AN ASSESSMENT OF THE EFFECTS OF NOVEL ANTI-INFLAMMATORY COMPOUNDS IN CELL BASED STUDIES

by

FADIA MOHAMED GAFRI

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University of Strathclyde,

Strathclyde Institute of Pharmacy and Biomedical Sciences

(SIPBS),

Glasgow, UK

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ABSTRACT

NF-κB and AP-1 are transcription factors with an evolutionary conserved role in the triggering and coordination of both innate and adaptive immune responses. Since they regulate a large number of inflammatory genes, they are considered as potential targets for anti-inflammatory drugs. In the current study, natural SU182 and synthetic alkaloid compounds, SU331, SU432 and minor groove binder compounds AIK18/85/1 and AIK18/70 obtained from the library associated with University of Strathclyde CRUK-Small Molecule Drug Discovery (SMDD) were investigated for possible anti-inflammatory effects at μM concentrations. In NCTC2544 human keratinocyte cells stably transfected with either NF-κB- or AP-1-linked luciferase reporter plasmids, tumour necrosis factor-α (TNF-α) and phorbol-12-myristate-13 acetate (PMA) well characterized stimuli for canonical NF-κB pathway induced NF-κB and AP-1 transcriptional activity respectively. However, all tested compounds inhibited NF-κB transcriptional activity; in particular AIK18/85/1 prevented the TNF-α-induced translocation of NF-κB (p65) to the nucleus assessed by indirect immunoflouresnce. This effect of AIK18/85/1 was also reflected in the significant reduction of nuclear extract NF-κB-DNA binding activity as detected by Electrophoresis Mobility Shift Assay (EMSA), but without affecting the degradation of IκBα protein induced by TNF-α. Furthermore, AIK18/70 also decreased TNF-α-induced NF-κB-DNA binding activity but neither affected the phosphorylation of p65 nor the degradation of IκBα. On the other hand, both SU331 and SU432 inhibited the IκBα loss and resultant NF-κB-DNA binding activity in a concentration dependent manner. Although none of these compounds inhibited TNF-α-induced phosphorylation of NF-κB (Ser536-p65), their mode of inhibition on NF-κB signalling was sufficient to prevent the expression of NF-κB dependent proteins such as COX-2 and iNOS in LPS stimulated RAW 264.7 macrophage cells. Intriguingly, in contrast to other compounds SU182 was only effective at the level of NF-κB and AP-1 transcriptional activities, but without affecting the expression level of iNOS and COX-2 enzymes.

Taken together these data indicate the potential for tested compounds to interfere with NF-κB signalling as IKK inhibitors or novel translocation inhibitor and thus

may considered to be useful leads for the development of novel anti-inflammatory and anticancer drugs.

PUBLICATIONS

Research papers:

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DEDICATIONS

To

My mother, soul of my father and Libyan heroes who martyred or were injured for liberty.

ABBREVIATIONS

AAT	Alpha-1-antitrypsin
AP-1	Activator protein-1
Akt	Protein kinase B (PKB)
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BCL-3	B-cell lymphoma 3
BH4	Tetrahydrobiopterin,
BSA	Bovine serum albumin
β-TrCP	Beta-transducin repeats-containing protein
CAPE	Caffeic acid phenyl ester
C/EBP	CCAAT-enhancer binding protein
cNOS	Constitutive nitric oxide synthase
COPD	Chronic obstructive pulmonary disease
COXs	Cyclooxygenases (1 and 2)
CRE	cAMP response element
CREB	cAMP response element binding protein
DAPI	4', 6-Diamidino-2-phenylindole
DCIC	3,4-dichloroisocoumarin

DHMEQ	Dehydroxymethylepoxyquinomicin
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DSS	Dextran sulphate sodium
DTT	Dithiothreitol
ECL	Enhanced chemilluminescence
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis (β-aminoethyl ether) tetraacetic acid
EMSA	Electrophoretic mobility shift assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal regulated kinase
FAD	Flavin adenine dinucleotide
FCS	Foetal calf serum
FMN	Flavin mononucleotide
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde phosphate dehydrogenase
GAS	γ-activated sites
h	Hours
HDACs	Histone deacetylaces

HMG-A1	High mobility group-A1
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
ICAM	Intracellular adhesion molecule
IFN-γ	Interferon γ
IRF-1	IFNγ regulatory factor-1
IκB	Inhibitor of Kappa B (α, β, γ, ε and ζ)
IKK	IκB kinase (α, β and γ)
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP-10	Immune protein 10
IRAK	IL-1 receptor-associated kinase
ISRE	Interferon-α stimulated response element
JAK	Janus tyrosine Kinase
JNK	c-jun N-terminal kinase
KC	Keratinocyte chemoattractant
kDa	kilo-Dalton
l	Liter
LH	Luteinizing hormone
LPS	Lipopolysaccharide
M	Molar

mA	milliampere
MAP kinases	Mitogen activated protein kinases
MCP-1	Monocyte chemotactic protein-1
MEK	MAP Kinase kinase
MEKK	MAP kinase kinase kinase
mg	milligram
MGBs	Minor groove binders
min	minute
MIP	Macrophage inflammatory protein (1α, 1β and 2)
ml	milliliter
MKP-1	MAPK phosphatase 1
mM	millimolar
MMPs	Matrix metalloproteases
MnSOD	Manganese superoxide dismutase
mRNA	Messenger ribonucleic acid
MSK	Mitogen and stress activated kinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation factor 88
NADPH	Nicotinamide adenine dinucleotide
NEMO	NF-κB essential modifier

NES	Nuclear export signal
NFAT	Nuclear factor of activated T cells
NF-IL6/C/EBP	Interleukin-6/CCAAT enhancer-binding protein
NF-κB	Nuclear factor kappa B
ng	Nanogram
NIK	NF-κB inducing kinase
NLS	Nuclear localisation sequence
nm	Nanometer
NMR	Nuclear magnetic resonance
·NO	Nitric oxide
nNOS	Nuronal nitric oxide synthase
NOSs	Nitric oxide synthases
NP-40	Nonidet P-40
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAGE	Polyacrylamide gel electrophoresis
PAMP$_S$	Pathogen-associated molecular Patterns
PAR-2	Protease- activated receptor-2
PBS	Phosphate buffered saline
P/CAF	P300/CBP-associated factor
PDGF	Platelet-derived growth factor
PDTC	Pyrrolidine dithiocarbamate

PEA3	Polyomavirus enhancer activator 3
PGs	Prostaglandins
PI3K	Phosphoinositide-3- kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylymethylsulphonyl fluoride
PPARs	Peroxisome proliferative activated receptors
PPI	Protein-protein interaction
PPRs	Pattern recognition receptors
RANKL	Receptor activator of NF-κB ligand
RANTES	Regulated upon activation in normal T cell expressed and secreted
RA	Rheumatoid arthritis
RASFs	Rheumatoid arthritis derived synovial fibroblasts
RHD	Rel homology domain
RIP	Receptor interacting protein
ROIs	Reactive oxygen intermediates
ROS	Reactive oxygen specicies
RT	Room temperature
SAHA	Suberoylanilide hydroxamic acid
SCF	SKP1–CUL1–F-box protein

SDS	Sodium dodecyl sulphate
SPR	Surface plasmon resonance
SOCS-1	Suppressor of cytokine signalling-1
SRs	Super repressors
SRC-1	Steroid receptor coactivator
STAT	Signal transducer and activator of transcription
TAB	TAK1-binding protein
TAD	Transactivation domain
TAK1	TGF-β-activated kinase 1
TNBS	2,4,6-trinitrobenzene sulfonic acid
TSA	Trichostatin A
TEMED	N, N, N', N'-tetramethylethylenediamine
TGF-β	Transforming growth factor β
TLCK	7-amino-1- chloro-3-tosylamido-2-heptanone
TLRs	Toll like receptors
TNF-α	Tumour necrosis factor-alpha
TNFR	Tumour necrosis factor receptor
TOLLIP	Toll-interacting protein
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone
Tpl-2	Tumour progression locus-2
TRADD	TNFR-associated death domain

TRAF	TNFR-associated factor (2 and 6)
TRAIL	TNF-related apoptosis induced ligand
UC	Ulcerative colitis
UV	Ultraviolet
V	Volt
VCAM	Vascular adhesion molecule
WB	Western blotting
wtNBD	Wilde type nemo binding domain peptide
YopJ	Yersinia outer membrane protein-J
-/-	Gene deletion
ºC	Celsius degree
µg	Microgram
µl	Microliter
µM	Micromolar

AMINO ACID ABBREVIATIONS

Symbol	3-letter code	Full name
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

1.1.1 Immunity and inflammation

Generally all living organisms constantly exposed to a variety of infectious, non-infectious, and environmental microorganisms. Most surfaces, including the cutaneous epithelia and the mucosal linings represent an efficient barrier against potential pathogens. The microorganisms that succeed in passing this first line of intrinsic defense will be eliminated by other defense mechanisms including the cellular innate immune response, through accumulation of immune cells (e.g. phagocytic neutrophils and macrophages) at the site of infection. In addition, humoral innate immunity, constituted in plasma proteins (e.g. complement components) also join at the site of infection (Janeway, 2004). Current perceptions do however identify inflammation as a reaction involving a variety of diseases including those of non-pathogenic origin. Inflammation in non-pathogenic disease recognised with the participation of circulating leukocytes and cells of the lymphoid organs in the inflammatory response along with sentinel cells (dendritic cells, endothelial cells, cardiomyocytes, fibroblasts, keratinocytes and mast cells) of all tissues (Linde et al., 2007).

Whilst the specific nature of the adaptive immune response is well accepted the potential of specific recognition within the innate immune response has only recently been established. A broad range of molecular patterns, called pathogen-associated molecular Patterns ($PAMP_S$) can be detected by the first line of immune defense, that activate the innate immunity and inflammatory response (Medzhitov and Janeway, 2000). This is mediated by pattern recognition receptors (PPRs), proteins which recognise microbial pathogens or ligands of specific molecular structures. The expression of highly active genes subsequent to the activation of innate immunity is able to induce rapid (minutes to hours) defensive immune responses (Janeway and Medzhitov, 2002).

1.1.1.1 Toll like receptors (TLRs)

Several cell types play an important role in innate immunity. Monocytes, macrophages and their tissue-differentiated derivatives, such as microglia in the nervous system, express different PRRs, various scavenger and Toll-like receptors (TLRs). TLRs are a major family of trans-membrane receptors which recognise PAMPs such as cell wall components and nucleic acids of a large array of infectious organisms (Akira and Takeda, 2004). In mammals there are at least 11 TLRs able to distinguish fine differences between ligands (Akashi-Takamura and Miyake, 2006), for instance, lipopolysaccharide (LPS) an endotoxin of gram negative bacteria is one of the best characterised microbial ligand for TLR4 (Rakoff-Nahoum and Medzhitov, 2007). Activation of these receptors results in the initiation of a number of major cell signaling cascades including the NF-κB, MAP kinases (Section 1.4, 1.7), JAK/STAT and PI3K/Akt pathway. As a result, TLR activation induces the expression of a broad number of genes, encoding proteins including cytokines, which in turn regulates initial leukocyte activation and tissue inflammation (Guha and Mackman, 2001). Thus, the initiation of defensive action against pathogens (limiting tissue damage and enhancing fast recovery and tissue healing) is dependent on the capability of each organism to regulate the activation of innate immunity and local inflammatory responses. Furthermore, identification of the TLRs has not only put inflammation in the context of the innate immune response but has also helped to increase the understanding of the cellular mechanisms involved.

1.1.2 The inflammatory response

In response to cellular injury or infection a complex molecular and cellular interactions directed to mediate a return to physiological homeostasis and tissue repair. This inflammatory response is composed of both local events and systemic activation mediated by cytokines (Licastro et al., 2005). The classical key features of inflammation are redness, heat, swelling and pain which reflect these events. Usually, inflammation becomes a chronic condition if damaged tissue is not restored or stable irritation persists. A slow accumulation of collateral damage caused by chronic inflammation sometimes silent (asymptomatic) for years, but can eventually lead to severe tissue deterioration (Licastro et al., 2005). Therefore, chronic

inflammation is linked to the development of diseases such as asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and psoriasis. Key cellular events which drive components of the inflammatory process, include the synthesis and release of cytokines and chemokines, complement proteins, matrix metalloproteases (MMPs), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Barnes et al., 1998).

Cytokines are large secreted molecules that act on the surrounding micro-environment through cell to cell signalling. They are components of a complex signalling network. Their effects on target cells may be inhibited or enhanced by other cytokines, hormones, cytokine-receptor antagonists and circulating receptors. Tumor necrosis factor α (TNF-α), Interleukin-1 (IL-1) and Interleukin-6 (IL-6) are the classical pro-inflammatory cytokines (Bidwell et al., 1999), which have the ability to activate both local and systemic effects (see review by Watkins et al. (1995)). Locally, they contribute to the activation of the inflammatory cells, and along with chemokines, induce the expression of adhesion molecules (selectins, ICAM and VCAM) (Walzog and Gaehtgens, 2000) on the surface of endothelial cells to cause local recruitment (Licastro et al., 2005) and adherence of monocytes (Szmitko et al., 2003).

Chemokines can mediate the recruitment of inflammatory cells (e.g. T cells, dendritic cells, mast cells, eosinophils and neutrophils) for example in the case of allergy and asthma (Lukacs et al., 1996). In addition, chemokines play a role in inflammatory cells differentiation and activation, by selectively activating Th1 or Th2 cells or by effects on epithelial or endothelial cells (Smit and Lukacs, 2006).

In addition, the regulation of expression and activation of MMPs are considered critical for leukocyte transmigration (Goetzl et al., 1996, Leppert et al., 1996, Xia et al., 1996). Many MMPs were found in inflamed tissues; in particular MMP-19 has been isolated from the inflamed synovium of patients with rheumatoid arthritis (Sedlacek et al., 1998). Up-regulated MMP-2, MMP-9, and MMP-7 have also been found in macrophages within multiple sclerosis lesions, which are expected to play a

role in the deterioration of the blood-brain barrier, leukocyte emigration, and tissue destruction in this condition (Anthony et al., 1997, Madri and Graesser, 2000). These data are consistent with in vivo studies which confirmed that MMPs are involved in rodent models of multiple sclerosis (Gijbels et al., 1994, Graesser et al., 1998, Hewson et al., 1995).

In addition to MMPs, two key proteins that also feature in the production of inflammatory mediators during inflammation are the enzymes cyclooxygenases (COXs) (Simmons et al., 2004) and nitric oxide synthase (NOS) (Redington, 2006, Gautam, 2007). These mediators will be discussed further (Section 1.1.11).

1.1.3 Nuclear Factor kappa-B (NF-κB)

Transcription is a critical regulatory event in the pathways leading to gene expression relevant to inflammation. Transcription factors regulate transcription by binding to specific nucleotide sequences of genes present within the promoter, enhancer, or other regulatory regions of DNA (Latchman, 1995). This results in either activation or repression of a number of genes. There are many transcription factors and signalling pathways that can regulate the development of cancer and other diseases such as asthma, inflammatory bowel disease (IBD), rheumatoid arthritis (RA), atherosclerosis, cystic fibrosis, diabetes, AIDS, and Alzheimer's disease (Sebolt-Leopold and English, 2006). Of these, the transcription factor NF-κB plays a crucial role because of the rapidity of its activation and that regulate large number of genes that are implicated in inflammatory diseases. In addition, NF-κB activation recently considered as the key element that links cancer to inflammation (Karin and Greten, 2005, Folmer et al., 2008) (Section 1.1.13).

1.1.3.1 Components of the NF-κB cascade

Nuclear factor κ-B (NF-κB), a nuclear transcription factor, was first identified in 1986 by Sen and Baltimore, as a nuclear factor bound to an enhancer element of the immunoglobulin kappa light chain gene in B lymphocytes. However, subsequent studies clarified that NF-κB is primarily a cytoplasmic factor expressed by most cell types (Sen and Baltimore, 1986). It comprises a family of ubiquitous proteins; these

proteins contain a Rel homology domain (RHD) (DNA-binding domain/dimerisation domain) with a nuclear localisation sequence (NLS), which is conserved from Drosophila to man (Aggarwal, 2006). The mammalian NF-κB family consists of five members: NF-κB1 (p50/p105), NF-κB2 (p52/p100), c-Rel, RelA (p65) and RelB (Figure 1.1). RelA, c-Rel and RelB subunits are synthesised in their mature forms and contain a transactivation domain (TAD) that interacts with the transcriptional elements. On the other hand, NF-κB1 and NF-κB2 are synthesised in precursor forms (p105 and p100) which contain C-terminal ankyrin repeats that are proteolysed by the proteasome to process the mature forms, p50 and p52, respectively. Both p50 and p52 contain a DNA binding domain but lack a transactivation domain (Lee et al., 2007). NF-κB proteins exist in resting cells as homo- or hetero-dimers. Whilst RelB forms only heterodimers, other NF-κB subunits can form both homo- and hetero-dimers. NF-κB proteins are characterised by the presence of a highly conserved 300 amino acid RHD that is located towards the N-terminus of the protein, which is responsible for protein binding, dimerisation, and interaction with specific inhibitory factors known as the Inhibitor of Nuclear Factor Kappa B (IκB) proteins (Baldwin, 1996, Ghosh and Karin, 2002, Lee et al., 2007).

1.1.3.1.1 IκB proteins

The IκB protein family comprises three groups: the typical IκB proteins IκBα, IκBβ and IκBε (Figure 1.1), which are mainly locate in the cytoplasm of resting cells and undergo stimulus-induced degradation and re-synthesis; the precursor proteins p100 and p105, which can be either processed to the NF-κB mature subunits p52 and p50, respectively, or can be degraded; and the atypical IκB proteins IκBζ (encoded by NFKBIZ), BCL-3 (B-cell lymphoma 3) and IκBNS (encoded by NFKBID), which are only expressed upon stimulation of cells, and mediate their effects in the nucleus. IκBα is the prototypical member of the IκB family of proteins (Ghosh and Hayden, 2008). Following cell stimulation by either LPS, TNF-α or IL-1β, it undergoes rapid ubiquitin-mediated proteasomal degradation that results in the release of the bound, cytoplasmic NF-κB dimers. Subsequently, free NF-κB travels to the nucleus and mediates gene expression, including that of IκBα, which responsible for the

termination of the NF-κB mediated transcriptional response by sequestring NF-κB dimers in the cytoplasm (Hayden and Ghosh, 2004).

1.1.3.1.2 IKK complex

The sequential activation of IκBs and release of NF-κB isoforms are predominantly dependent on the IκB kinase (IKK). This kinase consists of a multi-subunit complex containing two highly homologous catalytic subunits, IKKα and IKKβ, which phosphorylate IκB and a non-enzymatic regulatory subunit IKKγ also known as NEMO (NF-κB essential modulator), which is required for the activation of IKKα/IKKβ hetero-dimers. Studies employing mutational approaches showed that phosphorylation of IκBs occurs at two serine residues (Ser^{32} and Ser^{36} in IκBα, and $Ser^{19}$and Ser^{23} in IκBβ) (Zandi et al., 1997) in their N-terminal regulatory domain which targets them for rapid polyubiquitination and subsequent degradation by the 26S proteasome (Senftleben and Karin, 2002, Chen, 2005).

From these discoveries a general model of NF-κB activation can be described. Upon exposure of cells to cytokines such as IL-1β and TNF-α followed by the subsequent activation of their cognate receptors, targeting of a number of adaptor proteins and upstream kinases results in the activation of the IKKs, phosphorylation and degradation of IκBα and nuclear translocation of NF-κB (Figure 1.2). Within the nucleus NF-κB binds to DNA at its response elements and activates the expression of genes involved in inflammation, cell cycle, invasion, metastasis, angiogenesis and anti-apoptosis (Barkett and Gilmore, 1999). However, numerous other studies have identified different NF-κB pathways and multiple cascades which can act independently and a number of variations in this basic model have been identified. These are outlined in the following section.

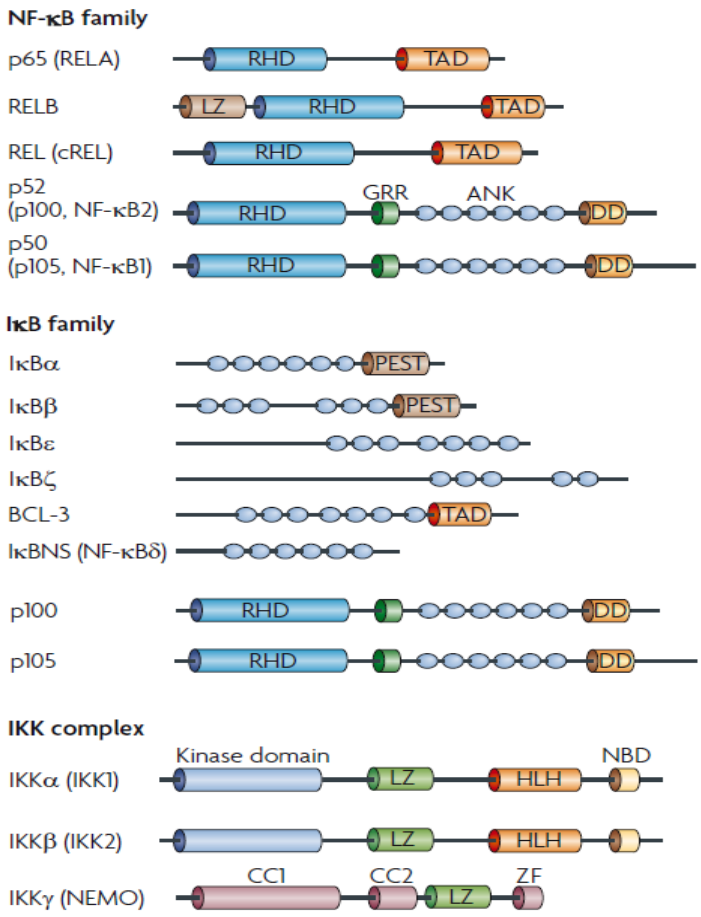


Figure 1.1: Members of the NF-κB, IκB and IKK protein families.
The classes of each protein family are indicated, and their alternative nomenclatures are provided in brackets. The precursor proteins p100 and p105 both act as IκB (inhibitor of nuclear factor-κB (NF-κB)) proteins and, when processed by the proteasome, as NF-κB family members. ANK, ankyrin-repeat; BCL-3, B-cell lymphoma 3; CC, coiled-coil; DD, death domain; GRR, glycine-rich region; HLH, helix–loop–helix; IKK, IκB kinase; LZ, leucine-zipper; NBD, NF-κB-essential-modulator binding domain; PEST, proline-, glutamic acid-, serine and threonine-rich; RHD, REL homology domain; TAD, transactivation domain; ZF, zinc-finger (Ghosh and Hayden, 2008).

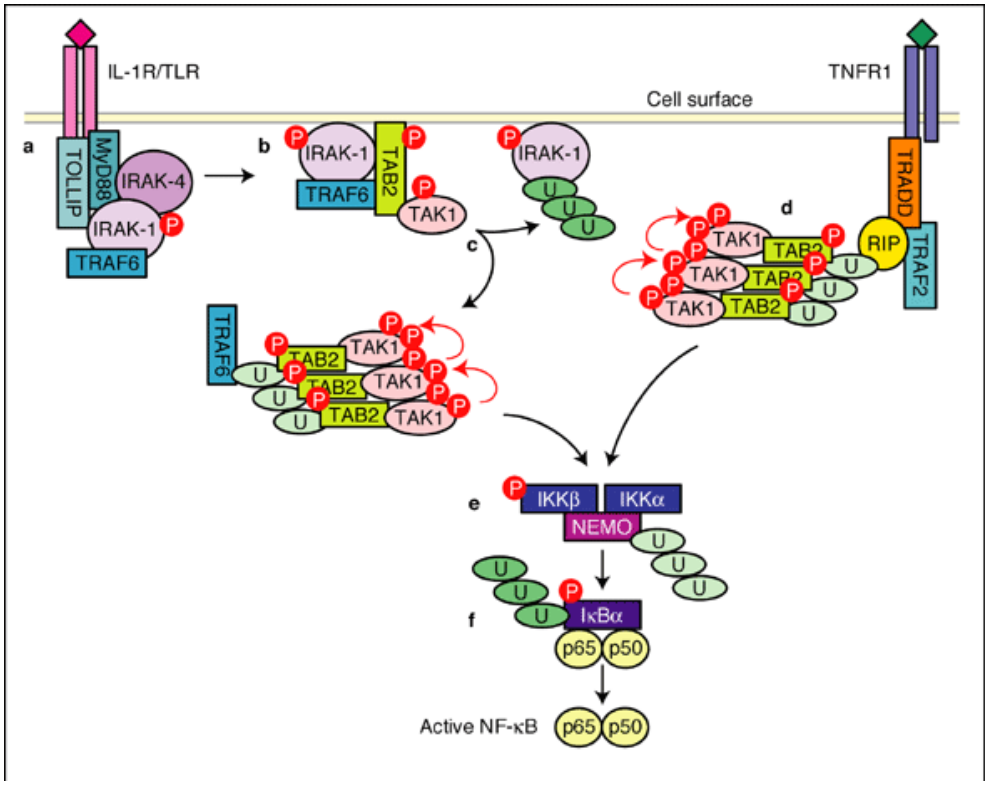


Figure 1.2: Activation of the IKKs. Following cell surface receptor activation, IKKβ activation via phosphorylation (either by TAK1 or an autoregulatory mechanism). Lys63-linked polyubiquitination of NEMO/IKKγ might play an important part in this process. Activated IKKβ phosphorylates IκBα, which is subsequently modified with Lys48-linked ubiquitin chains. This form of IκBα is subsequently transported to the proteasome, where it is degraded, thus liberating NF-κB for nuclear entry. Abbreviations: IκB, inhibitor of NF-κB; IKK, IκB kinase; IL-1R, interleukin 1 receptor; IRAK, IL-1R-associated kinase; MyD88, myeloid differentiation factor 88; NEMO, NF-κB essential modifier; P, phosphorylation; RIP, receptor-interacting protein; TAB, TAK1-binding protein; TAK1, TGF-β-activated kinase 1; TGF-β, transforming growth factor β; TLR, Toll-like receptor; TNFR, tumour necrosis factor receptor; TOLLIP, Toll-interacting protein; TRADD, TNFR-associated death domain; TRAF6, TNFR-associated factor 6 (Evans, 2005).

