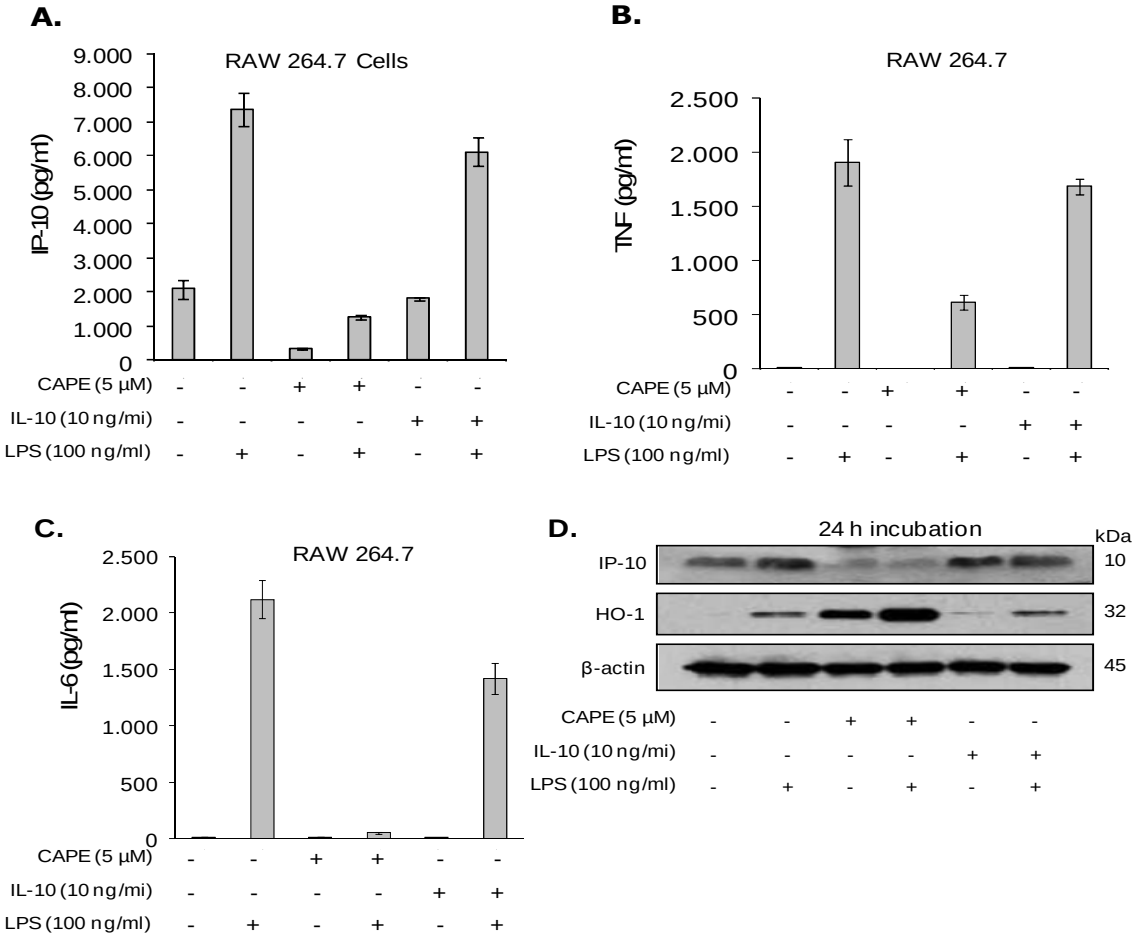

Figure 52. CAPE inhibits LPS induced cytokine expression in RAW 264.7 macrophages
CAPE inhibits LPS-induced IP-10 (**A**), TNF (**B**) and IL-6 (**C**) as well as intracellular IP-10 (**D**).