1.1.4 Pathways of NF-κB activation

Two principal signalling pathways have been identified that control the activity of NF-κB (Hayden and Ghosh, 2004). The classical (canonical) pathway (Figure 1.3) (Bakkar and Guttridge, 2010), as mentioned above, triggered in response to microbial products, stress and pro-inflammatory cytokines, depends mainly upon the activity of IKKβ and results in the nuclear translocation of NF-κB1(p50)/RelA(p65) and NF-κB1(p50)/c-Rel dimers. This signal is initiated from a variety of sources including certain cell surface cytokine receptors (e.g. TNFR), bacterial PRR (e.g. TLR4), intracellular redox sensing mechanisms and nuclear DNA damage signalling proteins. Once an inducer binds to its cognate receptor it will recruit adapter proteins such as TRADD, TRAF2 and RIP in case of activation with TNF-α, while activation with LPS and IL-1 results in the recruitment of MyD88, TRAF6 and IRAKs. Following this step, a free polyubiquitin chain binds to TAB2 to facilitate the autophosphorylation of TAK1 which in turn is responsible for the activation of the IKK complex. Within the activated IKK complex, the non-proteolytic K63-linked ploybuquitin chain binds to NEMO/IKKγ, whereas TAK1 phosphorylates IKKβ at amino acids residues serine 177 and 181. Upon phosphorylation mediated by IKKβ, IκBα is targeted for proteolytic ubiquitination, mainly by the K48-linked polyubiquitin chain. This is mainly conducted via the recruitment of Skp1, Cullin, F-box–containing (SCF) family of ubiquitin ligase that forms together with β-transducin repeat containing protein (β-TrCP) the SCF-β-TrCP ubiquitin complex, which then allows degradation of IκBα by 26S proteasome system (Figure 1.2, Figure 1.3) (Evans, 2005, Bakkar and Guttridge, 2010). This event represents the key step for the activation of the NF-κB p50/p65 hetero-dimer (Egan and Toruner, 2006, Scheidereit, 2006). Eventually, activated NF-κB p50/p65 is liberated from its sequestrant IκBα and translocates into the nucleus where ultimately interacts with specific promoter regions to facilitate transcription of relevant genes (Barkett and Gilmore, 1999). However, susceptibility to infections found to be increased during deficiencies in RelA and IKKβ, this occurs once the embryonic lethality associated with these deficiencies is prevented (Alcamo et al., 2001, Senftleben et al., 2001). Specifically, macrophages that lack IKKβ become very sensitive to LPS-induced apoptosis and thus are not functional (Bonizzi and Karin, 2004). Thus, the

importance of NF-κB function was essential in the suppression of LPS-induced apoptosis in macrophages (Hsu et al., 2004). Therefore, activation of the canonical pathway is essential for innate immunity; and also responsible for the inhibition of apoptosis under most conditions. Hence, IKKβ represents an attractive therapeutic target for the inhibition of NF-κB.

The other more recently described alternative NF-κB pathway (non-canonical) appears to be more prominent in lymphocytes (Figure 1.3) (Bakkar and Guttridge, 2010). This pathway is activated by specific lymphoid cytokines, such as B cell activating factor belonging to the TNF family (BAFF), CD40 ligand, and lymphotoxin-β (LT-β), leading to the recruitment of TRAF2, which activates cIAPs that in turn allow the accumulation of NIK (NF-κB inducer kinase) and its autophosphorylation. Subsequently, activation of IKKα results in IKKα-dependent proteolytic processing of p100 into the active p52 subunit through the K48-linked polyubiquitination (SCF-β-TrCP) and proteasome system (Bonizzi and Karin, 2004, Hayden and Ghosh, 2004). Usually, the p52 subunit dimerises with the NF-κB protein RelB, which translocates into the nucleus to induce the expression of a number of genes involved in lymphoid structure and function (Papa et al., 2006, Egan and Toruner, 2006). Indeed, analysis of macrophage populations from c-Rel$^{-/-}$ or RelB$^{-/-}$ mice has identified several functional defects. Specifically, it has been shown that RelB$^{-/-}$ macrophages are deficient in their ability to produce TNF-α but did not affect levels of IL-6, IL-10 and IL-12 while overproduce IL-1β (Caamano et al., 1999).

There are two kinases that can mediate the activation of the IKK complex, NIK and MEKK1. Recent evidence suggests that there is a potential of selective regulation of each IKK isoform but also some element of crossover. For instance, it has been reported that overexpression of NIK potently induces NF-κB (Malinin et al., 1997), mainly through the activation of the non-canoncial pathway (Scheidereit, 2006). On the other hand, overexpression of kinase deficient NIK mutants can inhibit IL-1β and TNFα induced-NF-κB activation (Malinin et al., 1997, Song et al., 1997). NIK can also interact with many key elements within the NF-κB signalling pathway, such as

TRAF2, TRAF6 and IKK. Furthermore, NIK has been shown to activate the non-canonical pathway (p52/RelB) through direct phosphorylation of IKKα (Perkins, 2006). In addition, MEKK1 also has been demonstrated to mediate the stimulatory effect of TNFα on IKK and NF-κB activation. This interaction has been confirmed in experiments with overexpression of MEKK1, since it activates both IKKα and IKKβ in transfected HeLa cells through direct phosphorylation of the IKKs in vitro (Lee et al., 1998, Gilmore and Herscovitch, 2006). Another serine/threonine kinase Tpl2 could also play role in IKK activation. When Tpl2 is overexpressed, it can activate IKK, ERK, JNK, and p38 MAP kinase pathways. It has been found that Tpl2 is mainly associated with IKKα and NIK (Lin et al., 1999).

Several intermediates are involved in NF-κB signalling triggered by T-cell receptor activation including PKCθ, CARMA1/CARD11, BCL10 and MALT1. PKCθ can directly interact with the IKK complex (Khoshnan et al., 2000), and may act as an adapter to link the other signalling components of the pathway, specifically CARMA1/CARD11, BCL10 and MALT1. Not surprisingly, NF-κB activation in response to antigen-receptor signalling was blocked when these genes were deleted (Ruland et al., 2001, Hara et al., 2003). Another protein that can also interact with IKK complex is the regulatory protein ELKS. When ELKS is targeted in knock-down experiments, the IKK complex failed to associate with IκBα and as a result the activation of NF-κB induced by TNFα or IL-1 is blocked (reviewed by Hayden and Ghosh, 2004).

There is a third pathway classified as the atypical pathway because its activation is independent of IKK. It can be activated by doxorubicin and UV light; both stimuli can cause DNA damage. Specifically, UV light induces IκBα degradation via the proteasome machinery, but in contrast to canonical pathway the targeted serine residues are located within a C-terminal cluster, which is recognised by p38-activated casein kinase-II (Tergaonkar, 2006). Alternatively, pervanadate treatment of T cells, or reoxygenation of hypoxic HeLa cells, results in phosphorylation of IκBα on tyrosine 42 (Imbert et al., 1996). In contrast to serine phosphorylation, the

subsequent proteolytic degradation is dispensable, but it is nevertheless sufficient to release the inhibitor from NF-κB (Aggarwal, 2006, Lee et al., 2007).

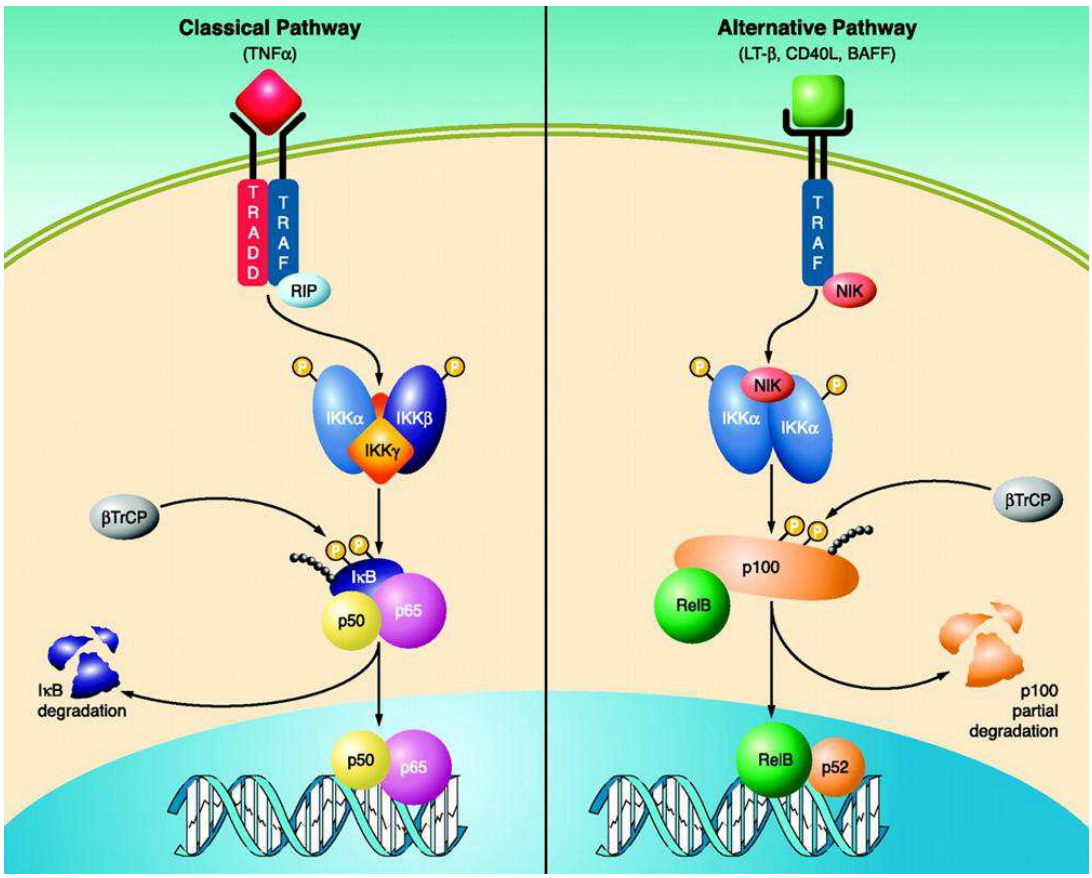


Figure 1.3: Classical and alternative NF-κB signalling pathways. The classical or canonical signalling pathway is illustrated using TNF-α stimulation. Signalling is initiated with the binding of TNF-α to its receptor and the subsequent sequential recruitment of the adaptors TRADD, RIP, and TRAF to the membrane. The following is IKK complex assembly that occurs between IKKα, IKKβ, and IKKγ, resulting in IKKβ phosphorylation and activation. IKKβ then phosphorylates IκBα to facilitate its polyubiquitination and immediate proteasomal degradation through β-TrCP. In turn, the classical p50/p65 hetero-dimer is freed to translocate into the nucleus and mediate transcription of NF-κB target genes. Alternative (non-canonical) NF-κB signalling is instead activated by ligands, such as CD40L, and lymphotoxin-β that triggers recruitment of TRAFs to the membrane-bound receptor and subsequently activates NIK. NIK then phosphorylates IKKα homo-dimer. Once active, IKKα phosphorylates p100, resulting in its processing to p52 subunit by β-TrCP. The formed RelB/p52 complex then translocate into the nucleus to promote NF-κB target genes that may be different from the canonical pathway (Bakkar and Guttridge, 2010).

1.1.5 Role of PKC and PKA signalling in IKK/NF-κB pathway

Early findings by Shirakawa and colleagues indicate that cytoplasmic NF-κB can be activated by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), and that both induce translocation of the active form of NF-κB and its binding to DNA (Shirakawa et al., 1989). A role of PKC has been proposed for phosphorylation of IκB (Baeuerle and Baltimore, 1988), that has later been found to be IKK-dependent. Such regulation was confirmed by using GF109203X, an inhibitor of conventional and novel PKC isoforms, which abrogates PMA/ionomycin-stimulated IKK complex in Jurkat and primary $CD3^+$ T lymphocytes (Trushin et al., 1999, Chen et al., 2001a). Previous studies have shown a role of PKCδ and PKCε in PMA-induced NF-κB-dependent transcription in both B-cells and fibroblasts (Krappmann et al., 2001, Hirano et al., 1995). Furthermore, these results are supported by studies involving the knockdown of PKCδ and ε in pulmonary A549 cells, which revealed an inhibitory effect on PMA-stimulated NF-κB-dependent transcription thus suggesting a role of novel PKC isoforms, particularly PKCδ and ε (Catley et al., 2004, Holden et al., 2008). However, PMA may also activate PKC/NF-κB via the Ras/Raf-1/ERK pathway (Chang et al., 2005). Recently, it has been found that in response to TNF-α stmulation PKCδ translocates into the nucleus and its activity is required for RelA/p65 transactivation, without involvement of NF-κB phosphorylation (Lu et al., 2009). On the other hand, in response to TNF-α stimulation the atypical PKCζ isoform can mediate phosphorylation of the p65 subunit at serine 311, which ultimately promotes the interaction between p65 and CBP (Duran et al., 2003).

One of the kinases that may also mediate post-translational modifications of the NF-κB (p65) subunit is PKA. It has been shown that phosphorylation of p65 at serine 276 by the catalytic activity of PKA can facilitate NF-κB -DNA binding, NF-κB interaction with the transcriptional coactivators CBP/p300 (Perkins, 2006, Oeckinghaus and Ghosh, 2009), as well as acetylation and displacement of histone deacetylase (Chen et al., 2001b, Zhong et al., 2002). However, the regulatory function of PKA has been confirmed as mutation of serine 276 to alanine is found to be lethal during the embryonic stage (Dong et al., 2008).

1.1.6 Role of NF-κB in inflammation

It is well accepted that the NF-κB signalling pathway plays important roles in inflammation, control of cell growth, apoptosis, cellular stress, and many other patho-physiological processes (Storz and Toker, 2003). NF-κB is activated by free radicals, inflammatory stimuli, carcinogens, tumor promoters, endotoxin, γ radiation, ultraviolet (UV) light, and X rays (Garg, 2002). Once activated, NF-κB induces the expression of more than 200 genes that have been shown to; suppress apoptosis and induce cellular transformation, mediate proliferation, promote invasion and metastasis, cause chemo-resistance and radio-resistance, and also inflammation. These cellular events link NF-κB to cancer, atherosclerosis, myocardial infarction, diabetes, allergy, asthma, arthritis, Crohn's disease, multiple sclerosis, Alzheimer's disease, osteoporosis, psoriasis, septic shock, AIDS, and other inflammatory diseases. Thus, agents that can suppress NF-κB activation, in principle, have the potential to prevent or delay the onset of or treat NF-κB-linked diseases (Aggarwal and Shishodia, 2004).

Specifically with regards to inflammation a large body of evidence has accumulated to support a role for the IKK/IκB/NF-κB axis in the regulation of a number of inflammatory conditions. This is through the regulation of multiple key intermediates which drive the inflammatory process, including cytokines, adhesion molecules, chemokines, COX-2, iNOS, MMPs and others. This has been studied using a number of different approaches for example the expression of dominant negative proteins involved in the pathway or siRNA induced knockdown of intermediates. For example, murine vascular smooth muscle cells transfected with p65 siRNA expressing plasmids have effectively blocked the 13-hydroperoxyoctadecadienoic **(**13-HPODE)-induced expression of both chemokine monocyte chemo-attractant protein-1 MCP-1 and TNF-α genes which are NF-κB dependent genes (Dwarakanath et al., 2004).

Knockout mouse models including tissue specific gene deletion has been particularly useful in defining the role of the NF-κB pathway in myeloid cells which are often resistant to transfection. As outlined previously, mice lacking the p50 subunit (NF-

κB1) developed normally in contrast to of p65 (RelA) knockout mice that died during embryonic development (Beg et al., 1995), but $p50^{-/-}$ mice were highly susceptible to bacterial infections with Staphylococcus and Listeria. Furthermore, these animals had immune compromised cell function (Sha et al., 1995), which appeared in the impaired ability of B cells to produce antibodies and to proliferate upon LPS challenge (Neurath et al., 1998). In contrast, however stimulated murine $RelB^{-/-}$ fibroblasts exhibited dramatic persistent induction of multiple chemokines (RANTES, MIP-1α, MIP-1β, MIP-2, IP-10, JE/MCP-1, and KC). Whilst In vivo, activated $RelB^{-/-}$ fibroblasts dramatically increased recruitment of granulocytes into tissue showing a role for RelB in the resolution of acute inflammation in tissues. The persistent overexpression of chemokines correlated with increased NF-κB binding as well as with increased p50, p65 (RelA), and IκBα expression (Xia et al., 1997). These studies have been supplemented using gene knockout models in the context of disease.

In addition a number of pharmacological tools have been developed to further investigate this pathway. These include IKK inhibitors, agents which prevent NF-κB translocation and inhibit NF-κB-DNA binding (Section 1.1.15).

In fact, many chronic inflammatory diseases are linked to dysregulated expression of pro-inflammatory cytokines (e.g. TNF-α and IL-1β), induction of pro-inflammatory enzymes (e.g. COX-2 and lipooxygenases), as well as the expression of adhesion molecules, MMPs and hyperproliferation of synovial fibroblasts. All of these factors are regulated by the activation of the transcription factor NF-κB. Thus, agents that suppress the expression of TNF-α, IL-β, COX-2, lipooxygenase, MMPs or adhesion molecules, or suppress the activation of NF-κB, all have potential for the treatment of arthritis (Khanna et al., 2007).

1.1.7 NF-κB in immuno-inflammatory diseases

In addition to cellular studies there are a number of different lines of evidence which support the involvement of NF-κB in the development and progression of a number of conditions underpinned by chronic inflammation. To date however, there are no

conclusive clinical studies including the use of drugs which can demonstrate conclusively a causal role of NF-κB. Nevertheless for conditions such as arthritis, colitis, asthma and others NF-κB is strongly implicated.

1.1.7.1 Rheumatoid Arthritis (RA)

Arthritis is a chronic inflammatory disease of unknown etiology affects the connective tissue preferentially and causing destructive joints. It has been considered as an autoimmune disease, and rheumatoid factors are detected in 80% of the affected patients (Geiler, 1996). It is mainly characterised by synovial hyperplasia, pathological immune phenomena and progressive destruction of the affected joints resulting in pain and stiffness. Various cell types are involved in the pathogenesis of RA in addition to synovial fibroblasts, T cells, antigen presenting cells and endothelial cells (Seemayer et al., 2001).

Molecular, cellular and animal studies have identified various interactions between the fibroblasts and other synovial and inflammatory cells in the joints. For example mast cells appear to contribute to RA pathology in mouse studies, as was shown in a mouse model of autoantibody-induced arthritis in which mast cell-deficient mice exhibited attenuated joint inflammation (Lee et al., 2002). Moreover, mast cells produce cytokines that are of great relevance in RA, including TNF-α and IL-1β (Sandler et al., 2007). In an earlier biopsy study it was also found that macrophages in RA are of importance, which has shown the degree of joint erosion is correlated with the number of synovial cells that expressed the macrophage marker CD14 (Yanni et al., 1994). Furthermore, blocking the predominant macrophage-derived cytokine TNF-α, has led to significant clinical improvement in short-term trials in RA patients (Elliott and Maini, 1994).

Among several nuclear transcription factors that are involved in the activation of cells in the proliferating rheumatoid synovia, NF-κB is highly involved. A study carried out by Aupperle and colleagues confirmed the role of IKK in primary fibroblast-like synoviocytes isolated from synovium of patients with RA and osteoarthritis. In both groups, have been shown that immunoreactive IKK protein

was abundant in these cells while IKKα and IKKβ were constitutively expressed at the mRNA level (Aupperle et al., 1999). Furthermore, in these cells induction of IL-6, IL-8, ICAM-1, and collagenase-1 expression was verified as being dependent specifically on IKKβ (Tak and Firestein, 2001). In particular IKKβ dominant negative expressing mutant cell populations were found to be resistant to TNF-α-induced nuclear translocation of NF-κB, and accordingly the presence of IKKβ was required for cytokine synthesis (IL-6 and IL-8) via NF-κB activation in rheumatoid arthritis synovial fibroblasts (RASFs) (Andreakos et al., 2003). In addition the p50 and p65 subunits of NF-κB were found in the majority of CD14-positive cells within the synovial tissue, as well as the endothelial cells through the synovium, in most RA patients. Thus, NF-κB is likely to play an important role in the expression of macrophage derived cytokines in rheumatoid synovia (Handel et al., 1995).

In addition NF-κB has shown to play a role in hyper-proliferation of synovial cells; whilst inhibition of NF-κB induced apoptosis in TNF-α or IL-1β stimulated synovial cells. This finding is confirmed by an in vivo study of induced arthritis in rats (Miagkov et al., 1998). Furthermore, intra-articular administration of NF-κB decoy oligonucleotides has prevented the recurrence of streptococcal induced arthritis in the joints, indicating a beneficial effects of local inhibition of NF-κB in arthritis (Tripathi, 2006).

Some of the effects outlined above may be related to the inhibition of COX-2 expression. COX-2 is strongly expressed in the synovial lining, and is linked to NF-κB activation in RASFs. A number of non-selective and selective COX inhibitors, including ibuprofen, rofecoxib, diclofenac, and meloxicam were found to be able to block IL-1β-induced prostaglandin E_2 production in RASFs (Kojima et al., 2003).

Many studies have shown that the inflammatory mediator iNOS is also involved in RA. iNOS has been found in synovium and cartilage of arthritis patients which leads to an increase in NO production. As a result, it increases synovial blood flow and modulates cellular function within synovium and articular cartilage, which may contribute to the pathogenesis of inflammatory arthritis (Grabowski et al., 1997).

However, inhibition of NOS using specific inhibitors was reported to decrease the disease in experimental RA (McCartney-Francis et al., 1993). A critical role for iNOS and NO has been suggested in RA associated with bone erosions, this was confirmed by using iNOS knockout mice which showed resistance to IL-1-induced bone resorption (van't Hof et al., 2000). Further evidences also showed that several other cell types were responsible for generating NO in the inflamed synovium, including osteoblasts, osteoclasts, macrophages, fibroblasts, neutrophils and endothelial cells (van't Hof and Ralston, 2001, Nagy et al., 2007, Nagy et al., 2010).

1.1.7.2 Colitis

One well described inflammatory bowel diseases (IBDs) is ulcerative colitis (UC), identified by a set of clinical, endoscopic and histological features (Kirsner, 1988) but still with an unknown aetiology. It is known by a superficial inflammation causing epithelial cell destruction (ulceration) mainly in the rectum and colon (Podolsky, 2002, Bouma and Strober, 2003). Expression of adhesion molecules is a feature of most chronic inflammatory diseases, which is responsible for recruiting inflammatory cells (e.g. neutrophils, eosinophils and T lymphocytes) from the circulation to the site of inflammation (Albelda et al., 1994). An increased levels of mucosal pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, IL-12 and IFN-γ in hapten reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model, have been shown to play an important role in sustained inflammatory responses (Elson et al., 1995, Strober et al., 1998, Boismenu and Chen, 2000). It has been shown that the expression of these proteins is dependent on NF-κB which is strongly activated in IBDs (Neurath et al., 1998, Rogler and Andus, 1998). Furthermore, the use of p65 (NF-κB) antisense oligonucleotides in a TNBS-induced colitis model efficiently inhibited NF-κB activation and cytokine production resulting in the prevention of mucosal inflammation (Fichtner-Feigl et al., 2005). Moreover, compounds which can down-regulate NF-κB like curcumin have been proved to be useful in the treatment of IBD (Sugimoto et al., 2002). Sulfasalazine salicylazosulfapyridine (SASP) can alleviate dextran sulphate sodium (DSS)-induced colitis in mice through blockade of p65 binding to ICAM gene promoter (Zhao et al., 2010). Medications currently available for IBD mitigate inflammation and reduce

symptoms, but do not provide a cure of long-term complications. Although these agents have shown some effectiveness in the treatment of IBD, they have serious side effects that limit their clinical use. Therefore, these studies indicate the potential of pharmacological intervention based on inhibition of components of the NF-κB pathway in particular at the level of the IKKs. This discussed further in Section 1.1.15.

1.1.8 Role of Mitogen Activated Protein Kinases (MAPK) in immunity and inflammation

It should be recognised that several other kinase/transcription factor cascades, in addition to NF-κB play a role in the genesis of inflammation. Induction of the inflammatory cascade by Gram-negative bacteria is triggered by binding of the LPS to membrane receptor TLR4, which leads to the recruitment and formation of the complex MyD88/IL-1R-associated protein kinase (IRAK)/TNFR associated factor 6 (TRAF-6) within the cell. Subsequently, the signal transduction events trigger activation of a family of Ser/Thr protein kinases known as the mitogen activated protein kinases (MAPKs). These consist of three major MAP kinase subfamilies (Akira and Takeda, 2004); the extracellular regulated kinases (ERK1/ERK2 ERK3/ERK4; and ERK5), c-jun N-terminal kinases (JNK1, JNK2 and JNK3), and isoforms of p38 MAP kinase (p38α, p38β, p38γ and p38δ). A substantial body of evidence has identified roles for these kinases in a large number of cellular responses and has implicated this pathway in a number of disease conditions (see reviews by Davis et al., 2000, Chen et al., 2001c, Chang and Karin, 2001, Roux and Blenis, 2004).

In relation to inflammation there is considerable evidence implicating a role for the MAP kinase cascade. Transient activation of ERK1/2, JNK, and p38 MAPK were observed in both immortalised and primary murine macrophages after stimulation mediated by TLR4 (Zhao et al., 2006). It has been reported that dual phosphorylation on tyrosine and threonine residues of p38 MAPK is a critical event in the activation of several transcription factors (e.g., NF-κB, activating transcription factor-2, Elk-1, and C/EBP). Both the JNK and p38 MAPK signalling pathways are also involved in

the regulation of LPS-induced expression of TNF-α by stabilising the TNF-α mRNA and affecting its translational silencing (Kontoyiannis et al., 1999). In addition, a targeted deletion of MAPK-activated protein kinase-2, one of several kinases that are regulated through direct phosphorylation by p38 MAPK, resulted in massive decrease in the production of TNF-α, and rendered mice resistant to LPS/D-galactosamine-induced shock (Kotlyarov et al., 1999). Another MAPK kinase, MAPK kinase kinase 3, has also been described as an essential signal transducer of the MyD88/IRAK/TRAF6 complex in TLR4 signalling. Furthermore, the role of MAPK signalling in inflammation has been ascertained when MAPK phosphatase-1 (MKP-1) deficiency exacerbates murine collagen-induced arthritis (CIA) (Salojin et al., 2006).

The therapeutic potential of down-regulating the MAP kinase pathway has been recognised by inhibiting the expression of pro-inflammatory proteins IL-6, IL-8, MMP-1 and MMP-3 in RASFs, following application of a specific inhibitor of p38 MAP kinase (Westra et al., 2004). In particular, both isoforms of the p38 MAP kinase α and γ (Korb et al., 2006, Kunisch et al., 2007) can modulate several pro-inflammatory pathways in RASFs and accordingly have been targeted in clinical trials. For example, microglia of p38α deficient mice showed a reduced cytokine response to LPS. Furthermore, orally administered p38 inhibitor abolished the increase of IL-1β levels in LPS-induced cerebral cortex of mice (Bachstetter et al., 2011). However, serious adverse effects such as skin infections and elevated liver function tests have prevented further development of therapeutic p38 MAP kinase inhibitors thus far (Hammaker and Firestein, 2010, Bonilla-Hernan et al., 2011).

1.1.9 Activator protein-1 (AP-1): role in inflammation

A key transcription factor downstream of the MAP kinase pathway and linked to inflammation is activator protein-1 (AP-1). AP-1 is a transcription factor comprises a family of proteins that bind specific DNA sequences, the TPA response elements (5`-TGAG/CTCA-3`) which present in the promoter region of set of genes implicated in cell proliferation, differentiation, inflammation and stress responses (Kerppola and Curran, 1995). AP-1 is composed of either homo or hetero-dimers of members of the

c-Fos (c-Fos, FosB, Fra-1 and Fra-2) and c-Jun (c-Jun, c-JunB and c-JunD) families. The subunits of AP-1 contain the basic-leucine zipper (bZIP) motif which mediates dimerisation, and DNA binding. In fact, Jun proteins can form homo- and hetero-dimers while the Fos proteins can only form hetero-dimers. Further bZIP transcription factors, such as members of CREB and ATF families also can form complex with AP-1 (Leevers et al., 1994). AP-1 is a pro-inflammatory transcription factor, its activation mediated by cytoplasmic transforming proteins Src and Ras via MAP kinase pathways, and it is induced by a variety of cytokines and ROS inducing agents such as UV light, hydrogen peroxide and mitogens (Angel and Karin, 1991). In fact, it has been shown that N-acetyl-cysteine and thiol-reducing compounds stimulate oxidative stress-mediated activation of AP-1 by asbestos (Janssen et al., 1995), as well as the that induced by phorbol esters. Paradoxically, N-acetyl-cysteine prevents the induction mediated by hydrogen peroxide (Collart et al., 1995). Moreover, AP-1 is considered to be a potent antioxidant transcription factor since it can be activated by many antioxidant compounds, including vitamin E (reviewed by Lavrovsky et al. (2000)). An earlier study has also shown that exposure of c-Fos and c-Jun to chemical reduction strongly activated DNA-binding (Xanthoudakis et al., 1992). However, results obtained by others suggest that molecular mechanisms of AP-1 activation are more complex.

Like NF-κB, AP-1 promotes transcription of genes linked to inflammatory diseases (Surh et al., 2001). Thus, AP-1 modulates several inflammatory conditions; this is obvious in the case of deleted epidermal JunB and c-Jun of adult mice which lead to a psoriasis-like disease characterised with psoriatic arthritis (Zenz et al., 2005). A role for AP-1 is also observed in hypercholesterolemic mice; AP-1 deficiency protects against the development of atherosclerosis (Osto et al., 2008). In addition in humans, AP-1 is activated in progressive and unstable atherosclerotic plaques and may function as a switch for the inflammatory tissue response and repair (Hansson and Hermansson, 2011). Furthermore, increased AP-1-DNA binding has been found in the synovium of RA and osteoarthritis patients. In mouse model of collagen-induced arthritis, NF-κB and AP-1 activation preceded both clinical arthritis and gene transcription of collagenases (metalloproteases) (Han et al., 1998), which play

an important role in the degradation of cartilage and bone (Firestein, 1991). These findings suggest that the interaction of AP-1 with NF-κB might be involved in the pathogenesis of RA and may explain the benefit of combined therapeutic strategies.

1.1.10 JAK/STAT pathway

Janus tyrosine Kinases (JAKs) and Signal Transducer and Activator of Transcription (STAT) are crucial elements of many cytokine receptor systems that regulate several cellular functions (e.g. cell growth, survival, proliferation, haematopoiesis and pathogen resistance). Specifically, JAK/STAT signalling involved in inflammatory responses and pro-inflammatory cytokine production, which observed in inflammatory cells like T-cells, neutrophils, and macrophages. The JAK protein belongs to a family of non-receptor PTKs comprising of JAK1, JAK2, JAK3 and TYK2 (non-receptor Protein Tyrosine Kinase-2). Upon ligand-mediated receptor multimerisation two JAKs undergo trans-phosphorylation. Subsequently, the activated JAKs phosphorylate downstream targets including STATs. In the resting state STATs reside in the cytoplasm, upon activation phosphorylated STATs dimers then translocate into the nucleus by importin nuclear import systems (Rawlings et al., 2004). To date seven different isoforms of STATs have been identified. They are present in many cell types, including cells of epithelial origin (Ihle, 2001). These include STATs (1 to 6, including STAT5α and STAT5β), which are encoded by distinct genes. The JAK/STAT signalling pathways are regulated by a variety of stimuli. For example, following activation of the interferon gamma receptor by IFNγ, STAT1 mediates a pro-inflammatory response (Ivanenkov et al., 2011, Darnell et al., 1994).

Initially, STAT1 was identified as a component of ISGF-3 (IFN-stimulated gene factor), the IFN-α-stimulated, ISRE-binding factor (Schindler et al., 1992). Then STAT1 homo-dimers was determined in GAF, the IFNγ-stimulated GAS-binding transcription factor (Shuai et al., 1992). The crucial role of STAT1 in response to both type I and type II IFNs has been confirmed by a gene targeting study (Meraz et al., 1996). This may explain an increased susceptibility to viral and bacterial infections in humans expressing STAT1 mutants (Chapgier et al., 2006).

Specifically, STAT1 target genes appear to promote inflammation and antagonise proliferation. By contrast STAT3 associated with pro-proliferative and anti-inflammatory activities. Therefore, balanced response can be achieved through activation of both STAT1 and STAT3 (e.g. members of the IFN-I and IL-6 families) (Schindler et al., 2007).

It has been previously reported that JAK/STAT activation is found in atherosclerotic lesions (Gharavi et al., 2007). Particularly, STAT1 mediates a role in atherosclerotic lesion development through macrophage apoptosis and foam cell formation (Lim et al., 2008). Recently, it was demonstrated that activated STAT1 and STAT3 in cultured vascular cells were under inflammatory, atherogenic, and immune stimulation (Ortiz-Munoz et al., 2009).

Several small molecules have been shown to regulate the JAK/STAT signalling pathway, which include compounds already on the market for use against various inflammatory conditions and others currently being evaluated in clinical trials. For example, leflunomide a widely used drug to treat rheumatoid arthritis (Smolen et al., 2011). Leflunomide significantly inhibits the IL-4 and IL-13 enhanced production of CCL26 in the human keratinocyte cell line, HaCaT (Kagami et al., 2005). In addition, it markedly inhibits the deposition of type I collagen in hepatic stellate cells (HSCs) as well as the proliferation of primary HSC. This mainly mediated through interruption of the proliferative signal transduction pathways such as the primary target JAK/STAT, related MAP Kinases and PI3K/Protein kinase B (Akt) signalling cascades. These off-target effects provide a novel insight into the mechanisms by which leflunomide may exert an effect in liver fibrosis (Si et al., 2007, Ivanenkov et al., 2011).

1.1.11 Inflammatory mediators

As outlined above there is a potentially exhaustive list of NF-κB dependent genes encoding different inflammatory mediators and proteins and these are reviewed in previous sections and in recent reviews (Bloemen et al., 2007, Ma et al., 2012). However two major inflammatory proteins which have been shown to be regulated

by NF-κB activation and used to assess the potency and efficacy of anti-inflammatory drugs are COX-2 and iNOS. The characteristics of these enzymes and their regulation are reviewed below.

1.1.11.1 Cyclooxygenase-2 (COX-2)

Cyclooxygenase enzyme (COX) or prostaglandin H_2 synthase is responsible for conversion of arachidonic acid to prostaglandin G_2 (PGG_2), which is in turn converted to PGH_2 by a peroxidase reaction that is also catalyzed by the COX enzyme. In 1976 Miyamoto et al. purified the COX enzyme which was defined as COX-1 and found to be constitutively expressed in several tissues and to mediate physiological functions such as platelet aggregation, cytoprotection of the stomach and regulation of renal blood flow. A second, inducible form of COX, known as COX-2, was identified by Simmons et al. in 1989, and shown to be expressed primarily in cells that mediate inflammation such as macrophages, monocytes and synoviocytes. Genes for both isoforms were found on different chromosomes; 9 and 1 for COX-1 and COX-2 respectively (Williams and DuBois, 1996). Prior to the discovery of the COX enzyme, in 1971 it was established that NSAIDs such as aspirin and indomethacin acted to inhibit the enzyme and to prevent PG synthesis (Vane, 1997, Vane and Botting, 2000, Botting, 2010), then subsequent biochemical studies revealed the mechanism of such an important effect.

The second isoform, COX-2, is not expressed in most normal tissues, but its expression is rapidly induced by many stimuli such as proinflammatory cytokines (IL-1β, TNF-α), LPS, mitogens and oncogenes (phorbol esters), growth factors (fibroblast growth factor, FGF; platelet-derived growth factor, PDGF; epidermal growth factor, EGF) and hormones (luteinizing hormone, LH) (Williams and DuBois, 1996). COX-2 is considered as an early response gene, which can be modulated at transcriptional, post-transcriptional, translational levels and protein stability of mediating transcription factors. Such multiplicity in the control of COX-2 expression implies the existence of several regulatory pathways of regulation of function (Cerella et al., 2010). Increased COX-2 expression appears to be attributed to both increased transcription and enhanced mRNA stability. Promoter regions of

the COX-2 genes characterised by the presence of canonical TATA box and multiple transcription regulatory elements; including NF-κB, NF-IL6/C/EBP, NFAT, CRE and AP-1 transcription factors (Chun and Surh, 2004). There are two NF-κB binding sites in the COX-2 promoter region which are indispensable for expression.

There is considerable evidence supporting a role for both NF-κB and MAP kinase pathways in COX-2 expression. It has been shown that NF-κB can regulate the expression of COX-2 in both LPS induced murine J774 macrophages (D'Acquisto et al., 1997) and in human colon adenocarcinoma cells (Kojima et al., 2000). Consistent with this mechanism of regulation it was reported that LPS induced COX-2 expression in either the J774 macrophages (D'Acquisto et al., 1997) or in rats (Liu et al., 1999) was suppressed with the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC). Earlier study by Kopp and colleagues has revealed the mechanism of anti-iflammatory action of higher concentrations of aspirin and sodium salicylate, that both can inhibit NF-κB activation in a dose-dependent manner by preventing IκBα degradation in LPS-induced human T cells (Kopp and Ghosh, 1994). In support of this finding aspirin (Weber et al., 1995) and sodium salicylate (Pierce et al., 1996) both reduced the expression of NF-κB dependent surface adhesion molecules such as E-selectin, ICAM-1, and VCAM-1 in TNF-α-induced vascular endothelial cells by inhibiting the nuclear translocation of NF-κB. In addition, inhibition of TNF-α or IL-1-induced inflammatory mediators in human RASF by aspirin was also found to be mediated by NF-κB inhibition (Sakurada et al., 1996, Lee and Burckart, 1998). All these broad inhibitory effects of aspirin and salicylates in addition to the suppression of COX-2 transcription (Xu et al., 1999) were linked to inhibition of NF-κB-dependent inflammatory mediators including COX-2 expression.

In relation to other transcription factors, LPS can induce COX-2 expression via AP-1 activation mediated through the MEK/ERK cascade, whilst CREB phosphorylation resulting from LPS-induced Tpl-2/ERK/MSK activation can also play a central regulatory role in the regulation of COX-2 (Eliopoulos et al., 2002). Furthermore, it has been reported that the MEK1/2 inhibitor PD98059 or the p38 MAP kinase inhibitor SB202190 both caused attenuation of LPS-induced COX-2 mRNA

expression and PGE_2 production in RAW264.7 macrophage cells (Lo, 2003). Consistently, previous data has identified the contribution of MAP kinases to COX-2 protein expression (Paul et al., 1999). This is possibly via the binding of transcription co-activator p300 to the transcription complex (CREB, AP-1, C/EBP and NF-κB) at the COX-2 promoter which control the initiation of its transcription (Deng et al., 2004, Tsatsanis et al., 2006).

1.1.11.2 Inducible nitric oxide synthase (iNOS)

In the early 1980s Furchgott and colleagues revealed the potential of a substance released from the endothelium which could relax blood vessels. This substance which was independent of cyclooxygenase activity and therefore not a PG was later identified as nitric oxide (NO). These seminal studies unveiled a major regulatory system important not only in normal physiological regulation but also in pathophysiology (Furchgott and Zawadzki, 1980). Since this discovery a considerable effort has been made to understand NO formation, function, regulation and pharmacological inhibition (Palmer et al., 1987, Furchgott, 1987, Ignarro et al., 1988).

The formation of NO requires the oxidation of L-arginine via two successive mono-oxygenation reactions to form an intermediate hydroxylated L-arginine and lastly L-citrulline. Two moles of O_2 and one and half moles of reduced nicotinamide adenine dinucleotide (NADPH) are consumed per mole of ·NO formed (Liu and Gross, 1996). This reaction is mediated via NOS, an enzyme which exists as three isoforms. The two constitutive isoforms (cNOS), one is neuronal NOS (nNOS) and the other is endothelial NOS (eNOS); both are responsible for the basal release of NO. However, the third NOS is a calcium-independent inducible isoform (iNOS), which is mainly expressed in response to cytokines and LPS stimulation, but could also be expressed constitutively in some tissues (e.g. lung) (Knowles and Moncada, 1994).

All NOS isoforms share amino acid homology of approximately 55%, mostly within the catalytic regions (Michel and Feron, 1997). Thus, provides similar catalytic activity of the three isoforms and all require molecular oxygen and NADPH co-

substrates, as well as several co-factors (e.g. tetrahydrobiopterin, BH4; flavin adenine dinucleotide, FAD; flavin mononucleotide, FMN). However, calmodulin the calcium regulating protein is required for catalysis by all isoforms, although an increase in Ca^{2+} is only necessary for eNOS and nNOS activation (Ruan et al., 1996, Rawlingson, 2003). NO synthesised by cNOS plays crucial role in several physiological functions. For instance, in the vascular system, it induces vasodilation, inhibits platelet aggregation, smooth muscle cells proliferation and migration, prevents neutrophil/platelet adhesion to endothelial cells, and maintains endothelial cell barrier function. Whilst NO acts as a neurotransmitter in the neural system (Ahern et al., 2002, Rosselli, 1997).

In contrast to either eNOS or nNOS increased expression of iNOS catalyses the synthesis of high concentrations of NO and plays a key role during the response to infection (Moncada et al., 1991). Thus, NO generated by the action of iNOS in white blood cells such as macrophages in response to gram negative bacteria acts as a cytotoxic agent against the invading microbe and mycobacterial infection (Bogdan, 2001, Deupree and Schoenfisch, 2009, Yang et al., 2009). A similar response occurs following infection with Leishmania (De Groote et al., 1995). Induction of iNOS is also important in the destruction of tumours (Xu et al., 2002), but in particular is linked to immunopathology (Nathan and Xie, 1994). In addition, iNOS has been linked to autoimmune and inflammatory diseases such as asthma, RA, and septic shock (Vane et al., 1994, Wong and Billiar, 1995, Paige and Jaffrey, 2007).

At a cellular level evidence supports a role for key signalling pathways including NF-κB and MAP kinases in the regulation of iNOS expression. Several transcription factor binding sequences were found in the promoter of the murine iNOS, and many of them are also present in the human iNOS promoter (Nunokawa et al., 1994). These include ten interferon-γ response elements (IFNγ-RE), IFNγ regulatory factor-1 (IRF-1) (Spitsin et al., 1996, Linn et al., 1997), three γ-activated sites (GAS), two consensus sequences for NF-κB binding, two AP-1 binding motifs, three interferon-α stimulated response elements (ISRE), four for NF-IL6, two TNF-α response elements

(TNF-α-RE), and a basal transcription recognition site (TATA box). Many of these elements are (Nunokawa et al., 1994, Papapetropoulos et al., 1999).

Molecular and pharmacological studies have supported the involvement of a number of signalling pathways in iNOS induction based on the structural features of the iNOS promoter. Expression of iNOS is transcriptionally regulated by cytokines such as TNF-α, IL-1β, IL-2 or IFNγ or LPS, agents which activate one or more of the signalling pathways which result in transcription factor binding to the iNOS promoter. For example IFNγ activates the JAK/STAT pathway whilst LPS activates the NF-κB pathway and several of the MAP kinases which in turn regulates the AP-1 complex. This may reflect the magnitude of iNOS induction in response to each agent and synergy between them (Lorsbach et al., 1993).

A number of signalling pathways inhibitors have been shown to prevent iNOS induction in response to several different agents. For examples glucocorticoids, and the antioxidants PDTC both inhibit iNOS expression mediated by NF-κB activation (Kleinert et al., 2004). In addition, an anti-inflammatory properties of green tea has also been reported which attributed to its effects on the expression of pro-inflammatory genes including iNOS via down-regulation of DNA binding of STAT1 transcription factor (Tedeschi et al., 2004).

However, iNOS could be induced by LPS through classical activation of the NF-κB pathway, and may also contribute to MAPK/AP-1 activation in murine macrophages (Chan and Riches, 2001, Surh et al., 2001). Moreover, it is also promoted by IFNγ-induced activation of JAK1/STAT1 signalling in macrophages (Lowenstein et al., 1993) and astroglial cells (Dell'Albani et al., 2001). Many studies have confirmed the inhibition of iNOS by plasmid DNA that directed to iNOS antisense RNA production in several cell types such as murine macrophages (Rothe et al., 1996), endothelial cells, and osteoblastic cells (Abe et al., 2003).

1.1.12 A role for NF-κB in cancer

Cancer is identified as a life threatening disease underpinned by dysregulation of the normal proliferative processes that regulate cell growth and survival. This fundamental process leads to loss of normal cell function within a local environment followed by metastatic spread. Prostate, lung and colorectal are the most common cancers for males. Substantial increases in incidence of prostate cancer have been reported in recent years for many countries around the world, including the UK. Current UK figures demonstrate that prostate cancer represents over 25% of all male cancers diagnosed in 2010. With the notion that prostate cancer age-standardised incidence rate is increased by around 10 % between 2001 and 2010 (Cancer statistics – registrations, England, 2009).

NF-κB has been implicated in carcinogenesis due to its role in cell survival, differentiation, as well as cell growth. Notably, NF-κB stimulation can drive expression of genes related to tumour growth and survival (Garg, 2002, Karin et al., 2002, Aggarwal, 2006). A number of human malignancies are characterised by elevation of NF-κB activity. Such activation of NF-κB for example reflected in up-regulation of cyclin D1 (Chen et al., 2001a), thus enhance survival via promoting cell cycle transition and anti-apoptotic genes (e.g. cIAP1, cIAP2, XIAP, Bcl-2 and Bcl-XL) (Barkett and Gilmore, 1999). Moreover, it was found that NF-κB was able to control the G_1-phase expression of key proto-oncogenes during the cell cycle which is regulated by the integrated activity of IκB kinases (IKKα and IKKβ), Akt and Chk1. This study also revealed that the recruitment and binding of NF-κB subunits to Cyclin D1, c-Myc and Skp2 promoters is highly coordinated along with RelA (p65) phosphorylation occurring through different phases of cell cycle. This occurs in concomitant with a switch from coactivator to corepressor recruitment, suggesting a link between NF-κB activation and DNA replication checkpoint (Barre and Perkins, 2007).

In addition, NF-κB is linked to tumour migration and angiogenesis, events crucial for metastatic spread (Helbig et al., 2003, Levine et al., 2003). Tumour cells with promoted NF-κB activity are characterised with constitutive expression of

angiogenic and angiostatic chemokines, and cytokines such as VEGF, IL-1 and IL-6, which are directly enhance tumour growth. In melanoma and breast cancer, the expression of the NF-κB dependent CCL5 (RANTES) correlated with the metastatic capacity of the tumour. Furthermore, it has been observed that induced expression of the chemokines CXCL1 and CXCL8 (IL-8) function as a positive feedback on the activation of NF-κB (Richmond, 2002). The importance of chemokine autocrine loops on the activation of NF-κB in melanoma tumour cells has been confirmed with the use of CXCL1 specific antibody, which inhibits constitutive NF-κB by half. In addition, dysregulation of NF-κB in melanoma may also be contributed to cytokines or alterations in the signal-transduction pathway (Shishodia and Aggarwal, 2004).

1.1.13 Link between inflammation and cancer

Recently, an attention has been directed toward chronic inflammation as a potential component in the progression of cancer. Cancer cells can express a number of cell surface antigens for example, adhesion molecules and chemoattractant proteins such as MCP1 which are cell markers relevant to inflammation. Cancer cells also express enzymes such as COX-2, iNOS and MMPs as a result of an inflammatory tumour environment. Indeed, about 20% of all human cancers are developed by chronic infection and inflammation, initiated from environmental insults such as infectious agents, or exposure to dietary carcinogens a well as hormonal imbalances (De Marzo et al., 2007). Chronic inflammation is strongly involved in the development of several cancers, including bladder, liver, colon, and gastric cancer. Such link has been found for example between clinical prostatitis (inflammation of the prostate) and risk of prostate cancer (Dennis et al., 2002). It has also been indicated that prolonged use of anti-inflammatory agents such as aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of prostate cancer (Jacobs et al., 2005, Wong et al., 2009).

More recent studies have also stressed the importance of immuno-surveillance in the development of cancer. Under normal conditions the adaptive immune system recognises alteration of genetic and epigenetic protein products in transformed cells. However, tumours can produce antigens due to accumulation of antigen-specific T

cells within the tumour, the draining lymph node, and the circulation of diagnosed patients (reviewed by Frey and Monu, 2008). The balance between the cancer development and different arms of the immune system are illustrated below (Figure 1.4).

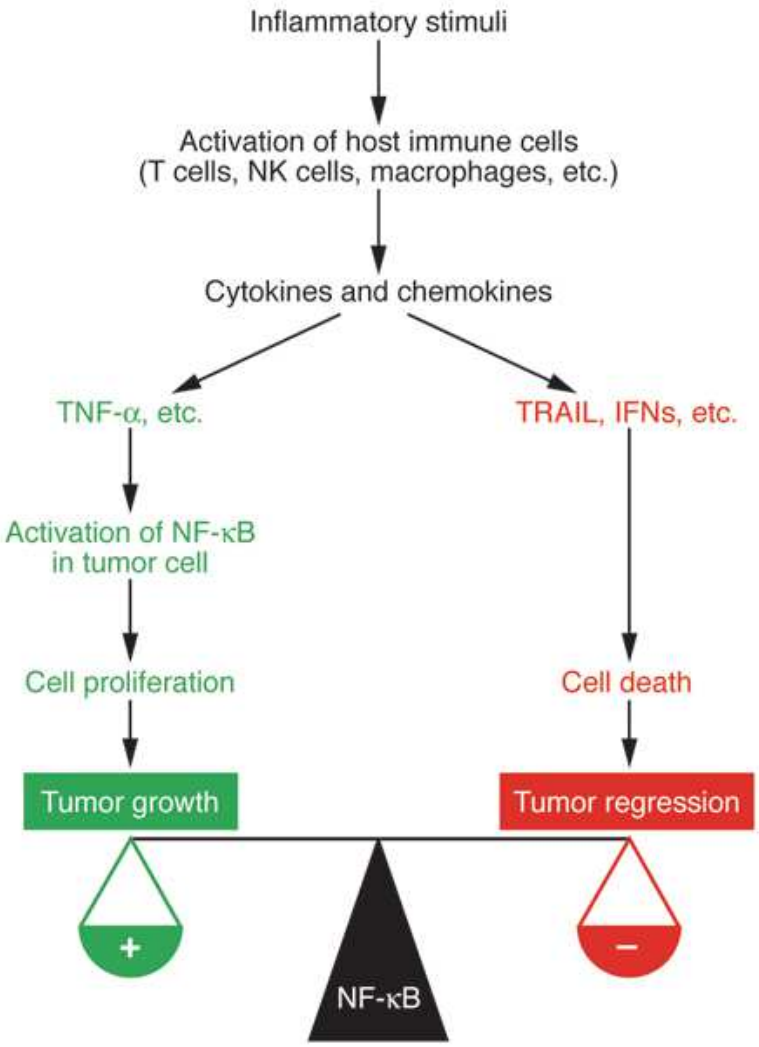


Figure 1.4: Role of NF-κB in inflammation linked tumour growth and regression. NF-κB within the malignant cells modulates the tumour response to inflammation. Activated NF-κB enhances tumour growth and confers resistance to TRAIL (tumour necrosis factor related apoptosis induced ligand) death cytokine. On the other hand, inhibition of NF-κB prevents inflammation-stimulated tumour growth and enhances inflammation-induced tumour regression mediated by TRAIL (Luo et al., 2005).

1.1.14 Pharmacological approaches to the inhibition of NF-κB activation

Since the discovery of NF-κB and more recently the IKKs, there has been a considerable research effort to develop inhibitors targeted to different components of the NF-κB pathway. This is required due to the relative lack of clinically effective medicines for chronic inflammatory diseases. Current medicines include NSAIDs, steroids and immunosuppressants. For example, both 5`-aminosalicylates and sulfasalazine first-line therapies have been used as maintenance agents for ulcerative colitis (Tamboli, 2007). Most recently the "biologics" have been used clinically and whilst these are promising drugs it is clear that multiple agents play a role in inflammatory diseases, therefore targeting the pathway involved in the action of a number of cytokines would be beneficial. In addition, there is a problem of side effects and loss in efficacy over time. Finally it should be noted that many well established drugs are clinically effective in inflammatory diseases and have the potential to inhibit intermediates of the NF-κB pathway. Therefore, overall there is a good rationale for developing inhibitors which target this pathway.

1.1.15 Classifications of NF-κB inhibitors

Given the complexity of the NF-κB pathway there are multiple sites of possible inhibition including intermediates upstream of the IKK complex, the IKK themselves, degradation of IκB proteins, the translocation of NF-κB to the nucleus and finally the binding and transcriptional activity of NF-κB itself.