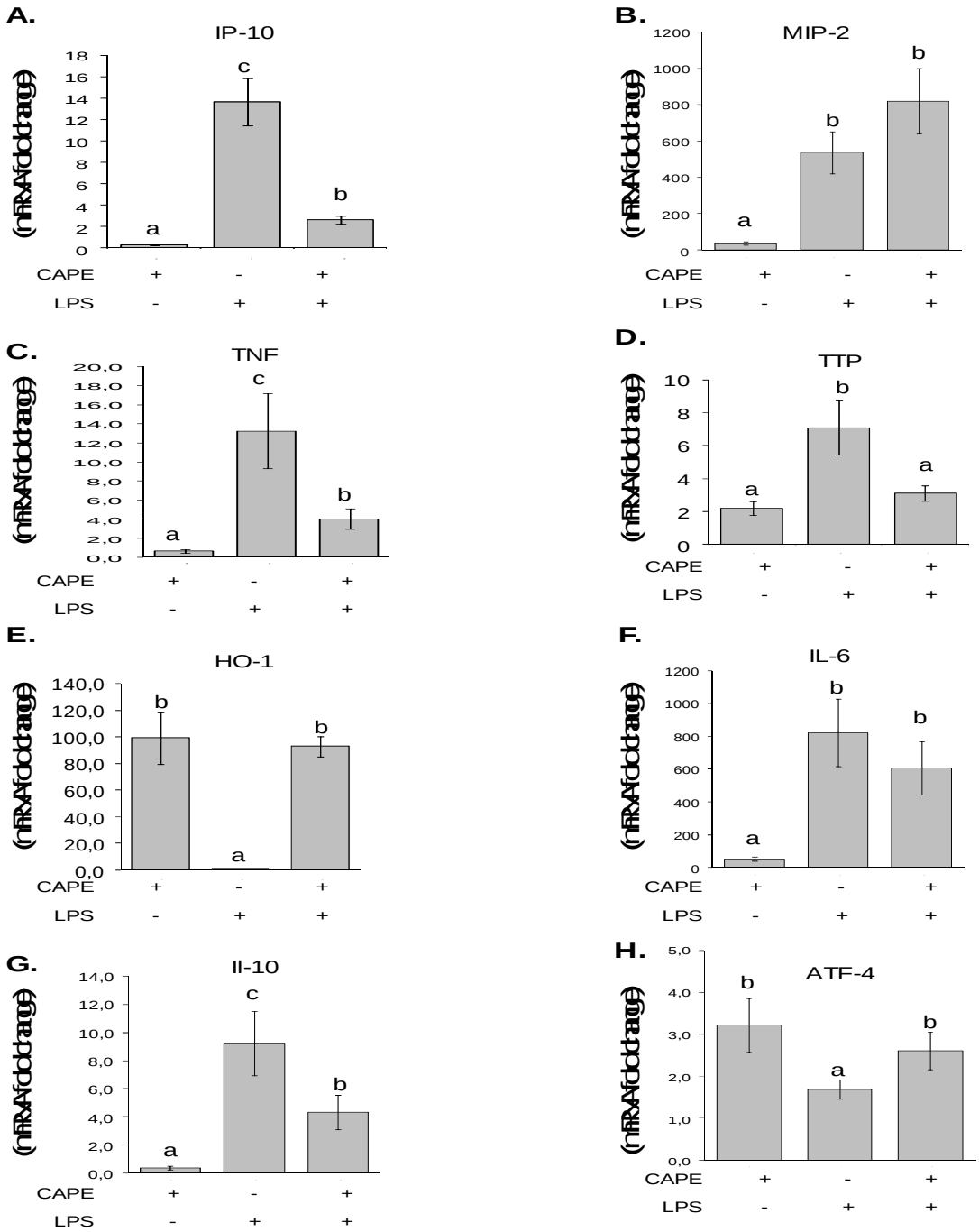

Figure 53. Effect of CAPE on LPS-induced mRNA in RAW 264.7 macrophages
CAPE inhibits IP-10 (**A**), TNF (**C**), TTP (**D**) and IL-10 (**G**) but not IL-6 (**F**); however, it induces MIP-2 (**B**), HO1 (**E**) and ATF-4 (**H**).

4.9 Effect of Enterodiol in IP-10 expression in RAW 264.7 macrophages and Mode K

Epidemiological studies suggest a possible protective effect of enterolactone and enterodiol against certain cancers, cardiovascular diseases, and osteoporosis [Bergman Jungestrom and others, 2007, Carreau and others, 2008, Clavel and others, 2005]. Given that

enterodiol is a metabolite of enterolactone the structural function effect was determined in Mode-K and RAW 264.7.

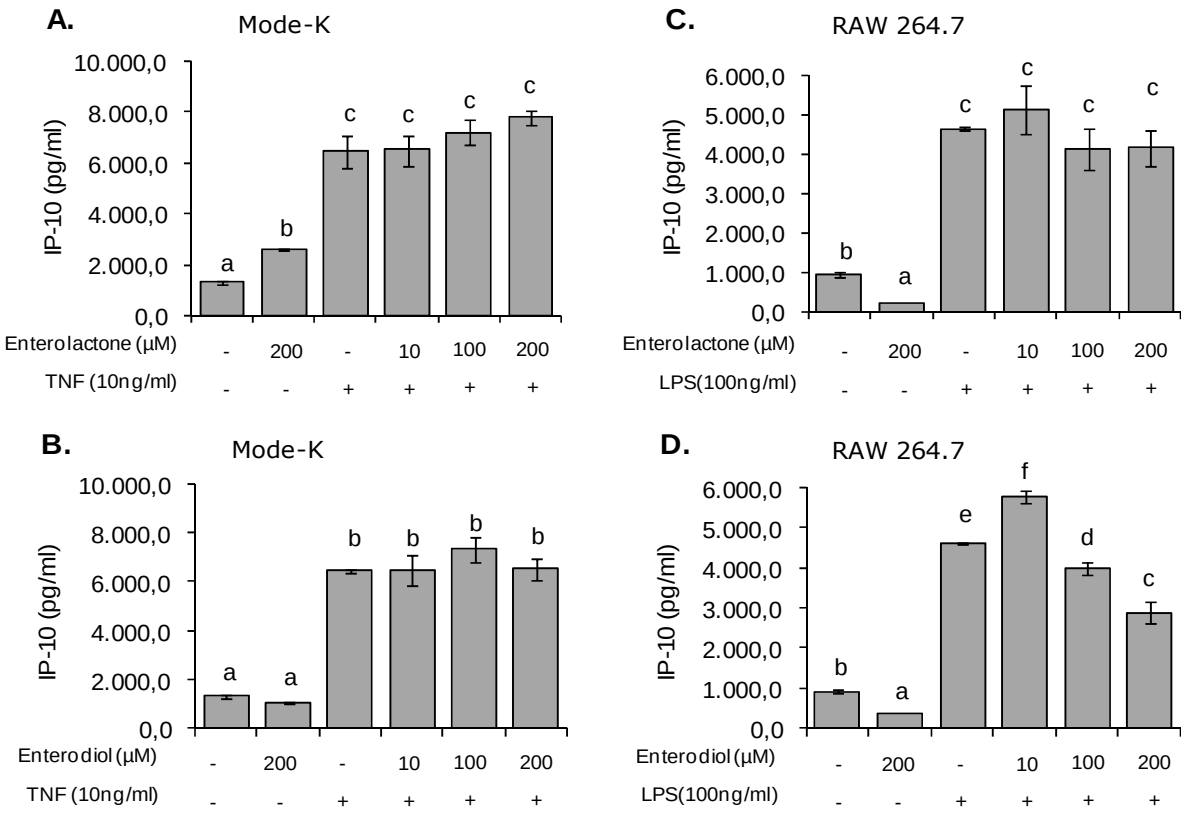

Figure 54. Effect of Enterodiol in IP-10 expression in RAW 264.7 macrophages
Cells were treated with or without Enterolactone, Enterodiol and TNF or LPS for 24 h. Enterolactone had on effect on IP-10 expression in Mode-K (**A)** and RAW 264.7 (**C**). Enterodiol had no effect on Mode-K (**B**) but dose-dependently inhibited IP-10 expression in RAW 264.7 (**D**)

CHAPTER FIVE
DISCUSSION

5.1 Functional and structure-activity effects of CAPE

In this study anti-inflammatory effects of CAPE on IP-10 expression are demonstrated by providing novel data on structure/activity relationship and molecular mechanisms underlying NF-κB pathway inhibition. IP-10 has been described as a chemoattractant, which binds to the CXCR3 receptors on monocytes and activated Th1 lymphocytes upon challenge with TNF or LPS [Dufour and others, 2002]. Recently, it has been shown that IP-10 protein expression by IEC is up-regulated in the $TNF^{\Delta ARE/+}$ mouse model of experimental ileitis [Hoermannsperger and others, 2009]. IP-10 expression was also shown to be higher following brain ischemia [Wang and others, 1998] and adipocyte maturation [Krinninger and others, 2011] and is an important signal for recruitment of inflammatory cells to the site of infection or tissue injury.