1.1.15.1 Inhibitors act upstream of the IKK complex

Since many cytokines like TNF-α and IL-1β share the property of activating NF-κB signalling through distinct cell-surface receptors, then blocking these receptors would be advantageous. This approach has been realised clinically by use of anti-TNF agents or antibodies that able to neutralise the TNFR. This type of upstream anti-NF-κB therapy has shown benefit in inflammatory diseases such as arthritis, inflammatory bowel disease (IBD) and Crohn's disease (Song et al., 2002, Gilmore and Herscovitch, 2006). Indeed, anti-cytokine biologic agents such as adalimumab

(recombinant human anti-human TNF-α mAb), infliximab (chimeric anti-human TNF-α mAb), and anakinra (recombinant form of human IL-1 receptor antagonist) have been utilised in the treatment of IBD (Podolsky, 2002) and in patients with autoimmune joint disorders (Setoguchi et al., 2006). Nevertheless, the available anti-cytokine biologics are almost proteins that possess the general disadvantages associated with protein drugs. These include costs, in addition to serious side effects such as infections (tuberculosis reactivation), risk of malignancy and potential immunosuppression (as they are contraindicated in infectious diseases, pneumonitis, and hepatitis B) (Saag et al., 2008). To date there are no small molecules currently available that target either cytokine receptors or receptor associated intermediates such as TRAF , FADD, NIK, RIP (Cao et al., 1996, Bradley and Pober, 2001).

1.1.15.2 Inhibitors of the IKK complex and its activities

As the IKK complex has been shown to be a site of convergence for numerous agents which activate the NF-κB pathway this has been studied with some intensity as a site of pharmacological regulation. Indeed, there are large numbers of products that have been shown to inhibit activation of NF-κB by acting at the level of the IKKs, either by inhibiting IκBα phosphorylation or an induced IKK activity in kinase assays. In addition, a constitutively active form of IKKβ (S177, 181E) has been used in vitro as a tool to identify direct inhibitors of IKKβ. However, the mechanism of action of IKK inhibitors in vivo was poorly investigated. Generally, chemical IKKα/β inhibitors are classified into three categories: ATP analogs which are specifically interact with kinases, allosteric IKK inhibitors, and compounds which interact with an activation loop of IKKβ through a specific binding with Cys residue (Cys-179). ATP analogs either natural products such as the β-carbolines (Gamble et al., 2012), or synthetic compounds such as SC-839, which is about 200 fold more selective to IKKβ over IKKα (reviewed by Gilmore and Herscovitch, 2006, Karin et al., 2004, Pande and Ramos, 2005). In addition SC-514, another ATP inhibitor, used as a tool to study NF-κB activation in RASF cells was also identified as a selective inhibitor for IKKβ over 30 other kinases and bound specifically at the ATP-binding site of IKKβ (Kishore et al., 2003). Another specific ATP competitor small molecule IKKβ

inhibitor AS602868 potentiated the pro-apoptotic effect of TNF-α in Jurkat leukemic T cells (Frelin et al., 2003).

In contrast, far fewer studies have utilised drugs with allosteric effects. For instance, the synthetic compound BMS-345541; (4(2'-aminoethyl) amino-1,8-dimethylimidazo(1,2-a) quinoxaline) (Blazkova et al., 2007), was identified as a potent specific inhibitor of both IKKα and IKKβ . It is non-competitively inhibits ATP by binding to an allosteric site on both kinases, which is about 10 times more selective to IKKβ (Burke et al., 2003). In addition, BMS-345541 suppresses NF-κB-dependent transcription of pro-inflammatory cytokines both in vitro and in vivo (Burke et al., 2003). Furthermore, its anti-inflammatory effects have been shown in different studies as an inhibitor of CIA (McIntyre et al., 2003), DSS-induced colitis (MacMaster et al., 2003) or in mice with neutrophilic lung inflammation (Everhart et al., 2006). Many other effects of BMS-345541 have also been reported such as an improved graft survival in a murine model of cardiac graft rejection (Townsend et al., 2004). As well as mitochondria-mediated apoptosis of melanoma cells due to BMS-345541-induced inhibition of IKK activity (Yang et al., 2006) and also multiple mitotic cell cycle transition in vitro (Blazkova et al., 2007).

Previous studies have demonstrated that several thiol-reactive compounds including metals (e.g. copper, gold, and zinc) can block IKK in LPS-induced macrophages by inhibiting NF-κB activation (Jeon et al., 2000). These metals, in addition to other anti-inflammtory compounds, such as parthenolide (Kwok et al., 2001), certain epoxyquinoids (Liang et al., 2006), cyclopentenone prostaglandins (Rossi et al., 2000) and arsenite (Kapahi et al., 2000) have also been shown to block IKKβ activity through interaction with Cys-179. This mostly occurs via a direct conjugation to the thiol group of this cysteine. Since Cys-179 is located between Ser 177 and Ser 181, within the kinase activation loop, that part of IKKβ is essential for phosphorylation induced by TNF-α and LPS (Perkins, 2006, Scheidereit, 2006).

Activation of IKK can also be inhibited using molecular intervention. For example, mutations introduced to the ATP-binding site or in the kinase activation loop of

IKKα and IKKβ resulted in creation of dominant-negative forms, which are able to inhibit NF-κB activity (Zandi et al., 1997, Ling et al., 1998, Mercurio et al., 1997). According to the roles of IKKβ and IKKα in canonical and non-canonical pathways respectively, overexpression of a dominant negative IKKβ inhibits LPS-induction of κB site-dependent transcription in THP-1 monocytic cells (O'Connell et al., 1998). In contrast, overexpression of dominant-negative IKKα or NIK can only block caspase-induced NF-κB activation (Shikama et al., 2003). Collectively, adenovirus-mediated delivery has allowed characterisation of IKKα and IKKβ-dependent cellular events and as such has served as experimental tool. For example, in cases like asthma (Catley et al., 2005) and cardiovascular conditions such as restenosis (Bu et al., 2005) IKKβ dominant-negative kinase may be therapeutically effective, where systemic delivery can be avoided (Gilmore and Herscovitch, 2006).

Alternatively, NEMO can also be targeted for inhibition of the IKK complex. This can be mediated by many ways, one approach by introducing a cell permeable amino-acid peptide sequence corresponding to the NBD of the IKKβ, which can inhibit both the binding of NEMO to IKK and the TNF-α-induced NF-κB pathway (May et al., 2000). This peptide (10 amino acids) has shown therapeutic potential in mouse models of inflammation (May et al., 2000, di Meglio et al., 2005). Another approach used to block NF-κB activation is by introducing peptides corresponding to the region of NEMO which are required for its oligomerisation (Agou et al., 2004). It has also been demonstrated that the IKK inhibitor wtNBD peptide, ameliorated colitis-related disease in mouse models of IBD (Shibata et al., 2007), highlighting the therapeutic potential of this approach.

1.1.15.3 Agents acting on IκBα (upregulation, ubiquitination and degradation)

Recent focus on the development of IKK inhibitors has tended to overshadow the potential of other intermediates in the NF-κB pathway to serve as sites of targeted inhibition. NF-κB pathway can be inhibited by retaining large amount of IκB proteins in the cytoplasm thus preventing resultant translocation of NF-κB into the nucleus. Effective molecules may act by enhancing synthesis of IκBα, or interrupting IκBα ubiquitination, while others inhibit IκBα degradation.

Few synthetic compounds have been found to up-regulate IκB expression and so mimic endogenous molecules and such molecules can inhibit NF-κB. This effect is one which is a feature of the normal anti-inflammatory function of the immune systems. This can occur by the inhibition of nuclear localisation of NF-κB through the action of endogenously expressed anti-inflammatory cytokines (IL-10, IL-11 and IL-13), which are able to increase the synthesis of IκBα mRNA and protein expression (Trepicchio and Dorner, 1998, Ehrlich et al., 1998, Lentsch et al., 1997). In the context of Alzheimer's disease, it has been shown that treatment of fetal cortical neurons with the β-amyloid molecule causes an increase in IκBα mRNA and protein levels and in turn reduces constitutive NF-κB activity. Furthermore, using antisense oligonucleotide to IκBα mRNA showed neuroprotection in cortical cells against β-amyloid toxicity (Bales et al., 1998). However, steroids are currently the only clinically used medicine which up-regulates IκBα as part of its mechanisms of action (Auphan et al., 1995).

The critical common steps before the NF-κB complex can translocate to the nucleus are the ubiquitination of IκBα by the ubiquitin ligase complex SCF-β-TrCP, and the subsequent proteasomal degradation of ubiquitinated IκBα (Scheidereit, 2006). Therefore, inhibition of ubiquitin-proteasome system (UPS) by IκBα stabilisation results in NF-κB suppression. There are also several inhibitors that have been reported to block degradation of IκBα. A large number of them are natural compounds of the flavonoid and triterpenoids such as curcumin, green tea polyphenols, celastrol and chalcones have shown efficacy as antineoplastic agents in various cancer types including cervical (Bazzaro et al., 2011, Anchoori et al., 2011), colon (Milacic et al., 2008) and oesophageal (O'Sullivan-Coyne et al., 2009) cancers, which are associated with proteasomal inhibition. Thus proteasomal malfunction might be linked with both anti-proliferative and anti-inflammatory activities (Dikshit et al., 2006b, Calabrese et al., 2008).

Direct inhibition of ubiquitination has been found to be a mechanism underpinning the effects of infectious agents. For example, The YopJ protein of the bacterial pathogen Yersinia stabilises IκBα thereby preventing NF-κB nuclear translocation

through deubiquitination for IκBα (Zhou et al., 2005). Another naturally occurring and clinically available alpha-1-antitrypsin (AAT) has been found to inhibit HIV replication in lymphocytes associated with decreased NF-κB activity. This effect is attributed to decreased IκBα polyubiquitination through lysine residue 48 (K48) and in contrast increased ubiquitination through lysine residue 63 (K63). Moreover, in presence of AAT, K63 linked ubiquitin-IκBα degradation has been shown to be slower than K48 linked ubiquitin-IκBα degradation, correlating an altered ubiquitination with a prolonged IκBα half-life (Zhou et al., 2011).

To date, drugs which inhibit ubiquitination have not been developed clinically. In an early study, Yaron et al. (1997) have blocked TNF-α-induced degradation of IκBα by micro-injecting phospho-peptides specific to the signal-dependent phosphorylation site of IκBα, which seemed to act as competitive inhibitors for binding to the ubiquitin ligase complex responsible for degradation of IκBα (Gilmore and Herscovitch, 2006). More recent report has identified a small molecule RO196-9920, which can inhibit TNF-α and LPS-induced IκBα degradation and consequent NF-κB activation by a direct inhibition of E3 ligase activity (ubiquitination) and subsequent degradation. This compound has also shown oral effectiveness in acute mouse models of inflammation (Swinney et al., 2002, Gupta et al., 2010).

Proteasome machinery can be interrupted by specific inhibitors, the proteasome inhibitors, they all share characteristic of cell permeability and can attain a dose-dependent NF-κB inhibition as a result of blocked proteasome-mediated IκBα degradation. They are several classes including the reversible inhibitors peptide aldehydes. They can inhibit the chymotrypsin like activity of the proteasome complex without altering the phosphorylated residues (Rock et al., 1994). For example MG101 or ALLnL (calpain inhibitor I), is a cysteine protease inhibitor, but with less potency compared to MG115 and MG132 (Palombella et al., 1994, Grisham et al., 1999). These inhibitors are widely used as a tool in studying the role of proteasome in different cellular functions (Myung et al., 2001).

A much more specific second class of proteasome inhibitors is lactacystin and its synthetic precursor, β-lactone. Through acylating the threonine residue in the active site of the proteasome subunit they can irreversibly block proteasome activity (Fenteany and Schreiber, 1998, Grisham et al., 1999).

Peptide boronic acids (dipeptidyl boronates) are representing the third class of proteasome inhibitors, comprise the PS-262, PS-273, PS-341, and PS-402. They are inhibitors of serine proteases, with the ability to block the chymotrypsin-like site in the 20S subunit core (Iqbal et al., 1995). Most effective inhibitor among these is PS-341 (bortezomib or Velcade®), which characterised by a higher efficacy in treatment of multiple myeloma, in addition to several hematologic and solid tumours (Adams and Kauffman, 2004). The ability of proteasome inhibitors to inhibit the activation of NF-κB results in the reduction of NF-κB dependent inflammatory mediators and leukocyte adhesion molecules in vitro and in vivo. Both peptidomimetic boronates and lactacystin were observed to possess anti-inflammatory effects in several rodent models and also effective in reperfusion injury, at doses that partially inhibit proteasome function (Lee and Goldberg, 1998a , Lee and Goldberg, 1998b).

Many inhibitors of serine proteases with chymotrypsin like specificity including DCIC, TPCK, and TLCK, can also block the proteasome function. However, uniquely these serine protease inhibitors can specifically block IκBα phosphorylation as well as degradation (Hwang et al., 2011). It should be noted that not all protease inhibitors can suppress NF-κB activation (Higuchi et al., 1995, Rossi et al., 1998).

1.1.15.4 Inhibition of NF-κB nuclear function

Another potential site of inhibition is the prevention of NF-κB function. This may occur either by inhibition of NF-κB nuclear translocation, DNA binding or transactivation. Many studies have used reduced protein-DNA binding in an Electrophoretic Mobility Shift Assay (EMSA) or reduced κB-site reporter gene activity as markers of NF-κB blockade. Moreover, attaining IκB proteins within cytoplasmic pool lead to inhibition of NF-κB nuclear function.

1.1.15.4.1 Inhibitors of NF-*κB nuclear translocation*

A number of NF-κB inhibitors function to block nuclear translocation. Cell permeable peptides containing the NLS of NF-κB p50 has been used to block nuclear translocation of p50-containing dimers. This thought to be mediated by saturation of the nuclear import system that facilitate the transport of NF-κB dimers containing p50 (Lin et al., 1995, Torgerson et al., 1998, Letoha et al., 2005). An example of used peptides is SN-50 (AAVALLPAVLLALLAP**VQRKRQKLM**P) (Lin et al., 1995), in addition to p50 it can prevent nuclear translocation of a number of transcription factors other than NF-κB (Torgerson et al., 1998, Gilmore and Herscovitch, 2006). In addition, inhibition of NF-κB nuclear translocation has also been shown by the anti-inflammatory and antitumour dehydroxy methyl epoxiquinomicin (DHMEQ) (Ohsugi et al., 2006, Umezawa, 2006). Whilst o,o`-bismyristoyl thiamine disulphide, has been reported to prevent nuclear translocation of NF-κB and HIV-1 transactivator (TAT), leading to suppression of HIV-1 replication (Shoji et al., 1998). Furthermore, a steroidal lactone, withaferin A, extracted from the plant Withania somnifera L. Dunal has been demonstrated to block filtrates of Pseudomonas aeruginosa (PAF)-induced NF-κB activation by specific inhibition of NF-κB (p65) subunit translocation (Maitra et al., 2009).

Effective molecular inhibition of NF-κB nuclear translocation can be attained using the super-repressors (SRs) IκBs mutants (Yan et al., 2010), which are permanently bind to NF-κB dimers and do not undergo phosphorylation or degradation. IκBα-SRs are the most important SRs of NF-κB, which may be characterised with mutations of serines 32 and 36 (phosphorylation sites) by alanine replacement, mutations of the lysine ubiquitination sites replaced by arginine, or deletion of proximal 40 amino acids. Thus, block phosphorylation and ubiquitination (Jobin et al., 1998, Bentires-Alj et al., 1999). In various in vitro cell cultures and in vivo transgenic and knockout mice studies, these molecules have been used effectively to block NF-κB activity (Wang et al., 1996, Van Antwerp and Verma, 1996, Bushdid et al., 1998). However, a limit in the potential clinical use of the IκBα-SR has been the associated development of squamous cell carcinoma in a murine model (van Hogerlinden et al., 1999, Gilmore and Herscovitch, 2006). This may be due in part to problems of

specificity, since IκBα-SR can crossly interact with the activity of NF-κB-independent proteins such as cyclin-dependent kinase 4 (Li et al., 2003), p53 (Chang, 2002), and histone deacetylases (HDACs) (Aguilera et al., 2004). Though full characterisation of IκBα protein-protein interactions (PPIs) remains to be pursued.

1.1.15.4.2 Inhibitors of NF-*κB*-DNA binding

Another potential site of inhibition involves disruption of protein-DNA binding. Several natural anti-inflammatory compounds including sesquiterpene lactones act by inhibition of NF-κB-DNA binding (SLs) (Zhang et al., 2005). Specifically, SLs may modulate NF-κB-DNA binding through direct interaction with Cys-38 in the DNA-binding domain of NF-κB (p65), as well as in p50 and c-Rel via analogous cysteine residues. Thus, mutations of these residues to serines render these NF-κB subunits resistant to inhibition by thiol-reactive molecules (Wagner et al., 2006). SLs and some epoxyquinoids may also target multiple steps within the NF-κB signalling pathway, it may target IKK complex in addition to NF-κB-DNA binding activity (Liang et al., 2006). Parthenolide, an example of compounds, that can suppress NF-κB-DNA binding activity via direct interaction with Cystein residue (Cys-179) of IKKβ (Kwok et al., 2001).

A molecular approach to specifically inhibit NF-κB-DNA binding is via decoy oligonucleotides (Decoy ONs). These are short synthetic fragments of DNA or RNA designed to specifically bind complementary sequences of proteins (i.e. transcription factors). When a decoy occupies the transcription protein-DNA binding site, it prevents subsequent binding to the promoter regions of target genes. On this bases, it has been reported that a double stranded ON containing a κB consensus sequence (NF-κB decoy ON) competed with the DNA for NF-κB binding (Bielinska et al., 1990), and displaced NF-κB dimer away from binding to specific gene promoters (e.g, decoy cis element) (Morishita et al., 1997, Kupatt et al., 2002). However, oligonucleotides modifications have been made in order to augment their stability as well as affinity for NF-κB in vivo (Isomura and Morita, 2006). An observed therapeutic efficacy of this κB-site decoy ON has been displayed in several animal models of inflammation. For instance, it reduced the influx of inflammatory cells and

cytokines in broncho-alveolar lavage fluid from ovalbumin-induced allergic mice (Desmet et al., 2004). Whilst the anti-inflammatory effects of NF-κB decoy ONs reported in several preclinical studies are promising for future clinical applications, systemic administration is still hampered by short half-life and non-specific distribution (De Stefano, 2011).

1.1.16 Antioxidants as inhibitors of the NF-κB pathway

In principle, oxidants, such as hydrogen peroxide can directly activate NF-κB in many cell types. In an inverse way, the induced activity of NF-κB in response to a various stimuli such as IL-1β, LPS, TNF-α, in some cells have been shown to be mediated by ROS (Gloire et al., 2006). Therefore, antioxidants may be a useful class of compounds for the inhibition of the pathway. However, the exact mechanisms of NF-κB activation by oxidants or its inactivation by antioxidants are not precisely known. It is likely that both interact with different key molecules in the NF-κB pathway. Several antioxidant compounds including thiol antioxidants (e.g., PDTC, NAC), vitamin C and E derivatives, calcium chelators (e.g., EGTA, lacidipine) and α-lipoic acid were able to inhibit hydrogen peroxide-induced NF-κB activation. Many of these agents are scavengers of reactive oxygen intermediates (ROIs) (Sen and Packer, 1996, Gilmore and Herscovitch, 2006). Whilst, the phenolic antioxidant Caffeic acid phenethyl ester (CAPE), can directly interfere with NF-κB-DNA binding, whereas this effect can be reversed by dithiothreitol (Singh and Aggarwal, 1995). Other agents like rotenone an inhibitor of mitochondrial electron transport that suppresses ROI production as well as overexpresses antioxidising enzymes, such as catalase and MnSOD, can inhibit TNF-α-induced NF-κB activation (Schulze-Osthoff et al., 1993, Manna et al., 1998). Exceptionally, it has been suggested that the NF-κB inhibitory activity of PDTC is not related to its antioxidant activity (Hayakawa et al., 2003).

As indicated above the site and mechanism of actions of antioxidants within the pathway remains unclear although certain sites of inhibition have been identified. In other cases antioxidants may alter the redox state of cysteine residues in the IKK activation loop leading to inhibition of its activity (Gloire et al., 2006). Furthermore,

interference of antioxidants with the DNA-binding loop may be affected by redox state. Accordingly, NF-κB-DNA binding can be inhibited via interaction with thiol-reactive metals (Shumilla et al., 1998).

1.1.17 Minor groove binders (MGBs)

Recently, more interest has been given to the potential use of minor groove binders (MGBs) and their derivatives as human chemotherapeutics mediated targeted delivery of DNA-modifying agents (Marchini et al., 2004, Pindur et al., 2005). This approach is based on the fact that MGBs including the antibiotic distamycin A can bind to AT-rich sequences within the minor groove of the DNA in a conformation and sequence specific manner (Dervan, 2001). Therefore, this property has been exploited to disrupt transcription factor-DNA binding to AT-rich sequences (Cozzi, 2000, Bell et al., 1997). In addition to effects on NF-κB, it has been shown that high mobility group A1 protein (HMGA1) binding to DNA was also blocked by the MGBs in vitro (Radic et al., 1992, Pellacani et al., 1999).

Distamycin A and polyamide based drugs are selectively able to interfere with the NF-κB binding specifically to a number of κB sites without affecting the full function of the transcription factor. This could represent a valuable therapeutic alternative to the existing small molecule inhibitors (Raskatov et al., 2012). A recent study has demonstrated that distamycin A can improve survival and reduced the LPS-induced hypotension in murine endotoxemia (Baron et al., 2004). This in vivo effect found to be correlated with reduced iNOS induction in tissues and in murine macrophages; this was attributed to MGBs specific interruption with NF-κB-DNA binding to a distinct AT-rich sequence of the iNOS enhancer. Furthermore, a recent in vivo study has displayed distamycin A, as a novel targeted approach in modulating liver and lung inflammation associated with murine endotoxemia by attenuating binding of HMGA1 to a specific AT-rich sequence of the P-selectin gene promoter (Baron et al., 2010).

1.1.18 Histone deacetylase (HDAC) inhibitors

In general, acetylation of particular lysine residues within the N-terminus of nucleosomal histones is mainly linked to the disruption of chromatin and transcriptional activation of genes. This process mediated by several transcriptional coactivators (e.g. CBP/p300, P/CAF, SRC-1) through their an intrinsic acetyl transferase activity (Roth et al., 2001). Many non-histone proteins undergo reversible acetylation as a post-translational modification, including non-histone structural chromosomal proteins, the HIV-1 Tat protein, non-nuclear proteins (α-tubulin), transcription factors, and nuclear import factors (human importin-α). Accordingly, acetylation can regulate and modify different functions of these nonhistone proteins such as DNA recognition, protein stability, protein-protein interaction (PPI) and subcellular localisation (Bannister and Miska, 2000, Quivy and Van Lint, 2004).

In the last decade, HDAC inhibitors have been shown to modulate the NF-κB activity in various cell types such as colon cancer cell lines and colonic macrophages (Segain et al., 2000, Luhrs et al., 2002). A study carried out on colon cancer cells showed that HDAC inhibitors butyrate and trichostatin A (TSA) suppress proteasome activity by down-regulating the expression of specific proteasome subunits. Eventually, this leads to inhibition of NF-κB activation through preventing the ubiquitin-mediated, proteasome-dependent degradation of IκBα (Place et al., 2005).

Butyrate and TSA, structurally unrelated HDAC inhibitors, decreased IL-8 expression and enhance cell differentiation in colonic epithelial cells (Huang et al., 1997). Furthermore, both inhibitors reduced NF-κB-dependent gene transactivation in RAW264.7 macrophages induced with receptor activated NF-κB ligand (RANKL), by suppressing NF-κB nuclear translocation (Rahman et al., 2003). In addition, HDAC inhibitors have shown inhibitory effects on bone resorption in a mouse model of RA (Chung et al., 2003). Moreover, oral administration of the HDAC inhibitor sodium valproate in DSS and TNBS-induced colitis models alleviated disease severity. This effect was also associated with suppressed production of inflammatory cytokines, as well as with increased lymphocyte

apoptosis and a dose-dependent increase in histone H3 acetylation (Glauben et al., 2006, Adcock, 2007, Blanchard and Chipoy, 2005). Recently, it has been reported that HDAC inhibitors, MS-275 and suberoylanilide hydroxamic acid (SAHA) exhibited anti-rheumatic effects by inducing growth arrest in RASFs. This action was in part mediated via suppression of LPS-induced NF-κB (p65) nuclear translocation and consequently inhibited pro-inflammatory cytokines secretion, as well as down-regulated angiogenesis and MMPs in E11 cells at sub-micromolar concentrations. Similar suppressive effects on pro-inflammatory cytokines have also been observed in THP-1 monocytic cells and inhibited NO secretion in RAW264.7 macrophage cells (Choo et al., 2010).

1.1.19 Natural products in therapy

Natural products are small molecules derived from diverse natural sources including bacteria, fungi, plants and others. Usually they are involved in treatment of most pathophysiological conditions, such as infectious, metabolic, neurological, cardiovascular and oncological diseases (Zhou et al., 2008). For example, plants provide an important source for discovery of potent small molecules to fight inflammatory diseases and cancer (Balunas and Kinghorn, 2005).

Although several promising molecules have been identified recently, there are still obstacles to overcome before their clinical use (Corson and Crews, 2007). Intriguingly, Phenolic compounds and terpenoids are representing the active ingredients in several plant extracts and also are major constituents of many fruits, vegetables and spices (Wagner and Elmadfa, 2003). These compounds have multiple properties, which are antioxidant, in addition to a variety of specific properties contribute to distinct interactions with several regulatory proteins. It has been suggested that most of therapeutically active plant derived molecules that used traditionally to treat inflammation or cancer could modulate NF-κB signalling (Salminen et al., 2008).

1.1.20 Natural compounds as NF-κB inhibitors

Two decades ago Kopp & Ghosh (1994) were identified the first plant-derived sodium salicylate and its semi-synthetic derivative aspirin as inhibitors of NF-κB. Since then, a large number of diverse natural products, have demonstrated NF-κB inhibitory activity (Bremner and Heinrich, 2002).

There are several different structural classes of natural products which have been shown to inhibit intermediates of the NF-κB pathway. Evidently, one class is the terpenoids which have been shown to affect upstream targets within the cascade, but they may also inhibit the MAP kinase pathway and subsequent activation of AP-1 (Kawai and Akira, 2007). The sesquiterpene lactone parthenolide derived from the anti-inflammatory medicinal plant Feverfew Tanacetum parthenium has been shown to inhibit pro-inflammatory signalling. Parthenolide specifically binds to and inhibits IKKβ, leading to the loss of NF-κB activation. Moreover, parthenolide-mediated inhibition of a constitutively active IKKβ mutant demonstrates that parthenolide acts directly at the level of IKKβ to inhibit NF-κB activation (Kwok et al., 2001). Furthermore, sesquiterpene lactones also regulate NF-κB-DNA complex formation. This binding is dependent on the cysteine residues of p50 and p65 subunits and it has been shown that Cys-38 in the DNA binding loop of p65 subunit is selectively targeted for such alkylating agents (Konaklieva and Plotkin, 2005, Salminen et al., 2008).