The activity of CAPE and several analogous compounds obtained by synthetic modification were first compared. Systematic variation of individual hydroxyl groups and modification of the ester functional group allowed us to pinpoint the essential structural features for sustained cytokine inhibition following CAPE treatment. It was found that catecholic hydroxyls are more reactive in comparison to methoxyl groups. Substitution of one of the hydroxyl in ring A (P3MC) resulted in reduced activity and methoxylation of both hydroxyls (PDC) rendered the compound inactive. The ability of P3MC to inhibit IP-10 expression but not SEAP activity when compared with CAPE can be attributed to methoxylation of the reactive hydroxyl group in ring A lowering the potency to reduce NF-κB-induced gene expression. Inhibition of TNF-induced IP-10 production by P3MC and CAPE depends on downstream events within the NF-κB signalling pathway as influenced by different NF-κB dimer combinations, whereas the SEAP assay is primarily under the control of κB binding repeats. Of course it cannot be excluded that other signalling pathways other than NF-κB play a substantial role in the inhibition of IP-10 production by P3MC. These observations in Mode-K cells are in agreement with a recent study showing that methylation of the hydroxyl residues in polyphenols resulted in a loss in potency against human leukemia cells [Landis-Piwowar and others, 2008]. In addition, Wang *et al.* [Wang and others, 2010] and Lee *et al.* [Lee and others, 2010] have also shown very recently that methoxyl derivatives

of CAPE are characterized by impaired or diminished cytoprotective and NF-κB inhibitory functions, respectively.

The importance of hydroxyl groups in mediating anti-inflammatory mechanisms was also supported by the fact that CAPE retained most of its biological activity after the ester was replaced with an amide (CAPEA), suggesting that the more stable amides are as active as the corresponding structurally flexible (less rigid and more volatile) esters. However, modification of CAPEA to CAT by further addition of a hydroxyl group to ring B resulting in the formation of a phenol ring led to a total loss of activity. One possible explanation is that the proximity of hydroxyl residues of the catechol structure facilitates formation of sterically important reactive intermediates [Rice-Evans and others, 2000, Rice-Evans and others, 1996]. The subtle structural differences between PDC and CAT may account for loss of activity as a result of: (i) increased polarity and, as a consequence, reduced cell membrane permeability; (ii) disturbed hydrophobic interactions in target protein binding sites. These results confirm the recent findings by Wang *et al.* and Lee *et al.* showing that the phenethyl moiety of CAPE determines activity, *i.e.*, caffeic acid or n-alkyl derivatives were characterized by loss of cytoprotection and anti-inflammatory activity [Lee and others, 2010, Wang and others, 2010]. Taken together, molecular similarity analyses revealed that the reactivity of the catecholic hydroxyl groups control the potency of CAPE, that is, its ability to interact with target proteins. Additional experiments confirmed that structural modifications similar to those described in IP-10 inhibition are also true for HO1 activation. This suggests that the catechol ring is extremely important in the functionality of CAPE, because the derived analogues were less potent than CAPE following methoxylation of the hydroxyl groups.

In addition to EGCG, quercetin, resveratrol Curcumin, lignans (enterolactone and enterodiol) and a handful of other small molecules feature in the public domain. Unfortunately, none have proven to be as effective as the many disease specific drugs; however, these molecules are useful in a sense, as they reflect the interests of drug design companies in developing selective and specific small molecule inhibitors from natural products.

5.2 CAPE versus NF-κB signalling

NF-κB has represented a paradigm for signal transduction and gene regulation for over 20 years because of its extensive involvement in key cellular processes, particularly in immune and inflammatory responses [Atreya and others, 2008, Baldwin, 1996, Hayden and

others, 2006]. An increasing body of literature illustrates that specific signalling in the nucleus is as equally important as cytoplasmic events for controlling the transcriptional activity of NF-κB. The mechanisms of these effects involve the synergistic interaction of Rel and non-Rel subunits within the NF-κB complex, participation of strictly nuclear proteins, crosstalk with other transcription factors, remodelling of chromatin structures, nuclear turnover of NF-κB, and other effects [Ghosh and Hayden, 2008, Schmitz and others, 2004, Tak and Firestein, 2001].

The study next aimed at providing novel insights into the molecular mechanisms underlying CAPE activity, focusing primarily on the functional effects of the p50/RelA heterodimer, which has been shown to transactivate many inflammatory genes [Curran and others, 2001, Hayden and others, 2006, Tak and Firestein, 2001]. The major regulatory step that prevents spontaneous NF-κB activity is the retention of the NF-κB/IκBα complex in the cytoplasm. Dissociation of this complex results in IκBα degradation and NF-κB liberation to the nucleus. Although this study contradicts one study on IκBα degradation in gastric epithelial cells [Abdel-Latif and others, 2005], it is in agreement with two previous reports which demonstrated that CAPE has no effect on IκBα degradation in osteoclasts [Ha and others, 2009] and U937 cells [Natarajan and others, 1996]. Lee *et al.* also obtained results that contradict these findings, *i.e.*, partial prevention of IκBα degradation by CAPE, but using higher concentrations (> 25 μM) in HCT116 cells [Lee and others, 2010]. The non-canonical NF-κB signalling pathway is as a result of p100 to p52 processing by the proteasome. The liberated p52 forms active p52/RelB complexes that induce transcriptional process responses differing from the IκBα-dependent canonical pathway. Processing of p100 and IκBα degradation regulate different NF-κB dimers and as a consequence non-canonical and canonical pathways regulate distinct NF-κB target genes that are stimuli specific. In CAPE did not alter p100 mRNA expression, yet induced transcripts of the p50 precursor p105. Consistent with a previous report that polyphenols affect recruitment of transcription factors to gene promoter regions [Ruiz and others, 2007], CAPE was found to inhibit NF-κB (p50/RelA heterodimer) binding to the IP-10 promoter. This is also in line with a genetic analysis study by Hoffmann *et al.*, who found that both p50 and RelA subunits are essential for IP-10 expression [Hoffmann and others, 2003]. Whereas CAPE inhibited p50 and RelA binding onto the IP-10 promoter, its failure to inhibit the coordinated degradation and re-synthesis of IκBα may be attributed to selective gene activation in the IκBα promoter [Hoffmann and others, 2002]. Importantly, Hoffmann *et al.* also showed that, whereas most