Other unrecognised sources of NF-κB inhibitors are marine bacteria, algae and invertebrates. To date a few marine compounds have been reported to inhibit NF-κB through targeting multiple components within the NF-κB pathway. They comprise compounds can target IKK, or specifically interfere with the proteolytic activity of the 26S proteasome leading to block degradation of IκBα, or inhibit the interaction of NF-κB with its DNA binding site (Folmer et al., 2008). For instance, many marine natural extracts can inhibit IκBα degradation (Nagle et al., 2004) include the sunscreen pigment scytonemin isolated from various cyanobacteria, the sesterterpene lactone cacospongionolide B isolated from the sponge Fasciospongia cavernosa and the sesterterpene lactone petrosaspongiolide M isolated from the sponge

Petrosaspongia nigra. Whilst, other products have been reported to act as 26S proteasome inhibitors such as the lactone-γ-lactam salinosporamide A isolated from the bacterium Salinispora tropica, the oxazole alkaloid mycalolide A isolated from a Mycale sp. sponge, and the sterol acetate agosterol C isolated from the sponge Acanthodendrilla sp. (Tsukamoto et al., 2003). It has also been shown that Fijian marine natural products inhibit TNF-α-induced IκBα degradation and the subsequent nuclear translocation of p50 and p65 dimers of NF-κB (Folmer et al., 2009).

1.1.21 Current anti-inflammatory drugs as NF-κB inhibitors

In context to an early discovery of the mechanism of action of aspirin as an inhibitor of PGs synthesis (Vane, 1997), the hunt for molecular targets remains open. In addition to the inhibition of COX, several NSAIDs, such as ibuprofen, sulindac aspirin, and indomethacin, have been shown to inhibit NF-κB activation in cell cultures (Kopp and Ghosh, 1994, Takada et al., 2004). Therefore, they are implicated in preventing expression of NF-κB-dependent inflammatory proteins like iNOS (Aeberhard et al., 1995) and COX-2 (Amin et al., 1995). However, aspirin in particular at high doses has also been shown to inhibit NF-κB activity via direct binding to inhibit the kinase activity of IKKβ, as result of lost ability to bind ATP (Yin et al., 1998). Moreover, aspirin has the ability to inhibit proteasome activity and then the degradation of IκBα (Dikshit et al., 2006a). This may explain the rationale of application of high doses of aspirin in diseases linked with increased NF-κB activity, including chronic inflammatory diseases (Gilmore and Herscovitch, 2006), diabetes (Yuan et al., 2001), heart diseases (Li and Fang, 2004) and cancer (McCarty and Block, 2006).

Glucocorticoids, such as dexamethasone, prednisolone and methylprednisolone, have been clinically used for their anti-inflammatory effects in diseases such as asthma or arthritis and in prevention of allograft rejection. Their effects and mechanism of action appear to be partially mediated by inhibition of NF-κB (Lee and Burckart, 1998). According to results obtained by De Bosscher et al. (2006), glucocorticoids, mediate their action via the glucocorticoid receptor (GR). They can block NF-κB by affecting several steps within the pathway, including up-regulation of IκBα,

inhibition of IKK activity, DNA binding, and transactivation in different cell types (De Bosscher et al., 2006). Very recently it has been shown that fluticasone propionate reduced both IKKα and IKKβ activity in TNF-α and cigarette smoke extract-treated peripheral blood mononuclear cells (PBMCs) or of asthmatic and COPD patients, with higher levels of inhibition for IKKβ than IKKα activity thereby reducing IL-8 expression (Gagliardo et al., 2011). Another recent study has demonstrated that pretreatment of epithelial intestinal cells with novel guggulsterone derivatives GG-50B and GG-52 decreased the TNF-α-induced phsophorylation of IKK and IκBα. In addition, GG-52 significantly alleviated DSS-induced colitis in murine models, providing an efficacy similar to that achieved by sulfasalazine or prednisolone (Kim et al., 2010).

An activated GRs could interact with NF-κB, which lead to inhibition of several pro-inflammatory genes that possess κB binding sites in their promoter region but lack the glucocorticoid response elements (GREs) (Ray and Prefontaine, 1994). The resulted NF-κB repression was in part due to induced transcription of IκBα gene and protein synthesis in some cell types (Auphan et al., 1995, Crinelli et al., 2000). Furthermore, it has been shown that in cells treated with dexamethasone, the NF-κB suppression occurs mainly at transcriptional level. It has been associated with down-regulation of TNF-α mRNA expression and inhibition of TNF-α production (Crinelli et al., 2000). Comparably, estrogen and certain selective estrogen receptor modulators (SERMs), such as raloxifene, can also inhibit NF-κB by facilitating the interaction of estrogen receptor with NF-κB (p65) subunit (Olivier et al., 2006, Gilmore and Herscovitch, 2006).

1.1.22 Immunosuppressive agents

A substantial number of drugs have been used in several clinical conditions, for example RA and colitis, despite of a relative lack of knowledge regarding their mechanism of action. This includes drugs such as sulfasalazines and cyclosporin A, which have been reappraised. One such example is the immunosuppressants which have been used not only to prevent allograft rejection but also in the diseases identified above.

Various immunosuppressants can target NF-κB by distinct mechanisms. Some can prevent NF-κB nuclear translocation via stabilization of IκBα; cyclosporin A (CsA) for example, counted as proteasome inhibitor via non-competitive inhibition of the chymotrypsin-like activity of the proteasome. It has been shown by in vivo study to block LPS-induced degradation of IκBα and processing of p105 (Meyer et al., 1997). In addition, CsA inhibits NF-κB nuclear translocation in an activated T cells (McCaffrey et al., 1994) by blocking the inducible degradation of both IκBα and IκBβ; whereas, the processing of p105 to p50 stayed unaffected in the same cells (Marienfeld et al., 1997). However, CsA may also target NF-κB through inhibition of calcineurin phosphatase activity (Frantz et al., 1994). Similarly, FK506 (tacrolimus) has been shown to block T and B cell proliferation, which attributed to the inhibition of calcineurin activity. In contrast to CsA, the effect of tacrolimus on NF-κB seems to be more specific for c-Rel. Indeed, tacrolimus specifically prevented c-Rel nuclear translocation in cells induced with phorbol esters and ionomycin (Sen et al., 1995). Thus, anti-NF-κB activity of tacrolimus was responsible for the effectiveness of application of tacrolimus ointment in keratinocytes as well as for its efficacy in psoriasis (Lan et al., 2005). On the other hand, the immunosuppressant deoxyspergualin can prevent NF-κB nuclear translocation by an interaction with the heat-shock protein Hsp70 (Tepper et al., 1995, Abe et al., 2003, Gilmore and Herscovitch, 2006) suggesting multiple sites of inhibition for this class of compound.

Certain immunosuppressants also act by interfering with the DNA binding or transcriptional activity of NF-κB (McCaffrey et al., 1994). For example, PG490 (diterpene triepoxide) can synergise with CsA to suppress NF-κB transcriptional activity (Gilmore and Herscovitch, 2006). Nevertheless, it has been suggested that PG490 could inhibit the expression of IκBα gene, it may also interfere with recruitment of coactivator proteins CREB /p300, or may prevent the interaction between p65 and RNA polymerase II (Qiu et al., 1999). Taken together these studies indicate that a component of the current anti-inflammatory drugs may involve an effect on NF-κB pathway.

1.2 AIMS AND OBJECTIVES

This introduction has outlined the central importance of the NF-κB pathway and its intermediates in the genesis of a number of diseases which are underpinned by chronic inflammation. This includes not only diseases such as asthma, RA and colitis but also cancer and cardiovascular diseases. Discovery of novel agents which could be used to block the NF-κB pathway would be clinically desirable. However, to identify such compounds and assess their potential a detailed analysis of the effects on all of the intermediates of the pathway is required. Therefore, in this thesis, a number of candidate compounds were assessed for anti-NF-κB activity. The rational of testing these compounds based on preliminary data obtained from experiments carried out in-house, which showed effectiveness of these compounds as inhibitors of IKK activity, as well as NF-κB transcriptional activity.

Accordingly, selected compounds were classified according to their chemical natures into alkaloids and minor groove binders/peptidomimetics. Compounds were examined against the NF-κB signalling pathway by testing: their effects on TNF-α-induced IκBα degradation, and NF-κB (p65) phosphorylation were assessed by Western blotting. In addition, subcellular localisation of NF-κB (p65) induced by TNF-α was assessed by microscopy. At the nuclear level, the effects of compounds on TNF-α-induced NF-κB-DNA binding activity also were investigated as well as further assessment of induced NF-κB transcriptional activity in response to different agents (TNF-α and PMA) assessed by reporter assay. Moreover, this study tends to investigate the effect of compounds on induced inflammatory biomarkers mainly mediated by NF-κB signalling. Finally, other off-targets will also be investigated to assess the selectivity of compounds; these targets include the MAP kinases, AP-1 activity and JAK/STAT1 signalling.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 GENERAL REAGENTS

All materials used were of highest commercial purity available and were supplied by Sigma Aldrich Chemical Company Ltd. (Poole, Dorset, UK) unless otherwise stated.

Amersham International Plc (Aylsbury, Buckinghamshire, UK)

ECL detection reagents

Bio-Rad Laboratories (Hertfordshire, UK)

Bio-Rad DC™ Protein Assay kit

Pre-stained SDS-Page molecular weight markers

Boehringer Mannheim (East Sussex, UK.

Bovine serum albumin (BSA, Fraction V)

Calbiochem®-Merck chemical Ltd (Nottingham, UK)

Mowiol.

MG-132 (Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal).

Corning B.V. (Netherlands)

Tissue culture plastic ware

FlukaChemie GmbH (Germany)

p-coumaric acid

GE Healthcare (Kent, UK)

Whatman™ 17 CHR Chromatography Paper

Amersham™Hybond-ECL Nitrocellulose membrane

Gibco®-Invitrogen Life Technologies Ltd (Renfrewshire, UK)

Versin (0.2% EDTA/PBS)

Modified Eagle`s Medium (DMEM 1X) + [4.5 g/L glucose, 0.11g/L sodium pyruvate].

Invitrogen (Paisley, UK)

Fetal calf serum (FCS)

Antibiotics:Penicillin, Streptomycin, Geneticin (G418), Blasticidin.

Molecular Devices Corp. (Downingtown, PA, USA)

MetaMorph Imaging Series software (version 7.0 or 7.6.4)

Nikon (Kingston upon Thames, UK).

Nikon TE-300 EP-1 upright fluorescence microscopy

40 x or 100 x oil-immersion Plan Fluor Objective Lens, NA=1.3

Perkin Elmer Life sciences(Cambridge, UK)

Easy Tides [γ^{32} P]- Adenosine 5`-Triphosphate (ATP)

Promega Corporation (Madison, WI, USA)

Gel Shift Binding 5X Buffer

NF-κB consensus oligonucleotide

Steady-Gio®Luciferase Assay Substrate

T4 Polynucleotide Kinase Buffer

T4 Polynucleotide Kinase

Roche diagnostics GmbH (Germany)

Dithiothreitol (DTT)

Roth GmbH (Karlsruhe, Germany)

Rotiphorese® Gel 30 (30% Acrylamide-0.8% Bisacrylamide)

Santa Cruz Biotechnology Inc. (CA,USA)

Human Recombinant TNF-α

StratechSientific Ltd (Newmarket Suffolk, UK)

DAPI (4,6-Diamidino-2-phenylindole, dihydrochloride) (17514-ABD)

Tocris Bioscience (Bristol, UK)

Cardamonin: (2E)-1-(2,4-Dihydroxy-6-methoxyphenyl)-3-phenyl-2-propen-1-one

University of Strathclyde -CRUK- Small Molecule Drug Discovery Initiative (SMDDI) (Glasgow, UK)

SU182, SU331 and SU432, AIK18/85/1 and AIK18/70

VWR International Ltd (Leicestershire, UK)

No. 0, (0.09-0.13mm thick), circular glass 13mm diameter coverslips

0.8-1.0mm thick glass microscopy slides

2.1.2 ANTIBODIES

Abcam plc (Cambridge, UK)

Mouse monoclonal anti-GAPDH (6C5)

Biosource International Inc. (USA)

Rabbit polyclonal anti-pJNK 1 & 2 (44-682G)

Rabbit polyclonal anti- pp38 (44684-G)

Cayman chemical (Michigan, USA)

Rabbit polyclonal anti-COX2 (160107)

Rabbit polyclonalanti-iNOS (160862)

Cell Signalling technology, Inc., (USA)

Rabbit polyclonal pp65 (ser-536)

Rabbit polyclonal p-STAT1α (Tyr 701)

Jackson ImmunoResearch Laboratories Inc. (PA, USA)

HRP-conjugated goat anti-rabbit IgG

HRP-conjugated anti-mouse IgG

Fluorescein (FITC) conjugated AffiniPure donkey anti-Rabbit IgG-711-095-132

Santa Cruz Biotechnology Inc. (CA, USA)

Mouse monoclonal anti-pERK (E-4)

Rabbitpolyclonalanti- IkBα (C-21)

Rabbit polyclonal anti-p38 (N-20)

Rabbit polyclonal anti-JNK-1 (FL)

Rabbit polyclonal anti ERK-1 (K-23)

Rabbit polyclonal NF-κB p65 (C-20)

2.2 METHODS

2.2.1 CELL CULTURE

All cell culture work was performed in a class II cell culture hood under aseptic conditions. All cells were grown in 75cm^2 flasks unless otherwise stated.

2.2.1.1 Reporter cells

Human skin epithelial cells NCTC2544 stably expressing PAR-2 (Clone G) (Kanke et al., 2001), and additionally transfected with NF-κB or AP-1 plasmid were maintained in complete medium M199 with Earl`s salt supplement (10% v/v FCS, 100U/ml penicillin, 100µg/ml streptomycin, 2mM glutamine). Antibiotics: 400µg/ml, 5µg/ml of geniticin and blasticidin respectively were added for selection pressure. Cells were split using versene (0.2 % (v/v) EDTA/BPS (pH 7.4)) and used at passages 7-20 for experiments.

2.2.1.2 Murine macrophages

Murine RAW264.7 macrophages, obtained from the European Type Cell Culture Collection (ECACC No.91062702) and cultured in Delbecco's modified Eagle's Medium (DMEM) containing 4.5g/L glucose and 0.11g/L sodium pyruvate

supplemented with 10% (v/v) Fetal calf serum (FCS), 2mM glutamine and 250U/ml penicillin and 250µg/ml streptomycin at 37°C in a humidified 5% CO_2:95% air incubator under standard conditions.

2.2.2 LUCIFERASE GENE REPORTER ASSAY

NCTC2544 reporter cells for NF-κB and AP-1 were plated in 96 well plates until near confluent and rendered quiescent for at least 18 h in an appropriate serum free medium. Cells were treated with different concentrations of test agents for 30 min prior to addition of appropriate concentrations of TNF-α, or phorbol 12-myristate 13-acetate (PMA). Stimulation was terminated by aspiration of the medium and subsequent washing with ice-cold BPS (pH 7.4). To each well (100µl) luciferase solution was added in a buffer containing (0.2mM luciferin substrate, and 1% (w/v) BSA in 10ml of lysis buffer [25mM Trisbase (pH 7.9), 8mM $MgCl_2$, 1mM DTT, 1% Triton X 100, 15% v/v glycerol, 1mM ATP]. The plate was left for 5 min at room temperature until read on a Wallac Trilux 1450 microbeta counter (liquid scintillation and luminescence counter, Turku, Finland).

2.2.3 WESTERN BLOTTING

2.2.3.1 Preparation of Whole Cell Extracts

Cells were exposed to vehicle,stimulant or test compound for anappropriate period of time. They were then washed twice with ice cold PBS before adding 200µl of pre-heated Laemmli's sample buffer (63mM Tris-HCL (pH 6.8), 2mM $Na_4P_2O_7$, 5mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50mM DTT, 0.007% (w/v) bromophenol blue). The cells were then scraped and the genomic DNA sheared repeatedly through syringe with a 21 guage needle in sterile Eppendorf tubes. The tubes were boiled for 4 min to denature proteins and samples were stored at -20 °C until use.

2.2.3.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Resolving gels were prepared containing an appropriate amount of 7.5% (w/v), 9% (w/v), 10% (w/v), and 11% (w/v) acrylamide solution (acrylamide 30%:N,N'-methylenebis-acrylamide 0.8% (37.5 : 1)), buffer (pH 8.8) of (1.5M Tris base and 0.4% (w/v) SDS), distilled water and 10% (w/v) ammonium persulfate (APS).

Polymerisation was initiated by the addition of 0.05% (v/v) N, N, N', N'-tetramethylethylenediamine (TEMED). The solution was poured between two glass plates assembled in a vertical slab configuration according to the manufacturer's instructions (Bio-Rad) and overlaid with 180 µl 0.1% (w/v) SDS. Following gel polymerisation the layer of 0.1% SDS (w/v) was removed and a stacking gel containing acrylamide: N,N'-methylenebis-acrylamide (37.5 : 1), buffer (pH 6.8) of (0.5M Tris base and 0.4% (w/v) SDS), 10% (w/v) ammonium persulfate and 0.05% (v/v) TEMED was poured directly to the resolving gel, and ateflon comb was immediately inserted into the stacking gel solution. After polymerization was complete the comb then removed and the polyacrylamide gels were assembled in a Bio-Rad Mini-PROTEAN®III™or Mini PROTEAN® Tetra cell electrophoresis tank, with reservoirs filled with electrophoresis buffer (25mM Tris base, 129mM glycine, 0.1% (w/v) SDS). Aliquots of samples (20-30µg/ml) were then loaded into the wells using a micro syringe. A pre-stained SDS-PAGE molecular weight marker of known molecular weights was run concurrently in order to identify the protein of interest. Samples were electrophoresed at a constant voltage of 120 V, until the bromophenol blue dye had reached the bottom of the gel.

2.2.3.3 Electrophoretic Transfer of Proteins to Nitrocellulose Membrane

The proteins separated by SDS-PAGE were transferred to nitrocellulose membranes by electrophoretic blotting following a standard protocol (Towbinet al., 1979). The gel was pressed firmly against a nitrocellulose sheet and assembled in transfer cassette sandwiched between two Whatman 3MM paper and two sponge pads. The cassette was immersed in blotting buffer (25mM Tris base, 19mM glycine, 20% (v/v) methanol) in a Bio-Rad Mini Trans-Blot ™ tank and a constant current of 300 mA was applied for approximately 2 h, whilst the tank was cooled by inclusion of an ice blotting buffer reservoir.

2.2.3.4 Immunological Detection of Protein

Following transfer of the proteins to the nitrocellulose membrane, the membrane was removed and any remaining protein blocked by incubation in a solution of 2% (w/v) BSA in NaTT [150mM NaCl, 20mM Tris base (pH 7.4), 0.2% (v/v) Tween-20] for

2 h with gentle agitation on a platform shaker. The blocking buffer was removed and membranes incubated overnight with antiserum specific to the target protein appropriately diluted in NaTT buffer containing 0.2% (w/v) BSA either at 4ºC on roller shaker or at room temperature as appropriate. On the following day membranes were washed in NaTT every 15 min for 1h and 30 min with gentle agitation. The membranes were then incubated fora further 2 h at room temperature with secondary horseradish peroxidase-conjugated IgG directed against the first immunoglobulin diluted to approximately 1:10000 in NaTT buffer containing 0.2% BSA. After six additional washes in NaTT as described before, immunoreactive protein bands were detected by incubation in enhanced chemiluminescence (ECL) for 90 sec. The membranes were place on a paper towel and mounted in an exposure cassette and covered withcling film, then exposed to Kodak X-OMAT LS film for the appropriate time under darkroom conditions and developed by a KODAK M35-M X-OMAT processor.

2.2.3.5 Stripping and Re-probing Nitrocellulose Membrane

The used nitrocellulose membranes were stored in a sealed container containing NATT buffer at 4ºC, until re-probing was required. Antibodies were then stripped from the nitrocellulose membrane by incubating it in 15ml stripping buffer (0.05M TrisHcl (pH 6.7), 2%SDS and 0.1M β-mercaptoethanol in ultra-pure water) for 1 h on incubator/shaker (60ºC) (Stuart Science Equipment) then it was discarded in a fume hood sink. Finally Blots were rinsed with NaTT three times for 10 min each time to get rid of stripping buffer. At this stage the blots are ready for the immunological detection protocol (Section 2.2.3.4).

2.2.4 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

2.2.4.1 Preparation of Nuclear Extracts

Cells were grown in 6 well plates until reached 80-90% confluence, quiesced overnight with serum free media. Cells were exposed to treatment (vehicle, stimulant, and/or test compound) for appropriate time. Stimulated cells washed with ice cold PBS twice then scraped and centrifuged in 500µl PBS at 13.000 rpm for 3 min. Nuclear extracts were prepared as previously described (Schreiber et al., 1989).

The pellet resuspended in 400µl buffer A(10mM HEPES (pH 7.9), 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT), and the following ingredients were added to buffer contents at day of experiment: 0.5mM PMSF, aprotinin 10µg/ml, leupeptin 10µg/ml, and pepstatin1µg/ml and incubated on ice for 15 min. 25µl of 10% (v/v) NP-40 was added to each sample and tubes vortexed at full speed for 10 seconds. Suspension were centrifuged at 13.000 rpm for 3 min, the produced pellets were suspended in 25µl of buffer B(20mM HEPES (pH7.9), 25% glycerol, 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT), and the following ingredients were added to buffer contents at day of experiment: 0.5mM PMSF, aprotinin 10µg/ml, leupeptin 10µg/ml, and pepstatin 1µg/ml, and samples were vortexed briefly to loosen cell pellet from the wall of eppendorf tube. Samples then agitated on a shaker at 4ºC for 15 min. Following this, samples were sonicated in an ice bath sonicator for 2 x 30 sec. At the end of this procedure samples were centrifuged at 13.000rpm for 15 min at 4ºC, and resultant supernatant were transferred to sterile eppendorf tubes and stored at -80 until required.

2.2.4.2 Determination of protein concentration by Bradford assay

The protein content of the nuclear extracts was quantified using BioRAD^{TM} protein assay kit based using Bradford method. For each assay performed, a standard curve was prepared using dilutions of BSA at concentration points (2, 5, 10, 20 and 30 mg/ml) as a protein standard prepared in nuclear extract buffer B. Duplicate aliquots of samples were made up to 800µl with water and completed with 200µl of concentrated protein dye reagent. Samples then were vortexed and allowed to stand for 15 min before reading the samples absorbance at 595nm using UV/Visible spectrophotometer.

2.2.4.3 Labellingof oligonucleotides with γ^{32}P-ATP

An oligonucleotide containing the consensus binding sequence for the transcription factor kappa B (NF-κB) (5`-AGTTGAGGGGACTTTCCCAGGC-3`), was labelled at the 5`- end via incubation with T4 polynucleotide kinase, nuclease free water and γ^{32}P-ATP (1µCi/µl) for 15-20 min at 37ºC. Termination of the reaction occurred by addition of 0.5M EDTA. Then the labeled oligonucleotide was diluted 1:10 with

TE buffer (10mM Tris base pH 8.0, 1mM EDTA). Efficiency of γ^{32}P-ATP incorporation into the oligonucleotide was determined by aliquoting spots of the labeled primer onto ED81 filters. Two of the filters were subjected to scintillation counting, to determine the total amount of label in each aliquote. From which the percentage of the label incorporation was determined:

Percentage incorporation= cpm incorporated X 100
cpm total

Removal of unincorporated oligonucleotide was achieved by running probe through G25 spin column at 1100x g for 4min. once the probe prepared kept at 4ºC until use.

2.2.4.4 NF-κB Transcription factors binding Assay

Non denaturing 6% EMSA gels were cast (10x TBE buffer, 2% (w/v) bis-acrylamide , 20% (w/v) acrylamide, 50% (v/v) glycerol,distilled water, 0.05% (v/v) TEMED, 10% (w/v) APS) and pre-run for 30 min at 100 V in 0.5% TBE running buffer (10xTBE buffer: 45mM Tris base, 1mMEDTA, 45mM boric acid(pH8.3)). During pre-run time of an empty gel, 5µg of nuclear extract samples were placed into eppendorf tubes and completed to a 7µl with Buffer B. 2 µl of gel shift binding 5xbuffer (20% glycerol, 2.5mM EDTA, 2.5mM DTT, 50mM Tris-HCl (pH 7.5), 250mM Nacl, 0.25 µg/ml poly (dI-dC). poly (dI-dc)), was added to samples, and left to incubate at room temperature for 20 min. 1µl (15000cpm) [γ32P]-labeled NF-κB consensus oligonucleotide was added to each sample and incubated for a further 20 min at room temperature. This step was terminated by addition of 1µl of 10x loading dye buffer (250mM Tris-HCl (pH7.5), 0.2% (w/v) bromophenol blue, 40% (v/v) glycerol). Then samples were loaded onto the non-denaturing gels, and run at 100 V for 45-50 min following this, gels were sandwiched between two cellophane sheets and dried for up to 2 h in gel dryer (70ºC). Dried gels were exposed to overnight autoradiography at -80ºC. Films were developed using Kodak X-Omat film developer. Scanning densitometry was performed to determine the level of binding of transcription factor.

2.2.5 SCANNING DENSITOMETRY

Blots were scanned on an Epson perfection 164054 Scanjet using Epson twain Scan jet Picture software. The captured images were then normalized to a control and quantified using Scion Image (Scion Corp., Maryland, USA).

2.2.6 INDIRECT IMMUNOFLUORESCENT MICROSCOPY

Cells were grown to approximately 70% confluence in 24 well plates on circular glass cover slips No.0. (0.9-0.13mm thick) (13mm diameter). Medium was replaced with serum free media, the next day cells were stimulated with an appropriate treatment. Then cover slips were washed with ice cold PBS twice, prior to fixation with 500µl ice cold methanol for 10min. Cover slips were washed twice with ice cold PBS, then were incubated with 500µl 0.25% triton x100 in BPS for 10 min at room temperature. After permealization of cells, three times of 5 min wash with ice cold PBS was applied, followed by blocking the nonspecific binding by adding 500µl of blocking buffer (1%BSA (w/v) diluted in 0.25% triton/PBS) for 30 min at room temperature. Then cover slips were incubated with primary antibody about 25µl/coverslip of rabbit polyclonal NF-κB (p65) (1:50 in blocking buffer) kept in a humidified chamber for overnight at 4ºC. Then cover slips were washed three times each for 5min with ice cold PBS followed by adding 25µl/coverslip secondary antibody Fluorescein (FITC) conjugated AffiniPure donkey anti-Rabbit (1:100) incubated for 1 h in the dark. Followed by 5min three times fixed washes with ice cold PBS, then at room temperature500µl/wellof 100ng/ml DAPI (4',6-diamidino-2-phenylindole) nuclear staining (1:2000 in PBS) was added in dark to cover slips for 5 min. Followed by twice wash with ice cold PBS, and cover slips were mounted onto glass microscope slides embedded in 15µLMowioland left to dry in cold room or at 4°C conditions overnight in the dark for visualisation by Nikon TE300-Ep-1 upright epifluorescence microscope (Nikon, Kingston upon thames, UK). Cells were imaged at x100 or x40 magnification with an oil-immersion Plan Fluor objective lens. Images were collected using a cool digital Cool Snap-HQ CCD camera (Roper Scientific, Photometrics, and Tucson, AZ). MetaMorph Imaging Series 7.0 (Molecular Devices Corp., Downington, PA, USA) was used for control of image acquisition, processing and modification of all image data. The background average

statistical correction editing function in MetaMorph was used to produce background corrected images. This was achieved by determining the average background level of fluorescence from regions of interest drawn adjacent to cells expressing fluorescence.

2.2.7 MTT ASSAY FOR CELL VIABILITY

Cells were seeded into 96-well plates at a density of 5×10^4 cells/well, and incubated with serum-free medium (in the case of NCTC2544 cells) in the presence of different concentrations of test compound. After incubation for 24 h, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5mg/ml) was added and incubation continued for another 2 h. Mitochondrial succinate dehydrogenase in live cells converts MTT to visible Formazan blue crystals during incubation. The medium was then decanted from wells cautiously and Formazan crystals solubilised in 100µl dimethylsulfoxide (DMSO) (Mosmann, 1983). The absorbance was measured at 570nm using microplate reader (DYNEX technologies, USA). The relative cell viability was calculated and compared with the absorbance of the untreated control group. All experiments were performed in triplicate.

2.2.8 MEASUREMENT OF NITRIC OXIDE (NO) PRODUCTION

The nitrite concentration in culture medium was measured as an indicator of NO production, according to the Griess reaction (Griess, 1879). RAW264.7 cells (5×10^5 cells/well) were cultured in 12 well plates in DMEM overnight and pretreated with different concentrations of test compound for 30 min. Cellular NO production was induced by adding a 1 µg/mL of LPS and incubating for 24 h. Next, 50 µl of supernatant medium was mixed with an equivalent volume of Griess reagent (1:1 mixture (v/v) of 2% (w/v) sulfanilamide, 0.2% (w/v) naphthyl ethylenediamine dihydrochloride and 5% phosphoric acid) and incubated for 10 min at room temprature. The absorbance of the sample was read on a Spectromax 190 plate reader at 540nm. The values obtained were compared with those of standard concentration curve of sodium nitrite (NaNO2; 10mM) dissolved in DMEM, and the concentrations of nitrite in the conditioned media of sample-treated cells calculated.

2.2.9 DATA ANALYSIS

Statistical comparison between groups were performed by byGraphPad Prism program using one way ANOVA followed by Dunnett's or knewman-keul tests as appropriate. Densitometry data generated from immunoblots were expressed as mean ±s.e.m. for at least three separate observations. Difference were considered significant when $p < 0.05$.

CHAPTER 3

EFFECTS OF A DNA MINOR GROOVE BINDER COMPOUNDS ON NF-ĸB SIGNALLING IN NCTC2544 CELLS AND RAW 264.7 MACROPHAGES

3.1 Introduction

Several well characterised signalling pathways are known to be involved in the development of many inflammatory diseases (Sebolt-Leopold and English, 2006), (Karin and Greten, 2005), (Folmer et al., 2008). The NF-κB pathway in particular, is activated by extracellular signals such as TNF-α (Osborn et al., 1989), IL-1β, LPS, UV light and phorbol esters (Sen and Baltimore, 1986). Signal transduction pathways linking, for example, the TNF-α receptor to NF-κB activation have been extensively studied (Ghosh et al., 1998) and have been reviewed in Chapter 1 of this thesis.

In order to assess the effect of NF-κB inhibitors several approaches using different assay systems can be used. A cell system in which activation of NF-κB can be studied is the human keratinocyte, a major cell type in the epidermis that plays a key role in skin inflammatory and immunological reactions due to their anatomical location (Barker et al., 1991). Keratinocytes are able to produce different inflammatory mediators and are actively engaged in skin inflammatory responses by attracting leukocytes and modulating their functions. These activities are particularly manifest during chronic inflammatory skin disorders, such as allergic contact dermatitis, psoriasis and atopic dermatitis (Berardesca and Distante, 1995).

NF-κB is regulated by two major kinases known as the inhibitory kappa B kinases or IKKs. In particular, IKKβ has been implicated in TNF-α-mediated inflammatory events in the skin (Pasparakis et al., 2002). IKK represents an intriguing target for the development of novel therapies in the treatment of inflammatory disorders since the transcription of many cytokine and adhesion molecules which mediate the pathogenesis in these disorders are dependent on NF-κB. An epithelial NCTC2544 cells stably expressing human proteinase activated receptor-2 (PAR2) (clone G) (Kanke et al., 2001) and NF-κB or AP-1 linked reporter plasmids (Macfarlane et al., 2005) were used in experiments described in this Chapter. As these pathways have been well characterised in this cell type it was chosen to evaluate the induction of NF-κB transcriptional activity mediated by TNF-α and PMA in the absence and presence of inhibitory molecules. In addition, the AP-1 reporter cells were used to assess the specificity of inhibition and the potential for off-target effects, because

AP-1 reporter cells were only responded to PMA but not to TNF-α, therefore, TNF-α stimulation was only limited to NF-κB studies. Subsequent to this, different steps involved in TNF-α mediated NF-κB pathway stimulation were investigated. At the cytoplasmic level, Western blot analysis was used to characterise the effect of TNF-α on different NF-κB pathway components such as IκBα degradation and NF-κB (p65) subunit phosphorylation. Furthermore, the characteristic of nuclear translocation of free cytoplasmic NF-κB (p65) subunit was investigated using indirect immunoflourescence. Finally the binding between heterodimers of NF-κB (p50/p65) and DNA was assessed by electrophoretic mobility shift assay (EMSA).

Using these approaches the effects of AIK18/85/1 (Figure 3.1) and AIK18/70 (Figure 3.18), DNA minor groove binders (MGBs), were examined to pinpoint the site and the mechanism of inhibition. AIK18/85/1 is structurally related to distamycin A which belongs to a class of naturally occurring oligopeptide antibiotics with varying degrees of antiviral and antitumor activities (Hahn, 1975). However, other studies (in vitro IKK assay) conducted in-house showed AIK18/85/1 to have IKK inhibitory activity and thus may be a multifunctional inhibitor. Furthermore, since RAW246.7 macrophages have been used widely for the screening of anti-inflammatory drug and evaluating the inhibitory effect of compounds on inflammatory mediators (Kim et al., 2009, Kim et al., 2008), AIK18/85/1 was examined for its effects on LPS-induced iNOS and COX-2 expression as key markers of an inflammatory response in this cell line.

3.2 Characterisation of the effect of TNF-α-induced NF-κB-linked luciferase reporter activity in NCTC2544 cells.

In order to activate the canonical NF-κB pathway, TNF-α (10ng/ml) was used to stimulate NF-κB-linked luciferase reporter activity in the human epithelial derived NCTC2544 cells (see Section 2.2). Initially a time course was established for TNF-α stimulation up to 6 h for optimum transcription (Figure 3.2a). After a delay of 1 h, activity rose to approximately 10 fold above basal value by 6 h (11.1 ± 1.9 fold $p < 0.01$). TNF-α also caused a concentration dependent increase in the transcriptional activity over the 1 - 20ng/ml range at 6 h stimulation (True et al., 2000) (Figure

3.2b). About 8 fold stimulation above basal values was the maximum induction achieved using 20ng/ml TNF-α (8.9 ± 0.7 fold, $p < 0.01$). Moreover, a significant increase in reporter activity was also observed at 10ng/ml (6.3 ± 0.6 fold, $p < 0.01$).

3.3 Characterisation of the effect of PMA-induced NF-κB-linked luciferase reporter activity in NCTC2544 cells.

In order to study the activation of the pathway by other agents, the PKC activator PMA was also used to stimulate NF-κB-linked luciferase reporter in NCTC2544 cells for up to 6 h. Again time course and concentration response curve for PMA-induced NF-κB reporter activity were established. The time course in Figure 3.3a showed that treatments of PMA at 4 and 6 h both resulted in significant induction, with more than a 100 fold increase at 6 h (120.5 ± 22.8 fold, $p < 0.01$). A concentration curve for PMA between 0.3-100nM for 6 h stimulation (Hatziieremia et al., 2006) gave a strong increase in reporter activity over the 3-100nM range. A maximal stimulation (greater than 100 fold) was achieved between 30-100nM, (141.2 ± 43.9 fold at 100nM, ($p < 0.05$); Figure 3.3b).

3.4 Characterisation of the effect of PMA-induced AP-1-linked luciferase reporter activity in NCTC2544 cells.

PMA was used to stimulate AP-1-linked luciferase reporters using human epithelial derived NCTC2544 cells (Section 2. 2) which had been stably transfected with AP-1 promoter plasmid (Macfarlane et al., 2005). Firstly, a PMA time course for up to 6 h (Figure 3.4a) was established as well as a concentration curve up to 300 nM (Figure 3.4b) in order to choose the optimal concentration for activation of AP-1 activity. The time course in Figure 3.4a shows that PMA caused a significant induction of AP-1 activity between 4-6 h (5.0 ± 0.3 fold increase at 6 h, $p < 0.01$). As shown in Figure 3.4b PMA promoted a concentration dependent increase in reporter activity after 6 h which was observed over the 1-300nM range. Statistically significant induction of AP-1 transcriptional activity was obtained at concentrations of 10nM and above. Stimulation with 30, 100 and 300nM caused significant AP-1 activation above the basal value of 4.7 ± 0.4, 4.8 ± 0.3, and 4.7 ± 0.6 fold, respectively ($p < 0.01$).

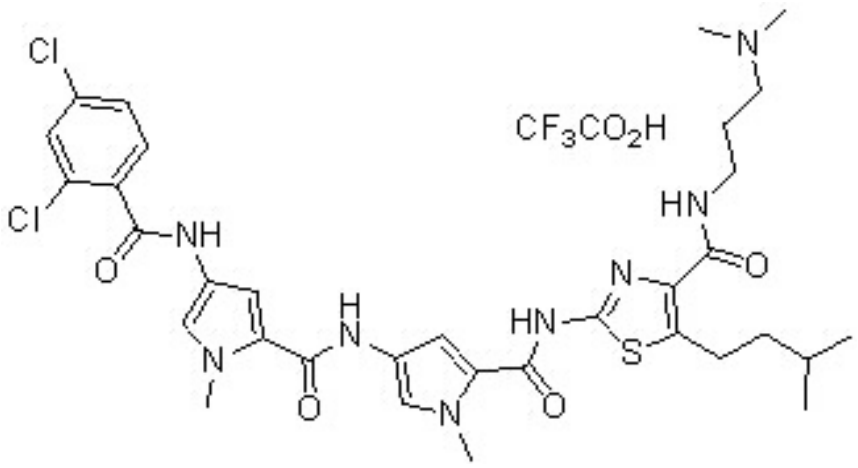
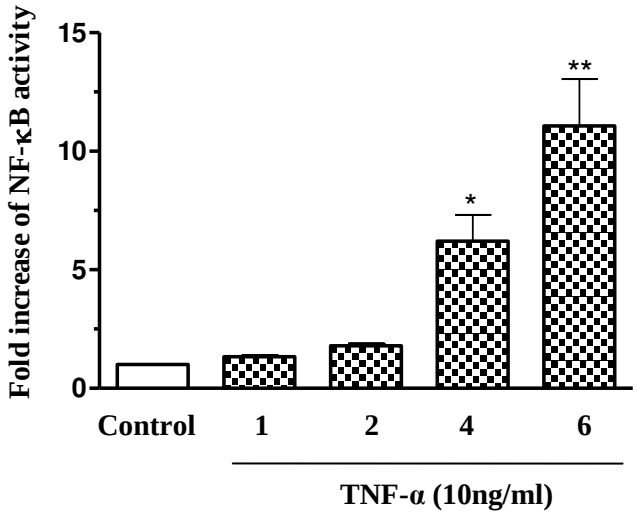


Figure 3.1: Chemical structure and systematic name of AIK 18/85/1. 2-[({4-[({4-[(2,4-dichlorobenzoyl)amino]-1-methyl-1H-pyrrol-2-yl}carbonyl)amino]-1-methyl-1H-pyrrol-2-yl}carbonyl)amino]-N-[3-(dimethylamino)propyl]-5-isopentyl-1,3-thiazole-4-carboxamide trifluoroacetate

a)



b)

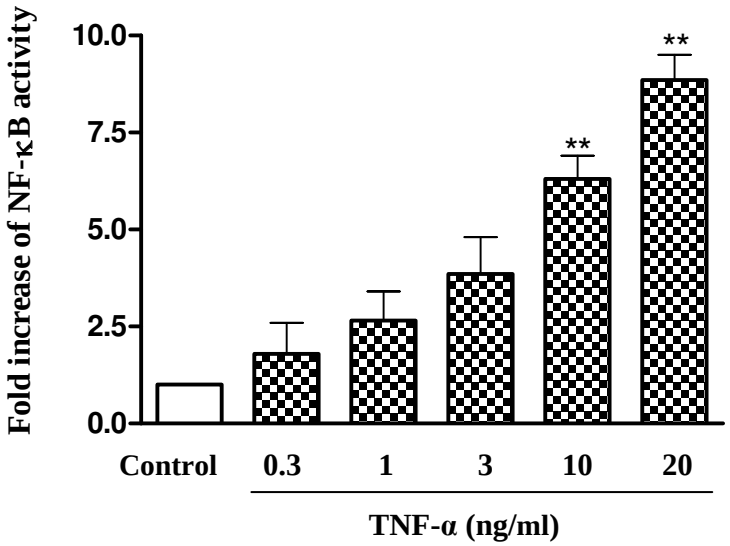
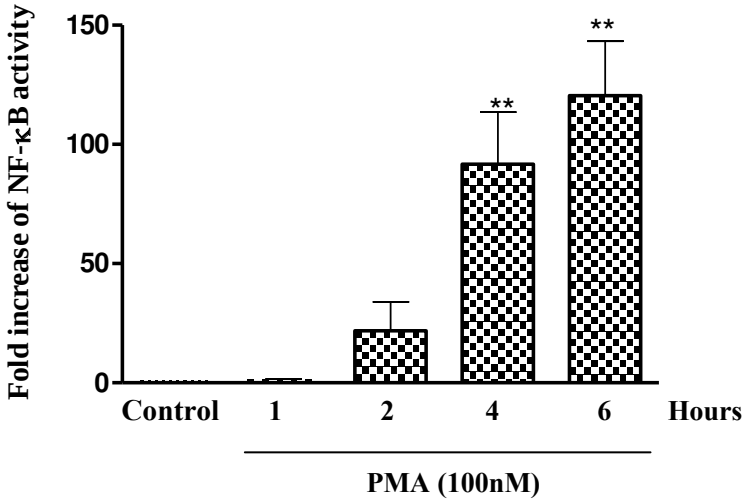


Figure 3.2: Time course and concentration response curve for TNF-α-mediated NF-κB-linked luciferase reporter activity in NCTC2544 cells. Cells were treated with TNF-α (10ng/ml) for up to 6 h (panel **a**), or with increasing concentrations of TNF-α (0.3-20ng/ml) for 6 h (panel **b**). NF-κB activity was assessed by luciferase assay as described in Section 2.2.2. Each value represents the mean ± s.e.m. of at least three independent experiments, * = $p < 0.05$ and ** = $p < 0.01$ compared with untreated control group.

a)



b)

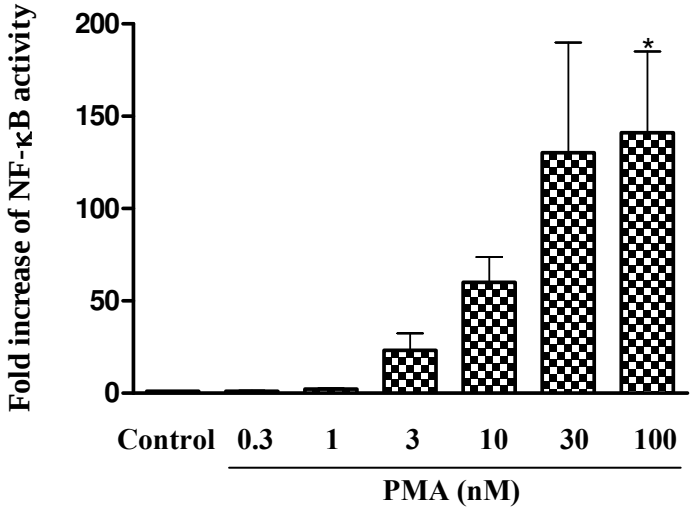
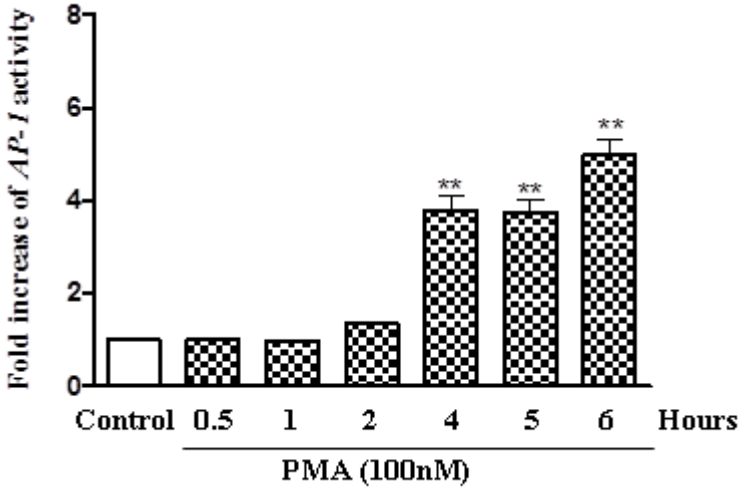


Figure 3.3: Time course and concentration response curve for PMA-mediated NF-κB-linked luciferase reporter activity in NCTC2544 cells. In panel **(a)** cells were treated with (100nM) PMA for up to 6 h. In panel **(b)** cells were treated with increasing concentrations of PMA (0.3-100nM) for 6 h. NF-κB activity was assessed by luciferase assay as outlined in Section 2.2.2. Each value represents the mean ± s.e.m. of three independent experiments, * = $p < 0.05$ and ** = $p < 0.01$ compared with untreated control group.

a)



b)

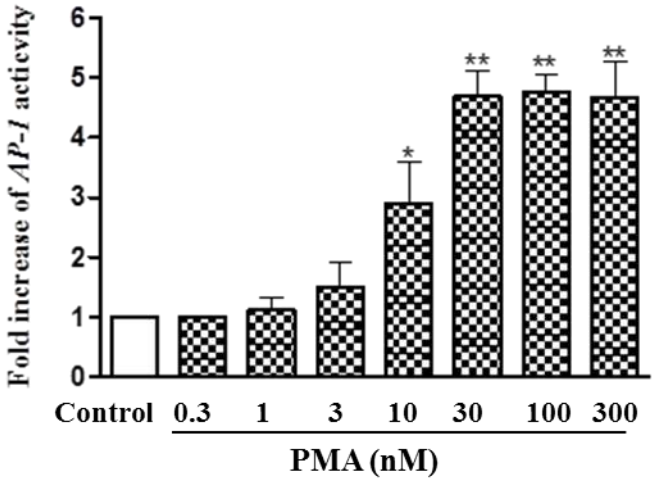


Figure 3.4: Time course and concentration response curve for PMA-mediated AP-1-linked luciferase reporter activity in NCTC2544 cells. In panel **(a)** cells were treated with 100nM PMA in 96 well plates for up to 6 h. In panel **(b)** cells were treated with increasing concentrations of PMA (0.3-300nM) for 6 h. AP-1 activity was assessed by luciferase assay as described in Section 2.2.2. Each value represents the mean ± s.e.m. of two and three independent experiments respectively, * = $p < 0.05$ and ** = $p < 0.01$ compared with untreated control group.

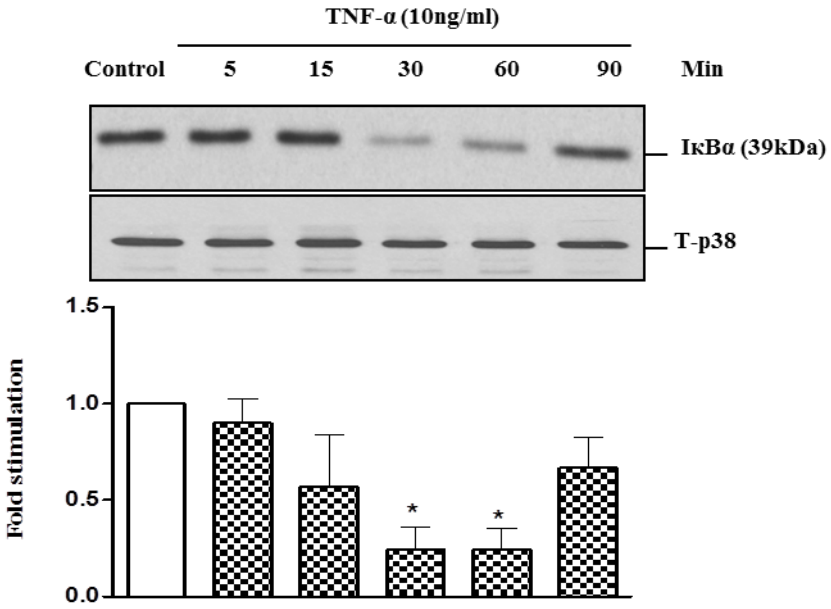
3.5 Characterisation of the effect of TNF-α-mediated IκBα degradation in NCTC2544 cells.

Since degradation of IκBα protein is a key step in the activation of canonical NF-κB signaling, the effect of TNF-α upon IκBα loss was also investigated in NCTC2544 cells. Stimulation resulted in rapid loss in IκBα which was maximal at 30 min and returned towards basal levels after 90 min as shown in Figure 3.5a. Examination of the concentration dependent effects demonstrated that TNF-α did not cause IκBα loss in concentrations between 1-5ng/ml, but caused a substantial degradation of IκBα at 10 and 20ng/ml (0.4 ± 0.2, 0.4 ± 0.3 folds), respectively (Figure 3.5b). From these results, TNF-α at a concentration of 10ng/ml was used to induce NF-κB signalling in all subsequent experiments.

3.6 Effects of MG-132 on TNF-α-induced NF-κB-linked luciferase reporter activity and TNF-α-mediated IκBα loss in NCTC2544 cells.