regulatory genes are RelA subunit-dependent, the p50 subunit is dispensable for some of these genes including the neutrophils chemokine MIP-2 that controls mucosal lymphocyte migration in IEC [Ohtsuka and others, 2001]. MIP-2 can be induced by many of the RelA-containing dimeric complexes including p50/RelA, p52/RelA, and P65/RelA. Recently it was shown that NF-κB and AP-1 activation is required for MIP-2 up-regulation and silencing of NF-κB alone may not be sufficient to reduce inflammation in acute pancreatitis [Orlichenko and others, 2010]. Data from the literature have also demonstrated that CAPE does not affect AP-1 promoter activity [Natarajan and others, 1996]. Taken together, it can be proposed that CAPE targets NF-κB DNA-binding sites of the p50/RelA heterodimer without compromising other essential NF-κB functions. This suggests that, although CAPE targets activity within the core of NF-κB binding in the promoter regions, it may in fact have selective effects, for instance, inhibition of IP-10 pro-inflammatory signals without completely blocking MIP-2, an acute phase response gene. Thus, in summary, NF-κB signalling is characterized by dynamic patterns of periodic NF-κB nuclear localization and target gene activation interspersed with nuclear exit and gene deactivation. Chromatin regulation likely plays an important role in modulating NF-κB target gene expression patterns. For instance, certain NF-κB target genes are primed whereas others are prevented from reactivation following a pioneering round of NF-κB activity, and these effects are associated with distinct chromatin modifications at the target genes [Foster and others, 2007, Foster and Medzhitov, 2009].

Whereas CAPE failed to attenuate TNF-driven inflammation in this study, recently Curcumin, Resveratrol and Simvastatin have been shown to exert anti-inflammatory effects in an ileitis (a hyper-acute Th1-type driven small intestinal inflammation) model [Bereswill and others, 2010]. Taken together it is plausible to conclude that there is need to carefully interpret results arising from different experimental IBD study models that exhibit distinct features as well as some form of genetic manipulation. In retrospect, we now know that epithelial cells and immune cells respond to CAPE in the same manner. Therefore, it is not surprising that CAPE exhibited adverse effects on RAW 264.7 and Bone marrow derived dendritic cells (BMDC) that are immuno-responsive.

5.3 Effect of CAPE on p38 MAPK, Nrf2 and HO1

Another important result of the present study is that CAPE potentiates phosphorylation and accumulation of p38 MAPK in the nucleus. Phosphorylation of p38 MAPK under conditions of oxidative/electrophilic stress has been shown to be synergistic to NF-κB activity

via phosphorylation of RelA by MSK1, a p38 MAPK substrate [Kefaloyianni and others, 2006]. However, based on the p38 MAPK inhibitor SB203580 findings, NF-κB-dependent IP-10 inhibition is not related to the p38 MAPK pathway activation. Although HO-1 and Nrf2 have been shown to interact with inflammatory pathways [Dinkova-Kostova and others, 2005, Pae and Chung, 2009], the combined effect of reduced NF-κB activity and increased anti-oxidative stress responses was not a prerequisite for the anti-inflammatory effects of CAPE. Using NAC to inhibit CAPE-induced HO-1 induction, it was revealed that IP-10 inhibition occurred independent of the presence or absence of HO-1 protein. Nrf2 deficient MEF also showed that Nrf2 is not required for inhibition of IP-10 protein expression by CAPE. Via knockdown of Nrf2 using shRNA in HCT116, Lee *et al.* had previously proposed that CAPE-mediated Nrf2 activation is associated with inhibition of the NF-κB pathways [Lee and others, 2010]. However, differences observed after specific knockdown were marginal at concentrations below 25 μM and related only to NF-κB reporter gene activity, whereas this data refers to the endpoint readout of interest, IP-10. Also, the findings go along the line of the work by Liu and co-workers [Liu and others, 2008], who found that a variety of phase 2 inducers, *i.e.*, chemicals inducing for instance NQO1, were capable of inhibiting nitric oxide production by mouse peritoneal macrophages, independent of macrophage origin (WT or $Nrf2^{-/-}$ mice).

Accumulating evidence indicates that MAPK, PI3K, PKA, PKC and tyrosine kinases contribute to HO1 induction by phosphorylating the transcription factors that regulate the ho1 gene in response to oxidative and or electrophilic induction. Conversely, pharmacological inhibition of the p38 MAPK phosphorylation by SB203580 did not alter the HO1 expression pattern following CAPE treatment. This indicates that in deed other mechanisms besides the Nrf2/Keap1 pathway are involved in HO1 induction. Though, the data shows enhanced phosphorylation status of p38 MAPK in response to CAPE it is difficult to create an association between P38 MAPK activation and oxidative stress response.

5.4 CAPE Attenuates inflammatory responses *in vitro*

Epithelial cells are in direct contact with the gut luminal content including bacteria and their metabolites and other chemical stimuli that trigger chemokine production in a dysregulated gut homeostasis. Experimental evidence has demonstrated up-regulation of the chemokine IP-10 in ileitis [Hoermannsperger and others, 2009], brain ischemia [Wang and others, 1998], and adipocyte maturation [Krinninger and others, 2011]. In this study, it has

been shown that CAPE abolishes expression of both TNF- and LPS-induced IP-10 expression in intestinal cells and embryonic fibroblasts from $TNF^{\Delta ARE/+}$ mice. In inflammation, many chemokines are expressed at different times following cell stimulation, thus failure by CAPE to inhibit MIP-2 gives a hint into the complexity of signals that are involved in leukocyte trafficking under inflammatory conditions. Hence, blockade of a single chemokine secreted by the epithelium should not be expected to be sufficient to suppress disease progression. Furthermore, a prerequisite for *in vivo* activity is that active compounds must reach target cells (distal epithelial cells in the present study). Whereas CAPE permeated IEC membranes at micromolar concentrations in the *in vitro* assays, this was most likely not the case *in vivo*, possibly due to rapid absorption in the upper GI tract and subsequent excretion or to degradation by intestinal microorganisms [Gonthier and others, 2006]. Contrary to these findings, it has been previously reported that CAPE attenuates peptidoglycan-polysaccharide-induced colitis in rats [Fitzpatrick and others, 2001]. However, the authors used an acute model of inflammation and daily intraperitoneal injection of CAPE (30 mg/kg body weight) for one week, which contrasts the dietary treatment (10 mg/kg, three times a week over six weeks) in a chronic inflammatory model of ileitis.

The observations in CAPE-based studies as a therapeutic molecule have huge variations in the resultant functional effects based on the dose dependence. In connection to this, it is difficult to establish the minimum inhibitory concentrations required for a specific target in a cell (the sub-optimal amount), since in most study models the apparent toxicity of CAPE is in the molecular concentration range of 1-100 µM. Hence, the resultant supra-physiological effects exhibited after administration of doses beyond 20 µM cannot be ruled out. Likewise cell line specificity in relation to dose dependence of CAPE is widely varied [Coban and others, 2008, Demestre and others, 2009, Lee and others, 2009, Lee and others, 2008, Natarajan and others, 1996, Onori and others, 2009, Orban and others, 2000]. All this variability could be due to the postulation that many factors contribute to the differences in cell type specificity of a compound, including cellular uptake and metabolism or “off target” (where compound target is unknown) interactions with other cell components [Feng and others, 2009]. Moreover, numerous studies show that activity *in vitro* does not necessarily mean that the compounds will be active *in vivo*. One of the requirements for *in vivo* activity is that the compounds “must” be able to enter cells and induce responses. Thus *in vitro* studies could be of limited use, if not validated in vivo where many factors come into play. The most common methods for predicting whether a small molecule binds to various drug targets

involves either high-throughput laboratory screening or virtual simulation compounds to check if they fit together with proteins, like “lock and key”. These computational methods can reveal both the ligand binding site and binding affinity, but all these require identification of the most appropriate target proteins via laboratory experimentation, a part of vital information that is largely missing on CAPE. In the transcription activation study using HEK 293 cells that do not express IRF1, the inhibitory effect of CAPE was somewhat diminished in comparison to IRF1 expressing cell lines, strongly implicating an IRF1 NF-κB association in IP-10 expression [Hoffmann and others, 2003].

5.5 Influence of CAPE in oxidative/electrophilic stress response

HO-1 is a stress response protein that has been shown to poses anti-inflammatory activity attributed to carbon monoxide (CO) production following heme degradation [Maines, 1997]. In addition, generation of HO-1 deficient mice suggests an anti-inflammatory role for HO-1, as these mice display increased inflammatory state. However, this anti-inflammatory effect exhibited by HO-1 was not observed in this study. Furthermore we were unable to show any role for HO-1 in NF-κB mediated .IP-10 expression. While, CAPE can elicit the induction of HO-1, which is cytoprotective against cellular oxidative/electrophilic stress it is difficult to see the physiological role of HO-1 protein in IP-10 inhibition.

Even though numerous studies show that, Nrf2 and p38 MAPK pathways are required for the induction of HO-1 by polyphenols, there is no evidence of an anti-inflammatory role for HO-1in IP-10 expression. Nrf2 interacts with its cognate DNA binding domain in the HO-1 promoter resulting in the up-regulation of *ho-1* gene transcription. In addition, other intracellular signaling molecules, including the MAPK family of proteins are involved in Nrf2 transcript activation [Farombi and Surh, 2006]. Depending on the specific stimulus and cell type p38 MAPK and/or ERK may be implicated in Nrf2-dependent HO-1expression, which has been shown to be less inducible in Nrf2-deficient mice [Khor and others, 2008]. Thus activation of p38 MAPK and ERK as observed in this study suggests their involvement in Nrf2 and HO-1 induction, albeit with no accompanying anti-inflammatory effects. For instance, just like CAPE, quercetin induced Nrf2- dependent HO-1 expression to protect human hepatocytes from ethanol-induced oxidative stress [Yao and others, 2007]. Following the remarkable induction of HO1 in Mode-K, it was investigated whether other cell lines respondent strongly to CAPE in terms of up-regulated HO1. Interestingly, activation of HO1 was not cell type-dependent given that the magnitude of induction was the same in other cell

types besides Mode-K, i.e., RAW264.7 macrophage and NRF2 MEF. However, the order of potency in the inductions was dose-dependent, whereby the RAW264.7 macrophage cell line was more sensitive while the MEF cell line was less sensitive in comparison to Mode-K cells.

5.6 De novo synthesis of Nrf2 is required for inducible expression of HO1

Cycloheximide (CHX) which completely blocks protein translation and not transcription significantly inhibited HO1 mRNA expression but not Nrf2 mRNA. This finding suggests that HO1 induction resulted primarily from transcriptional activation by the newly synthesized Nrf2 protein as opposed to the general assumption that it is dependent on the constitutive expression of Nrf2 [Nguyen and others, 2005]. Structural conformational changes in the Keap1 protein pool allows the newly synthesized Nrf2 to escape the Keap1gating where it binds to the ARE and induces expression of HO1 and other phase 2 enzymes, probably due to insufficient amounts of Keap1 that represses Nrf2 activity [Dinkova-Kostova and others, 2002, Dinkova-Kostova and others, 2005, Hayes and others, 2010]. Deficiencies of Nrf2 transcription factor in mice abrogate substantially the inducible expression of ARE-mediated detoxifying and antioxidant enzymes increasing the susceptibility of these mice to oxidative and/or carcinogenic compounds. To rescue this important homeostatic cellular defence system from oxidative stress, increased phosphorylation of c-Jun in $Nrf2^{-/-}$ but not $Nrf2^{+/+}$, with concomitant HO1 induction in the presence of CAPE was observed.