In order to verify the validity of NF-κB reporter assay for testing the inhibitory effects of compounds it was necessary to examine the effects of the MG-132 (Ortiz-Lazareno et al., 2008), a proteosome inhibitor which inhibits proteosomal degradation of IκBα in NCTC2544 NF-κB reporter cells. As shown in Figure 3.6a, TNF-α-stimulation caused 14 fold increases in NF-κB reporter activity at 6 h (14.1 ± 4.1 fold). Pretreatment with MG-132 essentially abolished reporter activity at all concentrations tested (1-30μM) (all $p < 0.01$). Since the cytosolic IκBα degradation is a proteosome dependent step in the TNF-α induced NF-κB pathway, the effect of MG-132 on IκBα loss was also examined. IκBα loss mediated by TNF-α stimulation (0.11 ± 0.005 fold) was significantly reversed by MG-132 in a concentration dependent manner (30, 20, and 10μM 1.1 ± 0.3, 1.0 ± 0.2 and 0.95 ± 0.14 fold respectively, all $p < 0.05$) as shown in Figure 3.6b. MG-132 inhibitory effect against TNF-α induced NF-κB-linked reporter activity was also very clear at all concentrations used during the experiment.

a)



b)

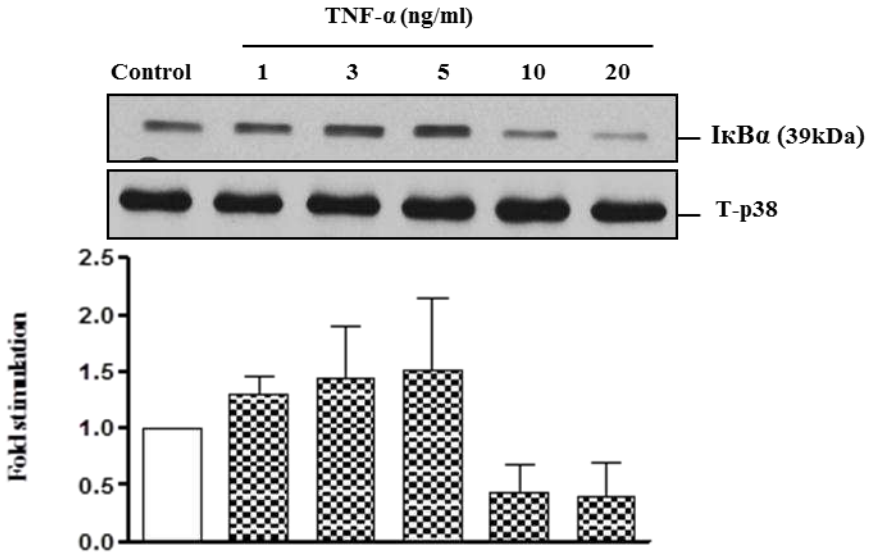
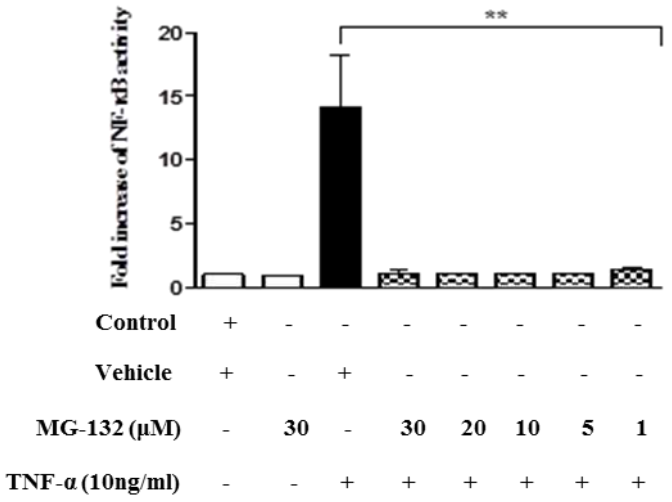


Figure 3.5: Time course and concentration response curve for TNF-α-induced IκBα loss in NCTC2544 cells. Panel **(a)** cells were stimulated with TNF-α (10 ng/ml) for a period of up to 90 min, and with increasing concentrations 1-20 ng/ml of TNF-α for 30 min (panel **b).** Whole cell lysates were prepared, separated by SDS-PAGE, then assessed for IκBα protein (39 kDa) and re-probed for total p38-MAP kinase as outlined in Section 2.2.3. The results are representative for three independent experiments and each value represents the mean ± s.e.m. * = $p < 0.05$ compared to control.

a)



b)

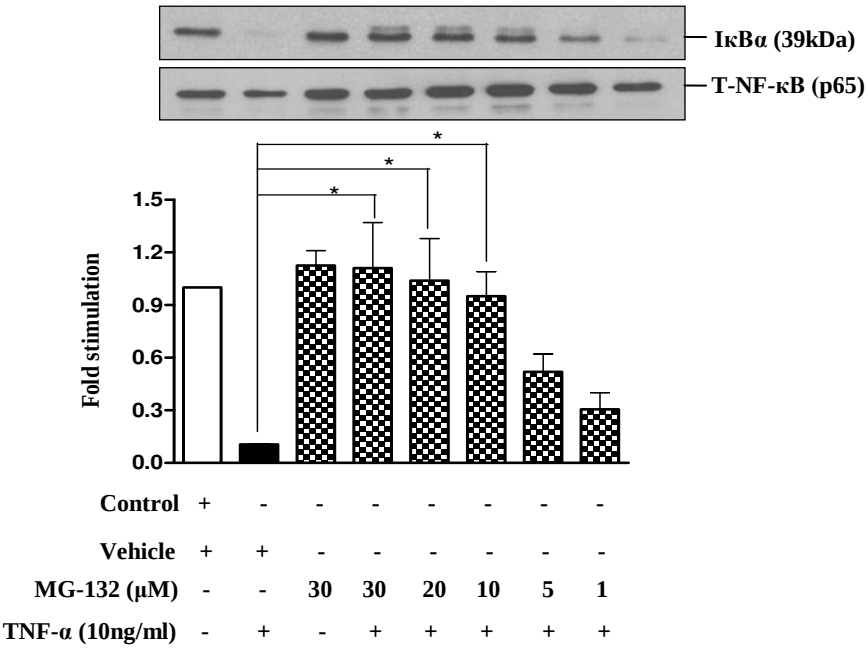


Figure 3.6: Effects of MG-132 on TNF-α-induced NF-κB-linked luciferse reporter activity and IκBα degradation in NCTC2544 cells. Cells were serum starved overnight and treated with MG-132 (1-30μM) for 30 min prior their stimulation with TNF-α (10 ng/ml) for 6 h in panel **a** and for 30 min in panel **b**. Treated cells then assayed for luciferase reporter activity as in panel **a** while in panel **b** whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for IκBα protein (39 kDa) and total-NF-κB (p65) as outlined in Section 2.2.3. Each value represents the mean ± s.e.m. of three independent experiments.* = $p < 0.05$, ** = $p < 0.01$ compared with stimulated control.

3.7 Effects of AIK18/85/1 on TNF-α, PMA-induced NF-κB- and PMA-induced AP-1-linked luciferase reporter activities in NCTC2544 cells.

The compound AIK18/85/1 (Figure 3.1) is one of the thiazole derivative peptidomimetics (DNA minor groove binder) that could be expected to have a broad range of effects on the transcriptional activity of different promoters. Having established that TNF-α (10ng/ml) stimulation in NCTC2544 cells caused an increase in NF-κB reporter activity, the effect of AIK18/85/1 (0.3-30μM range) on stimulated cells was examined. This concentration range was chosen according to IKK assays carried out in-house, showing that the IC_{50} of AIK18/85/1 and the other tested compounds are < 30 μM. Thus, in luciferase reporter assay concentration range between 0.3-30μM will be examined for all compounds investigated in this thesis. TNF-α (10ng/ml) produced 14 fold increase in NF-κB-linked luciferase activity in a reporter assay (13.6 ± 4.3 fold), whereas pretreatment with AIK18/85/1 compound prior to TNF-α addition caused a significant reduction in transcriptional activity. Inhibition was particularly marked at 30μM (1.9 ± 0.95 fold, $p < 0.05$), and a significant decrease in NF-κB transcriptional activity was also observed at 10μM (3.5 ± 1.04 fold, $p < 0.05$) of the compound as displayed in Figure 3.7a.

In addition AIK18/85/1 was tested against PMA stimulated NF-κB transcriptional activity. Whilst PMA alone gave a response of about 100-fold induction compared to untreated cells, AIK18/85/1 caused a significant and greater than 90% inhibition at a concentration of 30μM (from 102.9 ± 21.4 fold to 10.6 ± 4.5 fold, $p < 0.001$). Furthermore, AIK18/85/1 caused a marked significant inhibition of approximately 50-60% of PMA-induced NF-κB transcriptional activity at all concentrations applied ($p < 0.05$) (Figure 3.7b).

However, PMA-induced AP-1 transcriptional activity was greatly affected by the AIK18/85/1 compound as shown in Figure 3.7c. The AP-1 transcriptional activity induced by PMA was approximately 6 times above basal transcriptional activity (5.5 ± 0.34). AIK18/85/1 caused complete inhibition since it significantly reduced AP-1 activity to almost basal transcription level at 30μM (1.0 ± 0.06 fold, $p < 0.001$) and

to less than 50% of maximum activity using 10μM of the compound (2.6 ± 0.9 fold, $p < 0.001$).

3.8 Effect of AIK18/85/1 on IκBα degradation induced by TNF-α in NCTC2544 Cells.

In order to precisely indicate the site of action of AIK18/85/1, the compound was tested against IκBα degradation induced by TNF-α. According to results obtained in TNF-α-induced luciferase reporter activity, the range of concentration of AIK18/85/1 to be examined onward was limited within the effective concentrations 3-30μM. As demonstrated in Figure 3.8, a 30 min pretreatment of NCTC2544 cells with increasing concentrations of AIK18/85/1 (3, 10, and 30μM) prior to TNF-α stimulation for 30 min failed to reverse the IκBα loss caused by TNF-α. Thus, examinations of the effect of compound on subsequent steps in the signalling pathway become obligatory.

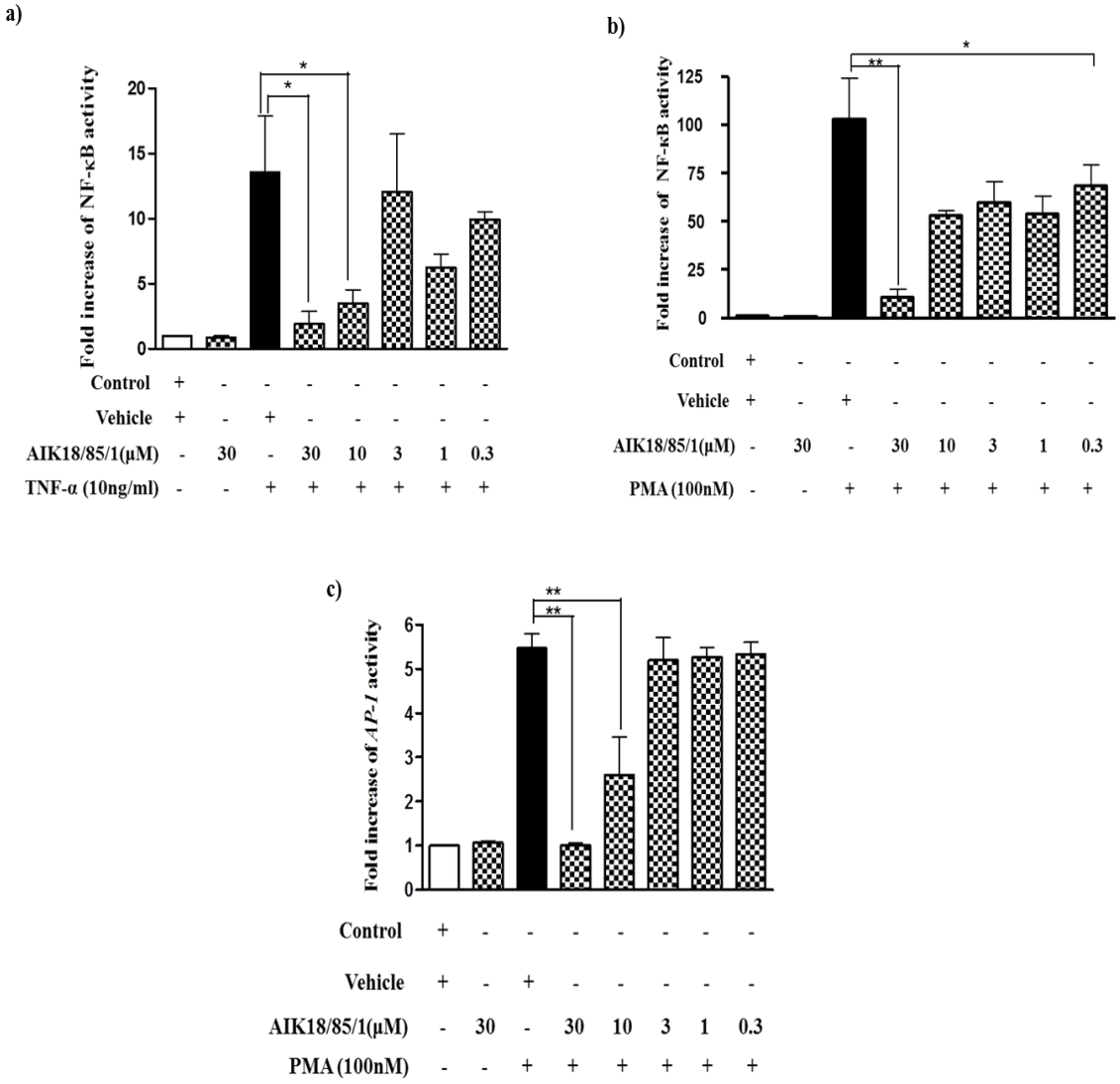


Figure 3.7: Effects of AIK18/85/1 on NF-κB- and AP-1-linked luciferase reporter activities in NCTC2544 cells. Cells were serum starved overnight then treated with AIK18/85/1 (0.3-30μM) for 30 min prior to 6 h stimulation with TNF-α (10ng/ml) in panel **a** and with PMA (100nM) in panels **b** and **c**. Lysed cells were then assayed for NF-κB transcriptional activity in **a** and **b** and for AP-1 transcriptional activity in **c** as outlined in Section 2.2.2. Each value represents the mean ± s.e.m. of three independent experiments, * = $p < 0.05$ and ** = $p < 0.01$ compared with stimulated control group.

a)

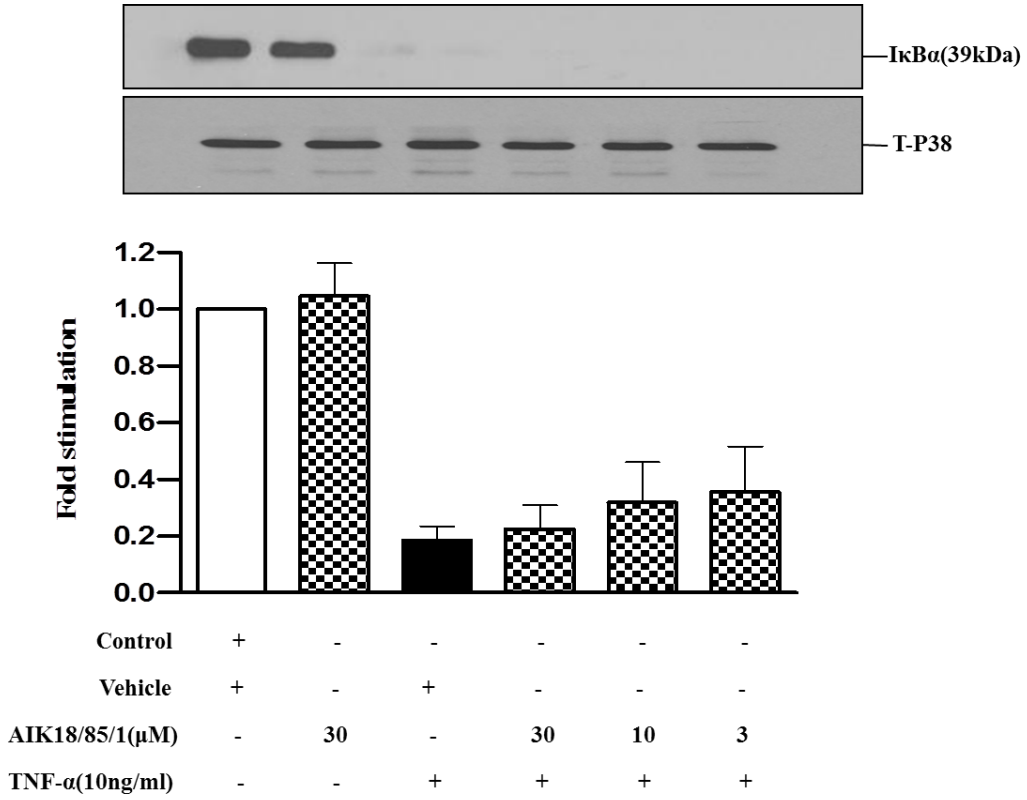


Figure 3.8: Effect of AIK18/85/1 on TNF-α-induced IκBα degradation in NCTC2544 cells. Cells were treated with AIK18/85/1 (3-30μM) for 30 min prior to stimulation with TNF-α (10ng/ml) for a further 30 min. Whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for IκBα protein (39kDa) and re-probed for total p38 MAPK (38kDa) as outlined in Section 2.2.3. The results are representative of three independent experiments and each value represents the mean ± s.e.m.

3.9 Characterisation of the effect of TNF-α on phosphorylation of NF-κB (Ser536-p65) in NCTC2544 cells.

NF-κB (p65 or RelA) is one of the NF-κB components that can be phosphorylated at different serine residues in response to TNF-α stimulation. Among them serine 536 can be phosphorylated due to upstream IKK activation (Sakurai et al., 2003). NCTC2544 cells exposed to TNF-α (10ng/ml) over a period of 2 h, were analysed for phosphorylation of NF-κB (p65) subunit by Western blotting. Figure 3.9 shows that TNF-α caused NF-κB (p65) activation as early as 10 min with a maximum stimulation being reached (16.1 ± 7.7 fold above the basal level) at 15 min. Phosphorylation then declined towards basal values by 60 min.

3.10 Effect of AIK18/85/1 on TNF-α-induced phosphorylation of NF-κB (Ser536-p65) in NCTC2544 cells.

The effect of AIK18/85/1 (3-30μM) on TNF-α induced NF-κB (p65) phosphorylation was examined. As shown in Figure 3.10, TNF-α stimulation lead to a strong increase in Ser536 phosphorylation of the p65 subunit. This response was not affected by AIK18/85/1 pretreatment at any of the concentrations tested. These data suggested that AIK18/85/1 was unlikely to have an inhibitory effect on IKKs as initially hypothesised. Experiments were conducted to pinpoint the sites of action of AIK18/85/1 downstream in the NF-κB pathway.

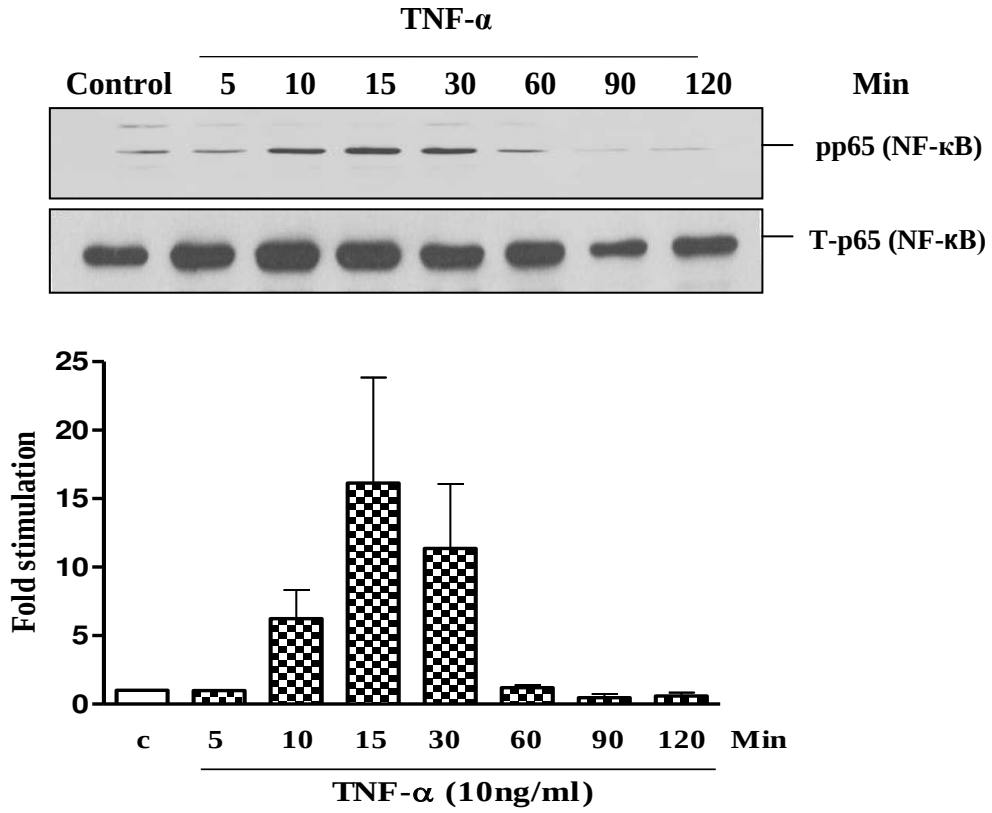


Figure 3.9: Time course of TNF-α-induced NF-κB (Ser536-p65) phosphorylation in NCTC2544 cells. Cells were treated with TNF-α (10ng/ml) over a period of 2 h. Whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for phospho-NF-κB (Ser536-p65) and blot was re-probed for total-NF-κB (T-p65) as outlined in Section 2.2.3. The results are representative for three independent experiments and each value represents the mean ± s.e.m.

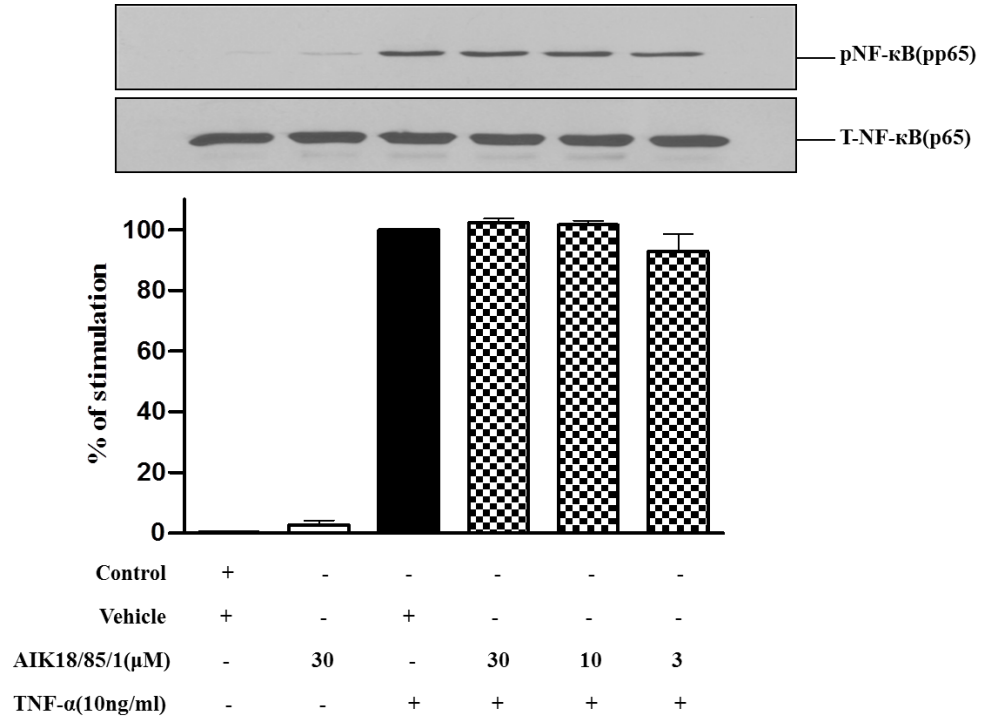


Figure 3.10: Effect of AIK18/85/1 on TNF-α-induced NF-κB (Ser536-p65) phosphorylation in NCTC2544 cells. Cells were treated with 3-30μM AIK18/85/1 for 30 min prior to stimulation with TNF-α (10ng/ml) for a further 30 min. Whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for phospho-NF-κB (p65-Ser536) or total NF-κB (T-p65) as outlined in Section 2.2.3. The results are representative of three independent experiments and each value represents the mean ± s.e.m.

3.11 Characterisation of the effects of TNF-α and PMA on NF-κB (p65) nuclear translocation in NCTC2544 cells.

The entry of freed NF-κB (p65) into the nucleus is a critical step in the NF-κB signalling pathway. Therefore, NCTC2544 cells were treated with either TNF-α (10ng/ml) or PMA (100nM) for up to 2 h (Gossye et al., 2009), then assayed by indirect immunofluorescence staining to detect the localisation of NF-κB (p65) within cell compartments (i.e. cytoplasm and nucleus). Figure 3.11a shows that TNF-α caused nuclear translocation of NF-κB (p65) at 30 min and subsequently returned back to the cytoplasm by 90 min. On the other hand, PMA-induced nuclear translocation was observed as late as 90 min (Figure 3.11b). These two time points were used to assess the effects of AIK18/85/1 on TNF-α and PMA responses in subsequent experiments.

3.12 Effect of AIK18/85/1 on TNF-α-mediated NF-κB (p65) translocation into the nucleus of NCTC2544 cells.

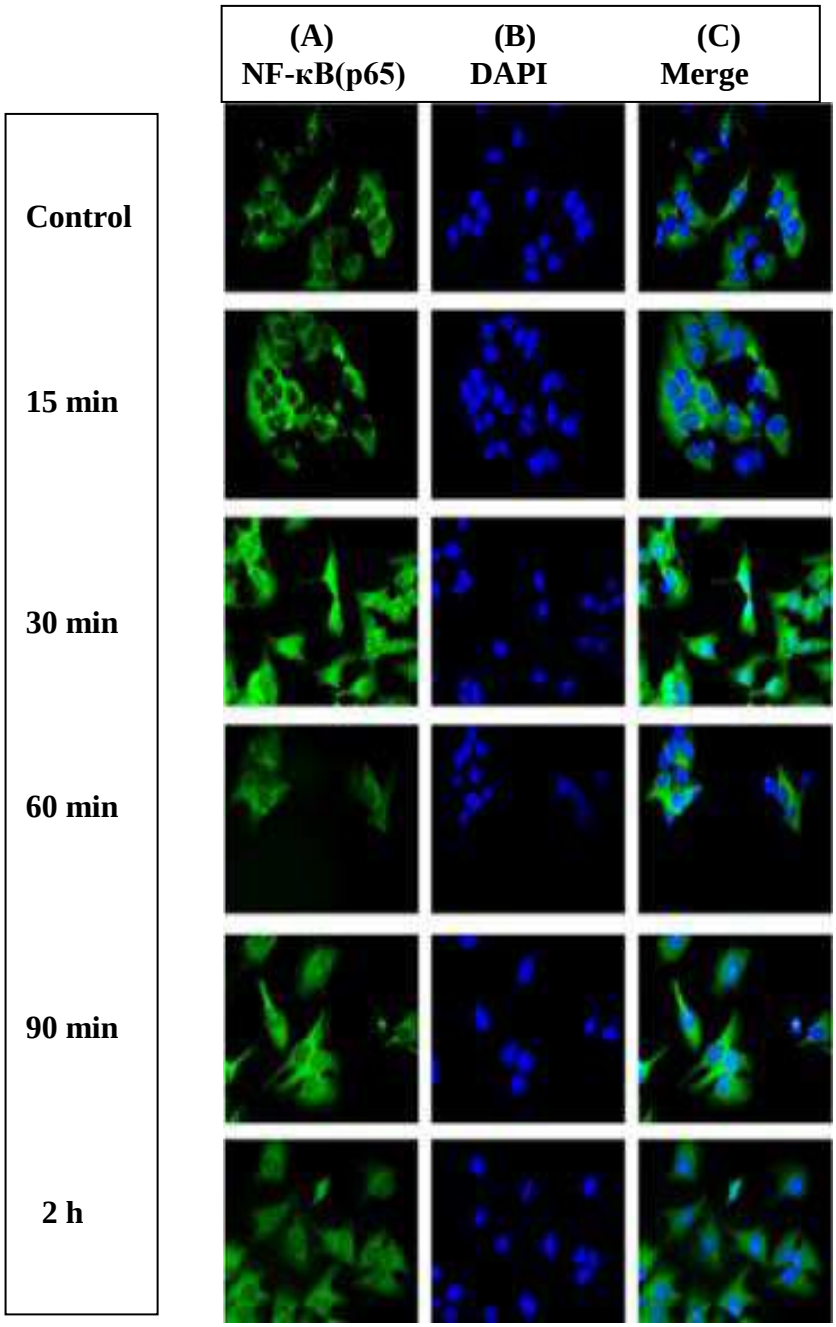
As can be seen in Figure 3.12, in resting cells NF-κB was found to be located strictly within the cytosol with only little co-staining along with DAPI in the nuclear compartment. TNF-α induced NF-κB (p65) translocation to the nucleus was marked after 30 min stimulation in NCTC2544 cells. In contrast pretreatment of cells with AIK18/85/1 resulted in a marked attenuation of p65 translocation in response to the TNF-α stimulation, particularly at 30μM. This effect compared favourably with the inhibitory effect produced by 50μM cardamonin (data not shown). However, a lesser effect of AIK18/85/1 on translocation was also observed at 10μM, when a punctuate staining was observed in the nuclear compartment.