Studies show that small molecules have protective effects in a cytokine environment because of their anti-inflammatory or antioxidant effects. Because of the significance of inflammatory cytokines to the cell, a lot of effort has been made in identifying genetic or small molecule approaches that protect cells from cytokine induced inflammation. When CAPE was added to $Nrf2^{-/-}$ a remarkable induction of HO1 even in the absence of Nrf2 was observed, indicating that HO1 has a very complex regulation and its gene expression is only partially dependent on Nrf2. Thus activation of the stress response element promoter in $Nrf2^{-/-}$ is significant and results in HO1 expression levels comparable to the $Nrf2^{+/+}$ cells. Cycloheximide (inhibits protein biosynthesis by blocking translational elongation) provides the ability to observe the half-life of a protein without confounding contributions from transcription or translation. Hence, the modest amounts of Nrf2 that can be produced in the presence of cycloheximide would not be expected to grossly alter HO1 expression; hence blockade of protein translational machinery implicates de novo synthesis of Nrf2 in *ho1* gene induction. This appears specific to HO1, because other non Nrf2-inducible genes like IP-10

and Nrf2 itself were still expressed following translational inhibition, indicating that the basal Nrf2 protein pool was not sufficient enough to induce HO1 mRNA transcription [Nguyen and others, 2005].

Nrf2 mutants are more sensitive than the wild type to CAPE. Deletion of the *nrf2* gene greatly alters cellular physiology by increasing the susceptibility of this phenotype to oxidative and electrophilic compounds. However, the sensitivity of $Nrf2^{-/-}$ cells, unlike that of the wild types is rescued by c-Jun over expression. In particular, c-Jun is rapidly turned over and phosphorylated, most likely reflecting a failure to activate the typical Nrf2- dependent antioxidant enzyme NQO1 under the control of ARE promoter. Over expression of c-Jun as a compensatory mechanism then induces HO1 enzyme as a response to the inducer compounds, albeit with no consequential cellular cytoprotective effects, indicating that this rescue of CAPE toxicity is less efficient than in wild type cells. Activating protein 1 (AP-1) is complex heterodimer of c-Jun and c-Fos that mediates HO1 gene activation in response to many regulatory factors including cytokines, growth factors, bacterial and viral infections, cellular stresses and extra-cellular signals; thus, activation of HO1 is not actively dependent on Nrf2 alone. The rapid induction of c-Jun in Nrf2 deficient cells has not been observed before, probably because previous experiments did not focus on other HO1 inducible pathways like AP-1. Hence for the first time this study identifies Nrf2 and c-Jun as primary targets of CAPE in HO1 induction.

5. 7 Benefits and limitations of CAPE in the study

Examples of emerging evidence linking conventional foods to health benefits include cancer risk reduction. Cruciferous vegetables reduced risk of several types of cancer in experimental and epidemiologic studies [Le Bon and Siess, 2000, Moriarty and others, 2007, Nagini, 2008, Sumiyoshi and Wargovich, 1990]. Tomato products rich in lycopene may reduce the risk of prostate, ovarian, gastric, and pancreatic cancers [Kavanaugh and others, 2007]. Citrus fruit may reduce the risk of stomach cancer [Bae and others, 2008]. For heart health, dark chocolate improved endothelial function [Faridi and others, 2008], and tree nuts and peanuts reduce the risk of sudden cardiac death [Kris-Etherton and others, 2008]. For intestinal health maintenance, fermented dairy products (probiotics) may improve irritable bowel syndrome [Quigley, 2007]; and for urinary tract function, cranberry juice reduced bacteriuria [Nowack, 2007]. Our improved understanding of human dietary requirements is as a result of developments in many scientific disciplines, including food science, nutrition,

chemistry, biochemistry, physiology, and genetics. Novel research in proteomics, metabolomics and nutrigenomics has further helped in the identification of the mechanisms of action by which the bioactive food constituents promote health including CAPE. A sound scientific basis for the relationship between functional foods and health benefits is important in the design and development of health claims. Numerous scientific studies have developed experimental approaches that can be used in the identification of potential relationships between bioactive food components and health benefits. Given that CAPE is the principle active component of the honeybee propolis, bee keepers can be educated on the importance of harvesting the wax for propolis extraction and subsequent use in processing into health promoting bio-products. However, evidence from this study is not sufficient enough to promote CAPE as a functional food product and more studies are needed.

This study was limited to the use of one experimental mouse model of ileitis (TNF that spontaneously develops inflammation in the first 4 weeks of birth. Thus, the failure by CAPE to ameliorate inflammation in the ileum could be attributed to this pathophysiological condition that is genetically inherent. It would be interesting to carryout further experiments in Interleukin-10 (IL-10) knock out mouse model of colitis which shows similarities to human inflammatory bowel disease given that the the disruption of IL-10 gene is not so severe when comparedto the TNF knock in.

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Inflammatory responses are important in many human diseases, and strategies directed at modulating the associated pathways have been successfully applied in the treatment of several chronic disorders with polyphenols. It is now evident that the catechol moiety controls CAPE potency and that slight change in structure does not necessarily mean small changes in activity. In addition, it has been shown that CAPE exhibits its anti-inflammatory effects by disrupting the p50/RelA NF-κB heterodimer binding to the IP-10 promoter and that IRF1 is a requirement in the NF-κB- mediated IP-10 expression. Although feeding experiments in $TNF^{\Delta ARE/+}$ mice failed to prevent TNF-driven Crohn's disease ileitis, the anti-inflammatory potential of CAPE was confirmed in ileal tissue explants and embryonic fibroblasts derived from $TNF^{\Delta ARE/+}$ mice. CAPE induces HO1 expression through the Keap1/Nrf2 signalling as well as phosphorylation of c-Jun protein in the AP-1 signalling pathway. In summary, knowledge about cellular and immune responses to CAPE treatment is broad, but somewhat superficial and more studies are needed to improve our understanding. Minimal information is available regarding mechanisms of action in different diseases and data on pathway interactions is largely missing with regard to activation and inhibition. Hence, even with the existence of highly efficient small molecules like CAPE, it is necessary to search for new combinations via molecular similarity studies to improve on therapeutic efficacy.

6.2 Recommendations

Structural modification of plant based molecules has led to the development of useful inhibitors in the treatment and management of chronic illnesses. The huge range and variability of available data coupled with overlapping roles of the NF-κB pathway components in different settings indicates that there is more to learn about CAPE effects on many of these physiological processes. One of the emerging themes underlying the anti-inflammatory, anti-oxidative and pro-apoptotic effects of CAPE is the ability to regulate signalling pathways that are largely mediated by the ability to interact with a variety of cells of the epithelial and immune systems. However, there are still some unanswered questions about how these processes are regulated, especially how different NF-κB, IκB and IKK isoforms, IRF1, Nrf2/Keap1 and other transcriptional cofactors are affected. CAPE has been shown to function under different disease conditions, but the information is somewhat scanty

on mechanistic effects. Nonetheless, the general agreement is that CAPE actively inhibits NF-κB and leads to deregulation of inflammatory gene expressions in varied physiological and pathological processes, including glucose homeostasis, host defence against infection, inflammation and cancer. Moreover, each of the functions described indicates that distinct mechanisms of action are involved and may act in concert to increase the biological effects observed *in vitro*, although the cellular localization and downstream molecular events following CAPE interaction with these cells is largely unknown. Therefore determination of the molecular mechanisms and target protein identification would be important follow-up steps that would address various questions including, functional effects, target selectivity, and the structural requirements and features essential for maintaining specific biological activity both *in vitro* and *in vivo*.

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6.4 Appendices

Appendix I: Publications

Original paper

- Mapesa, J. O., Waldschmitt, N., Schmoeller, I., Clavel, T., Hofmann, T., Mahungu, S. and Dirk Haller (2011). Catechols in Caffeic Acid Phenethyl Ester are essential for inhibition of TNF-mediated IP-10 expression through NF-κB-dependent but HO-1- and p38-independent mechanisms in mouse intestinal epithelial cells. *Molecular Nutrition & Food Research,* **55**: 1-12.
- Mapesa, J. O., Schmoeller, I., Clavel, T., Mahungu, S. and Dirk Haller. Identification of Nrf2 and c-Jun as primary targets of Caffeic Acid Phenethyl Ester in HO1 induction (**Manuscript**).
- Mapesa, J. O., Schmoeller, I., Clavel, T., Mahungu, S. and Dirk Haller. Caffeic acid phenethyl ester inhibits TNF-induced IP-10 expression via regulation of NF-B and IRF-1 in intestinal epithelial cells (**Manuscript**).

Poster presentation

- Mapesa, J. O., Schmoeller, I. and Dirk Haller (2010). Anti-inflammatory effects of Caffeic Acid Phenethyl Ester (CAPE) and CAPE analogues on murine intestinal epithelial cells – Structure activity relationship. *Proceedings of the 47th Scientific Congress of the German Society for Nutrition,* Jena, Germany.

Appendix II: Technical equipment

Equipment	Company
Desk-top centrifuge	Sigma
Thermomixer	Eppendorf
CO_2 incubator	Kojair
Laminar flow cabinet	Kojair
Weighing scale	Denver Instrument
Light cycler	Roche
Light microscope	Leica microsystems
Spectrophotometer Nanodrop ND-1000	PEQLAB
Thermocycler	Thermo electron corporation
Water bath (Thermo HAAKE DC30)	Thermo scientific
Orbital shaker	Thermo scientific

Biophotometer	Eppendorf
pH meter	Inolab
Multistainer Leica ST5020	Leica microsystems
Leica RM2255	Leica microsystems
Leica EG1150C	Leica microsystems
Refrigerator/Freezer	Thermo electron corporation
GS-800 Calibrated Densitometer	Bio-rad
Variable mode imager (Typhoon TRIO+)	GE Healthcare
UV-imager (Syngene-bio imaging)	SYNGENE
Power pack HC	Bio-rad
Microwave	Triple distribution systems
Fluoroskan Ascent	Thermo Labsystems
Luminoskan Ascent	Thermo Labsystems
Multiskan spectrum	Thermo Labsystems
Vortex	Bio-rad

Appendix III: Chemicals and reagents

Agarose	Roth, Karlsruhe
Ammonium persulphate (APS)	Roth, Karlsruhe
Bromophenol blue	Roth, Karlsruhe
CHAPS	Roth, Karlsruhe
Chloroform	Merck, Darmstadt
dNTP mix (RT PCR)	Invitrogen
DMSO	AppliChem
MG132 (proteasomal inhibitor)	Sigma
Cycloheximide (CHX) (Protein synthesis inhibitor)	Sigma
SB203580 (p38 MAPK inhibitor)	Sigma
Bay11-7082	Sigma
Epigallocatechin gallate (EGCG)	Sigma
Enterodiol (ED)	Sigma
Enterolactone (EL)	Sigma
Carbon monoxide releasing molecule 2 (CORM-2)	Sigma
Actinomycin D (RNA transcription inhibitor)	Sigma
Tunicamycin	Sigma
Dulbecco's Phosphate buffered solution (PBS) Cat No: D8537	Sigma

Dulbecco’s modified eagle medium (DMEM) Cat No: 41965-039	Invitrogen
Iscoves modified Dulbecco’s medium (IMDM) Cat No: E15-819	PAA
Bovine serum albumin (BSA)	PAA
L-glutamine	PAA
Antimycotic/antibiotic (AA)	PAA
Insulin transferring selenium (ITS) supplement Cat No: F01-015	PAA
Epidermal growth factor (EGF) Cat No: 13247-051	Invitrogen
Dithiothreitol (DTT)	Roth, Karlsruhe
Diethylpyrocarbonate (DEPC)	Roth, Karlsruhe
ECL detection kit	Amersham
EDTA	PAA
Ethanol	Roth, Karlsruhe
5x First strand buffer	Invitrogen
Glycerol	PAA
Glycin	PAA
IFN-γ	Invitrogen
TNF	Invitrogen
Isopropanol	Roth, Karlsruhe
IP-10 ELISA kit	R&D systems
IL-6 ELISA kit	R&D systems
MIP-2 ELISA kit	R&D systems
TNF ELISA kit	R&D systems
Methanol	Roth, Karlsruhe
MMLV-reverse transcriptase	Invitrogen
Milk powder, blotting grade	Roth, Karlsruhe
Pre-stained protein marker	Fermentas
Ponceau S	Sigma
RNase Out	Invitrogen
Random hexamers	Invitrogen
Sodium chloride	Roth, Karlsruhe
Sodium dodecyl sulphate	Roth, Karlsruhe
TEMED	Roth, Karlsruhe
Urea	Roth, Karlsruhe
Thiourea	Roth, Karlsruhe
Tris	Roth, Karlsruhe
Boric acid	Roth, Karlsruhe

Trizol	Roth, Karlsruhe
Trypan blue stain	Roth, Karlsruhe
Polyacrylamide 30 % (37.5:1)	Roth, Karlsruhe
0.25 % Trypsin-EDTA Cat No: 25200-056	Invitrogen

Appendix IV: Western blot buffers and solutions

PBS (without Ca and Mg)	**Laemmli buffer**
7.95g NaCl	12.5 ml 1.5M Tris·HCl, pH 6.8
1.44g $Na_2HPO_4 \cdot 2H_2O$	8 ml 10 % SDS
0.2g KCl	7.9 g Glycerol (99%)
0.2g KH_2PO_4	100 mM DTT (or 5ml β-mercaptoethanol)
Add H_2O up to 1l	0.5 % Bromophenol Blue
Adjust to pH=7.4	Add H_2O until 50ml
(Filtrate and autoclave)	
1.5 M Tris·HCl, pH 8.8	**0.5 M Tris·HCl, pH 6.8**
45.43 g Tris in 250 ml H_2O	15.14 g Tris in 250 ml H_2O
Adjust to pH=8.8 with HCl	Adjust to pH=6.8 with HCl
10% SDS	**25 % APS (Ammonium Persulfate)**
100 g SDS in 1l H_2O	500 mg APS in 2 ml dH_2O. Aliquot. Store at -20°C
10 x Running-buffer	**Semi-dry-blotting-buffer**
30 g Tris	3.03 g Tris
144 g Glycin	14.4 g Glycin
10 g SDS	200 ml 100 % Methanol
Add H_2O until 1l	Add H_2O until 1l
10x TBS (Tris Buffer Saline)	**TBST (Tris Buffer Saline-Tween)**
24.2 g Tris	100 ml 10 x TBS
80 g NaCl	1 ml Tween 20
Add H_2O until 1l	Add H_2O until 1l
Ponceau S	**5 % Non fat skim milk**
5 g Ponceau S	1.5 g Milk powder in 30ml TBST
10 ml 100%AcH	**Membrane stripping buffer**

Add H_2O up to 1l	0.2 M Glycine·HCl,pH 2.5, 0.05 % Tween20

Appendix V. SDS-PAGE preparation scheme

10 % Resolving Gel Buffer (Running Gel)	Volume
ddH_2O	4.1 ml
30 % PA [(37.5:1 Acrylamide/Bisacrylamide) Rotiphorese]	3.3 ml
1. 5 M Tris-HCl, pH 8.8	2.5 ml
10 % w/v SDS	100 µl
25 % APS	50 µl
Temed	5 µl
TOTAL VOLUME	**10 ml**

10% Stacking Gel Buffer (Loading Gel)	Volume
ddH_2O	2.05 ml
30 % PA [(37.5:1 Acrylamide/Bisacrylamide) Rotiphorese]	1.65 ml
0.5M Tris-HCl, pH 6.8	1.25 ml
10 % w/v SDS	50 µl
25 % APS	25 µl
Temed	5 µl
TOTAL VOLUME	**5 ml**

Appendix VI. Cell lysis buffer components

Composition	**20 ml bidest water**
Urea (7M)	8.4 g
Thiourea (2M)	3.04 g
CHAPS (2 %)	0.4 g
DTT (65 mM)	0.202 g
Protease inhibitor cocktail	1 tablet
Phosphatase inhibitor cocktail	1 tablet

Appendix VII. Antibodies used

Primary antibody	Dilution	Company
Acetylated Histone 3 (ac-H3)	1:2000	Cell signalling
CHOP	1:2000	Cell signalling
c-Jun	1:2000	Cell signalling
Cleaved caspase 3	1:2000	Cell signalling
Histone 3 (H3)	1:5000	Cell signalling
HO1	1:5000	Stressgen
IP-10	1:500	R&D
IκBα (#9242)	1:5000	Cell signalling
Keap1	1:1000	Santa cruz
Mouse anti-rabbit IgG (Light-chain specific)	1:2000	Cell signalling
Nrf2	1:1000	Santa cruz
p38 MAPK	1:2,000	Cell signalling
Phospho-IκBα (Ser32/36)	1:2000	Cell signalling
Phosphor-c-Jun (Ser73)	1:2000	Cell signalling
Phospho-RelA (ser536)	1:2,000	Cell signalling
Phospho-p38 MAPK (Thr180/Tyr182)	1:2,000	Cell signalling
p65/relA	1:2,000	Cell signalling
Ubiquitin	1:2000	Cell signalling
β-actin	1:5,000	MP biomedicals
Secondary antibody		
ECL anti-goat IgG HRP	1:2,000	Cell signalling
ECL anti-mouse IgG HRP	1:5,000	Cell signalling
ECL anti-rabbit IgG HRP	1:4,000	Cell signalling

Appendix VIII. Electrophoretic Mobility Shift Assay (EMSA) buffer composition

Homogenisation buffer (10 ml)	Low salt buffer (LSB) (10 ml)
10 mM HEPES (pH 7.9)	10 mM HEPES (pH 7.9)
1.5 mM $MgCl_2$	1.5 mM $MgCl_2$
10 mM KCl	20 mM KCl

0.5 mM DTT
0.5 % NP-40
1 mM PMSF
Phosphatase inhibitor cocktail (1 tablet)
Protease inhibitor cocktail (1 tablet)

0.5 mM DTT
0.2 mM EDTA
1 mM PMSF
Phosphatase inhibitor cocktail (1 tablet)
Protease inhibitor cocktail (1 tablet)
25 % Glycerol

High salt buffer (HSB) (10 ml)

10 mM HEPES (pH 7.9)
1.5 mM $MgCl_2$
1.2 M KCl
0.5 mM DTT
0.2 mM EDTA
1 mM PMSF
Phosphatase inhibitor cocktail (1 tablet)
Protease inhibitor cocktail (1 tablet)
25 % Glycerol

5x binding buffer (10 ml)

50mM TrisHCl pH 7.5
250 mM KCl
5 mM EDTA
5 mM DTT
75 % v/v Glycerol
Poly (dI-dC) (0.5 µg/µl end concentration)

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poly(dI-dC) Amersham Cat No: 27-880-01; 10 A_{260} units