3.13 Effect of AIK18/85/1 on PMA-mediated NF-κB (p65) translocation into the nucleus in NCTC2544 cells.

Parallel to the effect of AIK18/85/1 on TNF-α induced NF-κB (p65) nuclear translocation, the action of the compound on PMA-mediated translocation was also examined. PMA alone stimulated a strong, almost complete translocation of p65 to the nucleus with good co-localisation with DAPI. In contrast to the effect on TNF-α-

induced p65 translocation, AIK18/85/1 did not show any effect at the concentrations utilised (3-30μM).

a)



b)

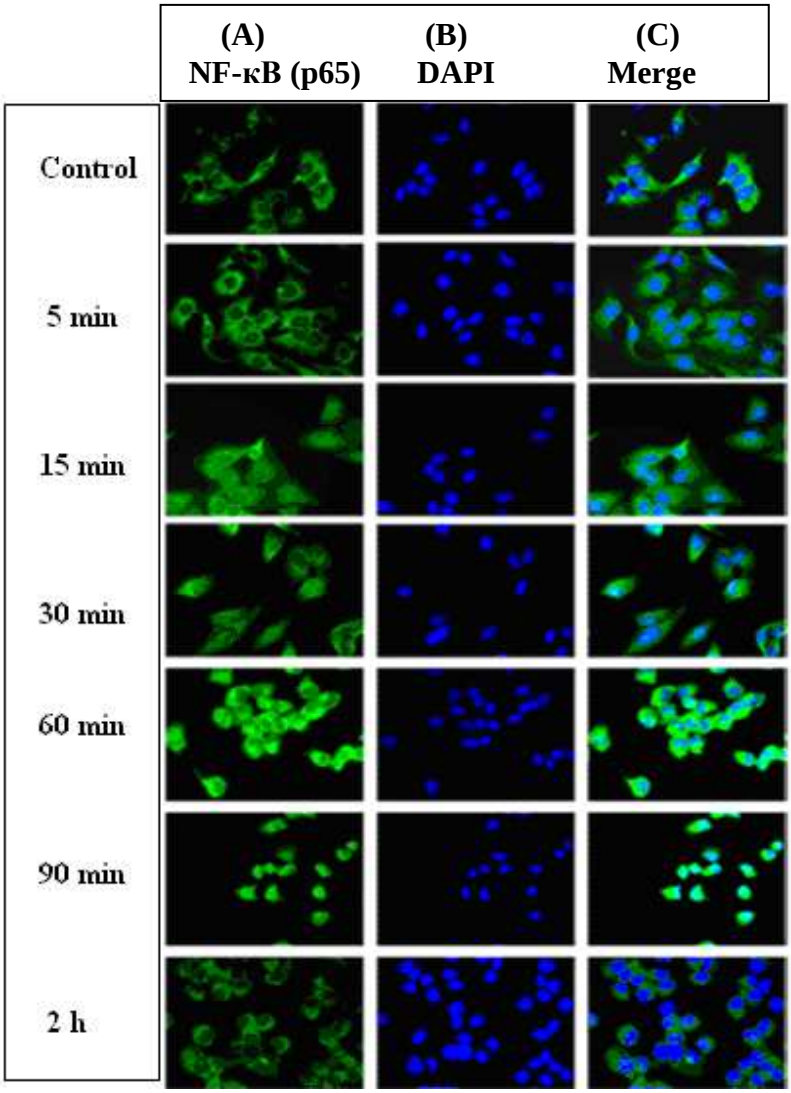


Figure 3.11: Time course for TNF-α and PMA-induced NF-κB(p65) nuclear translocation in NCTC2544 cells. Quiescent cells grown on cover slips were incubated for varying times over a period of 2 h with TNF-α (10ng/ml) in panel **a** or PMA (100nM) in panel **b.** Nuclear translocation of NF-κB was then evaluated by indirect immunofluorescence. **(A)** NF-κB localisation was visualised using an anti-NF-κB (p65) antibody and FITC-linked secondary antibody (green). **(B)** Nuclei were visualised with DAPI counterstaining (blue). **(C)** Merge of the two fluorescence images (green and blue). Images were acquired using fluorescence microscopy at 40x magnification (scale bar = 25μm). The results are representative of three independent experiments.

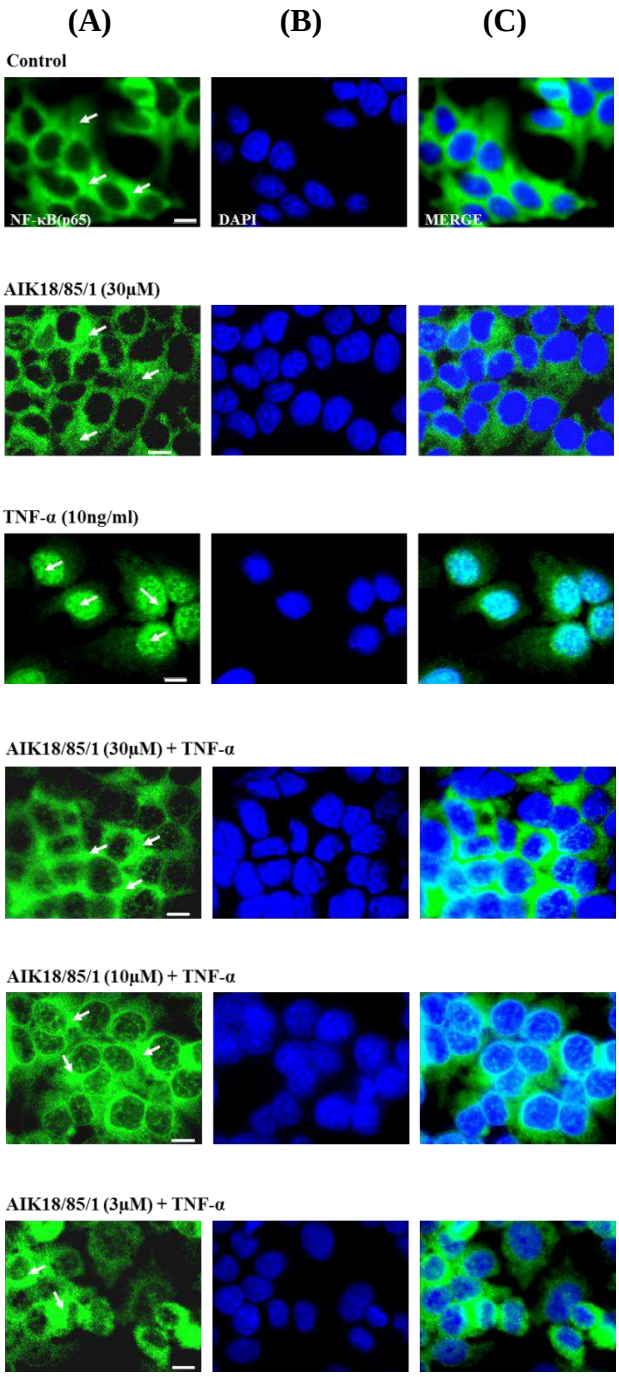


Figure 3.12: Effect of AIK18/85/1 on TNF-α-induced NF-κB (p65) translocation into the nucleus in NCTC2544 cells. Quiescent cells grown on cover slips up to 70% confluence were treated with AIK18/85/1 at concentrations (3-30μM) for 30 min prior to TNF-α stimulation for a further 30 min then evaluated by indirect immunofluorescence **(A)** NF-κB localisation was visualised using an anti-NF-κB (p65) antibody FITC-linked secondary antibody (green). **(B)** Nuclei were visualised by DAPI counterstaining (blue). **(C)** Merge of the two fluorescence images (green and blue). White arrows indicate the areas of p65 localisation. Images were acquired using fluorescence microscopy at 100x magnification (scale bar =10 μm). The results are representative of three independent experiments.

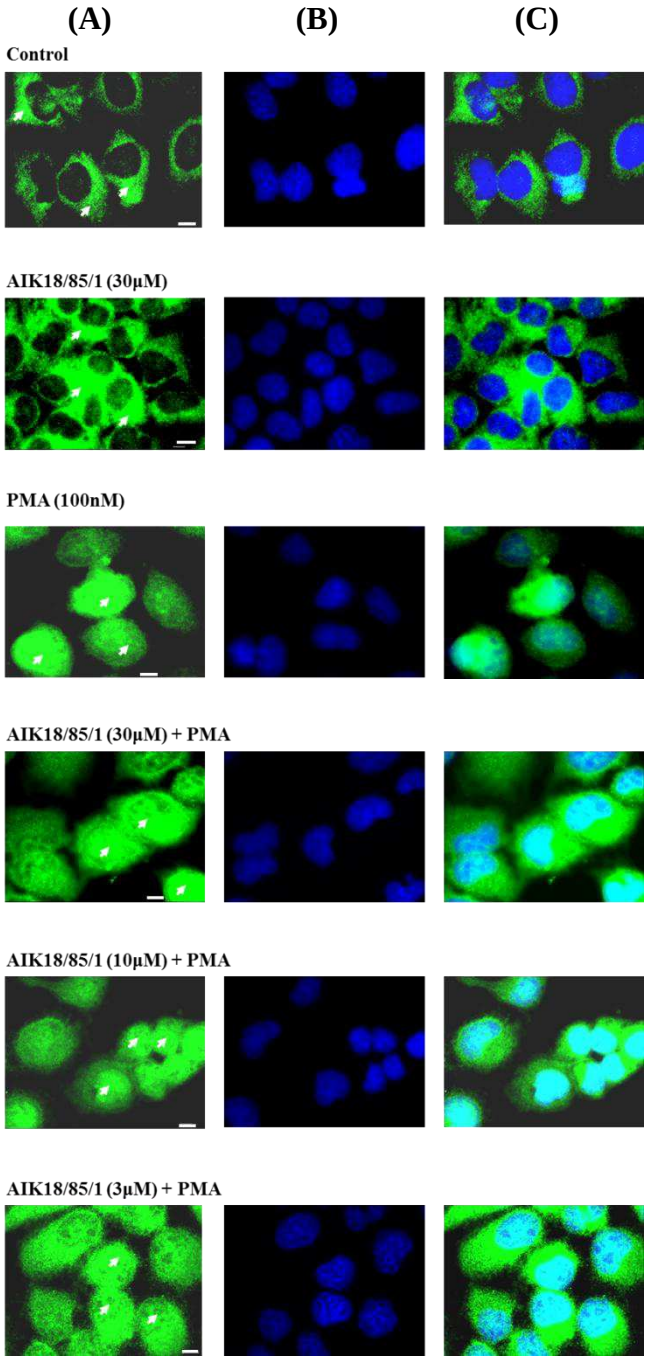


Figure 3.13: Effect of AIK18/85/1 on PMA-induced NF-κB (p65) translocation into the nucleus in NCTC2544 cells. Quiescent cells grown on cover slips were treated with AIK18/85/1 at concentrations (3-30µM) for 30 min prior to PMA (100nM) stimulation for a further 90 min then evaluated by indirect immunofluorescence **(A)** NF-κB localisation was visualised using an anti-NF-κB (p65) antibody and FITC-linked secondary antibody (green). **(B)** Nuclei were visualised by DAPI counterstaining (blue). **(C)** Merge of the two fluorescence images (green and blue). White arrows indicate the areas of p65 localisation. Images were acquired using fluorescence microscopy at 100x magnification (scale bar =10 µm). The results are representative of at least three independent experiments.

3.14 Characterisation of TNF-α and PMA-induced NF-κB-DNA binding activity in NCTC2544 cells.

Activation of NCTC2544 cells by TNF-α will promote dissociation of NF-κB hetero-dimer (p50/p65) from IκBα within the cytoplasm; this will be followed by translocation to the nucleus and interaction with specific κ binding sites on the promoters of several genes. This interaction was observed in Figure 3.14a when cells were stimulated with TNF-α (10ng/ml) for a period of up to 90 min, nuclear extracts prepared and then analysed by EMSA. Appearance of the NF-κB-DNA binding complex was gradual, starting as early as 5 min following stimulation with TNF-α, with a maximum stimulation reached at 30 min. A reduction of binding to DNA was observed at 60 min and a complete dissociation of the complex at 90 min. In addition, Figure 3.14b demonstrated that NF-κB-DNA binding activity was also induced by PMA (100nM) over a period of 2 h. A maximum increase in NF-κB-DNA binding was achieved at 90 min followed by dissociation of NF-κB-DNA binding complex beyond this time. Accordingly, the maximum time points of stimulation for both TNF-α 30 min and PMA 90 min were used to examine the effect of compounds on nuclear extract induced NF-κB-DNA binding activity (Tse et al., 2005, Holden et al., 2008).

3.15 Effects of AIK18/85/1 compound on TNF-α- and PMA- induced NF-κB-DNA binding activity in NCTC2544 cells.

Figure 3.15 shows the effect of AIK18/85/1 on NCTC2544 cells pretreated with AIK18/85/1 for 30 min prior to TNF-α (10ng/ml) stimulation for a further 30 min TNF-α (10ng/ml) stimulation, which is the optimum time to obtain maximum NF-κB-DNA binding activity (Figure 3.14a) (Tse et al., 2005). TNF-α stimulation resulted in a strong increase in DNA binding which was reduced by AIK18/85/1 in a concentration dependent manner. In order to confirm the inhibitory effects of compound, in this experiment the used range of concentration was limited between 10-50μM. Since 3μM was not effective as detected in previous experiments, it is replaced by 50μM in order to show concentration dependent effect. Inhibition was optimal between 30 and 50 μM reducing NF-κB-DNA binding activity by 59.7 % ±

9.0 ($p < 0.01$) at 50μM, 54.1 % ± 19.4 ($p < 0.05$) at 30μM and 41.2% ± 11.9 at 10μM.

As shown in Figure 3.16 similarly AIK18/85/1 was also able to produce significant inhibition of PMA-induced NF-κB-DNA binding (Holden et al., 2008). The degree of inhibition by AIK18/85/1 was similar to that observed in TNF-α-stimulated cells. AIK18/85/1 reduced the PMA response by 71.2% ± 11.2 at 50μM, 53.87% ± 20.0 at 30μM and 51.3% ± 17.8 at 10μM (all $p < 0.05$).

3.16 Effect of AIK18/85/1 on TNF-α pre-induced NF-κB-DNA binding activity in NCTC2544 cells.

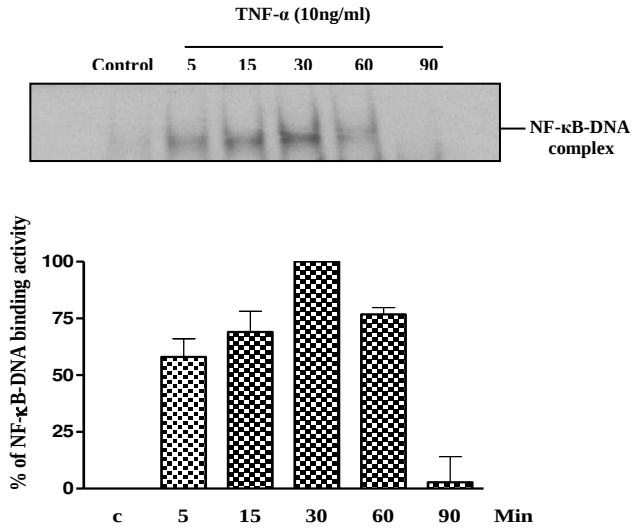
The inhibition of NF-κB-DNA binding observed by EMSA may reflect a reduction in the translocation of the NF-κB (p65) subunit to the nucleus as demonstrated by indirect immunofluorescence in Figure 3.12. However, the ability of NF-κB to bind to DNA could be modified by small molecules through physical interaction. Indeed, minor groove binders are well recognised to be able to interfere with protein-DNA binding. Therefore, the ability of AIK18/85/1 to physically interfere with the EMSA reaction was also investigated. This was determined by incubating nuclear extracts from TNF-α stimulated NCTC2544 cells with AIK18/85/1 then adding radiolabeled NF-κB oligonucleotide to the reaction mixture (Figure 3.17). In control conditions (TNF-α stimulated) strong specific DNA complex formation was observed however, there was no inhibitory effect of AIK18/85/1 at any of the concentrations tested (10-50μM). It was clearly illustrated that AIK18/85/1 was unable to produce physical interference at the DNA level in nuclear extracts, since the complex formed due to TNF-α stimulation was not altered by addition of compound. However, NF-κB-DNA binding was effectively abolished when 50x excess of unlabelled NF-κB oligonucleotide which added to an induced extract confirming that the complex was specific and could be disrupted by competition.

3.17 Effects of novel variant minor groove binder AIK18/70 on TNF-α-induced IκBα degradation, NF-κB (Ser536-p65) phosphorylation and NF-κB-DNA binding activity in NCTC2544 cells.

Having assessed the effects of AIK18/85/1 on different intermediates within the NF-κB signalling pathway, it was worthy to find out if this phenomenon was a characteristic of minor groove binders in general. Therefore, the effect of another structurally related minor groove binder AIK18/70 was assessed (Figure 3.18). Consistent with AIK18/85/1 when NCTC2544 cells were pretreated with AIK18/70 prior to TNF-α stimulation, it did not inhibit the induced IκBα loss as shown in Figure 3.19a. Similarly there was no effect on TNF-α stimulated NF-κB (p65-Ser536) phosphorylation when used over a similar concentration range to AIK/18/85/1 (Figure 3.19b).

The effect of AIK18/70 on NF-κB-DNA binding activity was also examined (Figure 3.20). In these experiments AIK18/70, similar to AIK18/85/1, strongly inhibited TNF-α induced NF-κB-DNA binding activity and reduced the maximum response to 17.9% ± 5.7 ($p < 0.01$) and 44.2% ± 13.868 ($p < 0.05$) at 50 and 30μM respectively. Figure 3.21 also demonstrated that AIK18/70 again showed similar effect to AIK18/85/1 when incubated with induced nuclear extracts in an EMSA reaction. No disruption of the DNA binding complex was observed with any concentration of the compound utilised. These data does not support the concept that minor groove binders share the ability to interact with DNA at specific consensus sequences.

a)



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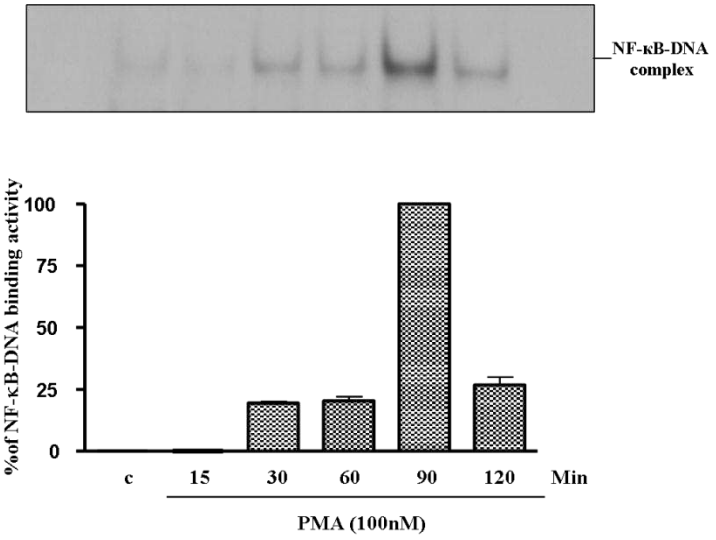


Figure 3.14: Time courses of TNF-α- and PMA-induced NF-κB-DNA binding activity in NCTC2544 cells. Cells were activated with TNF-α (10ng/ml) for a period up to 90 min in panel **a** or with PMA (100nM) in panel **b** for up to 2 h. Nuclear extracts were analysed by EMSA as outlined in Section 2.2.4. NF-κB-DNA binding complex bands are expressed as % of maximum binding activity. Results are representative of three independent experiments and each value represents the mean ± s.e.m.

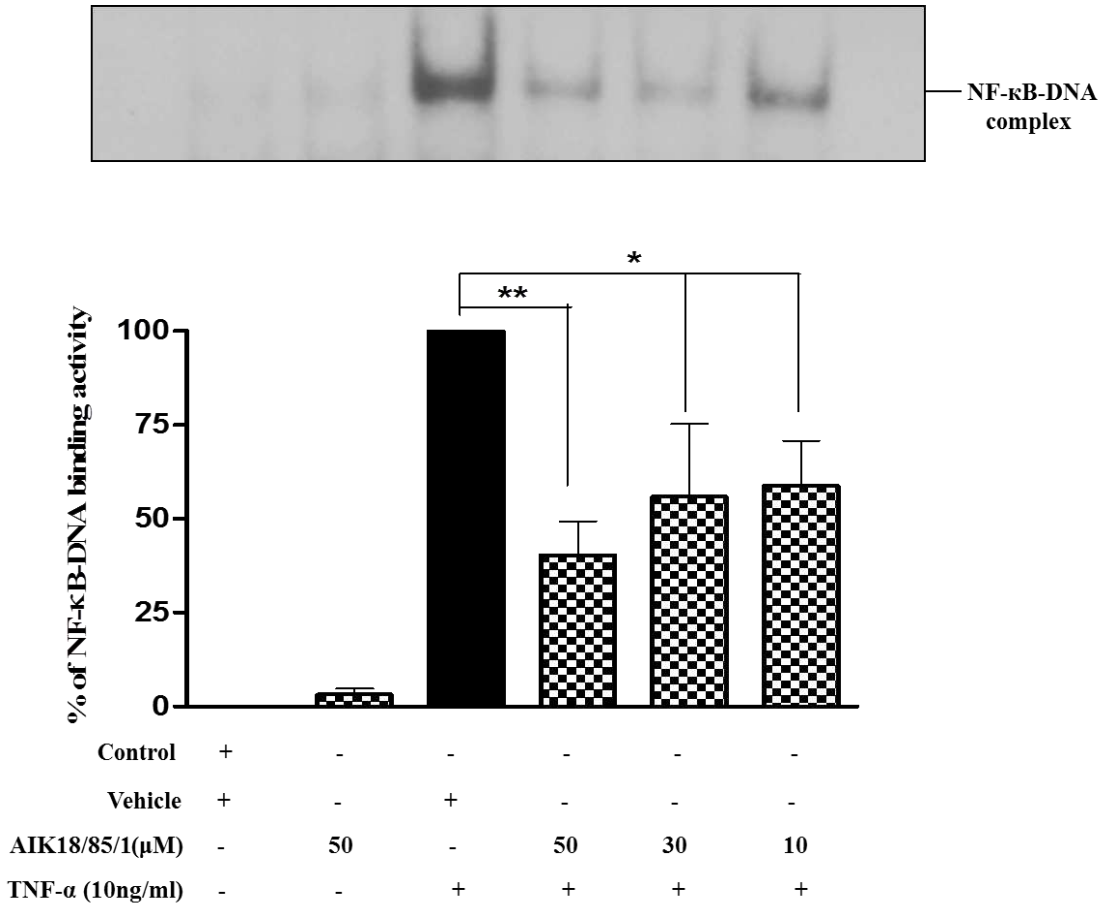


Figure 3.15: Effect of AIK18/85/1 on TNF-α-induced NF-κB-DNA binding activity in NCTC2544 cells. Cells were grown in 6 well plates as duplicates, serum starved overnight, treated with 10, 30 and 50μM AIK18/85/1 for 30 min prior to stimulation with TNF-α (10ng/ml) for a further 30 min. Vehicle (DMSO) was added to control untreated wells in parallel to TNF-α. Nuclear extracts were analysed by EMSA as outlined in Section 2.2.4. NF-κB-DNA binding complex bands are expressed as % of maximum binding activity. Results are representative of three independent experiments and each value represents the mean ± s.e.m., * = $p < 0.05$, ** = $p < 0.01$.

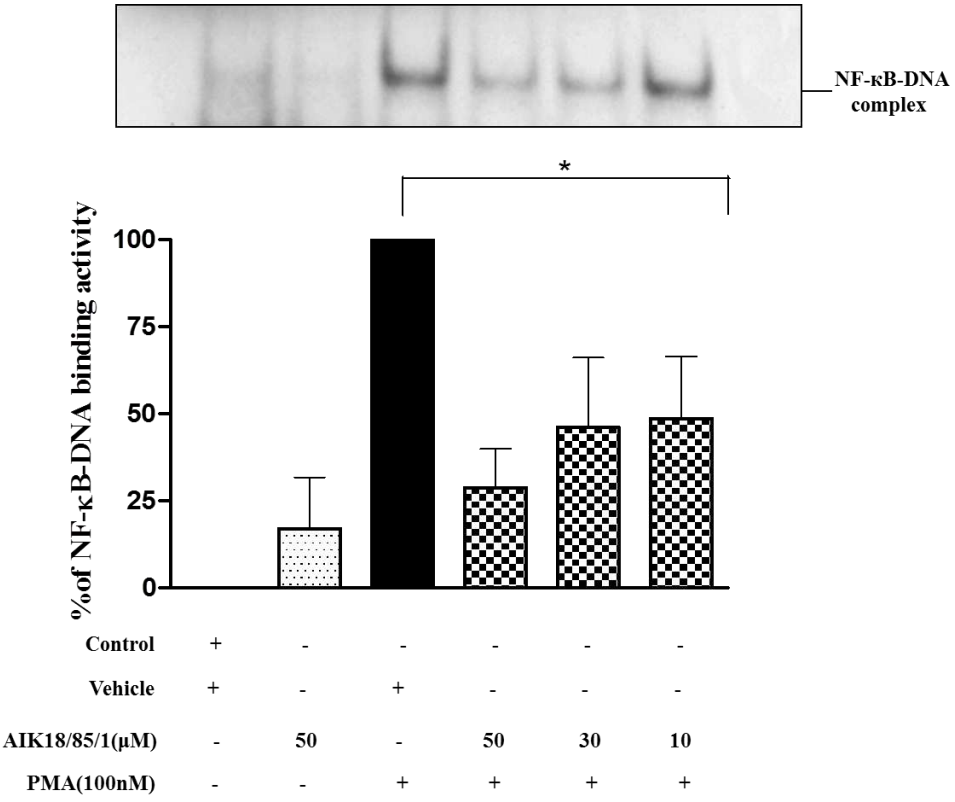


Figure 3.16: Effect of AIK18/85/1 on PMA-induced NF-κB-DNA binding activity in NCTC2544 cells. Cells were grown in 6 well plates as duplicates, serum starved overnight, pretreated with 10, 30 and 50μM AIK18/85/1 for 30 min prior to the addition of PMA (100nM) for a further 90 min. Vehicle (DMSO) was added to control untreated wells in parallel to PMA. Nuclear extracts were analysed by EMSA as outlined in Section 2.2.4. NF-κB-DNA binding complex bands are expressed as % of maximum binding activity. Results are representative of three independent experiments and each value represents the mean ± s.e.m., * = $p<0.05$.

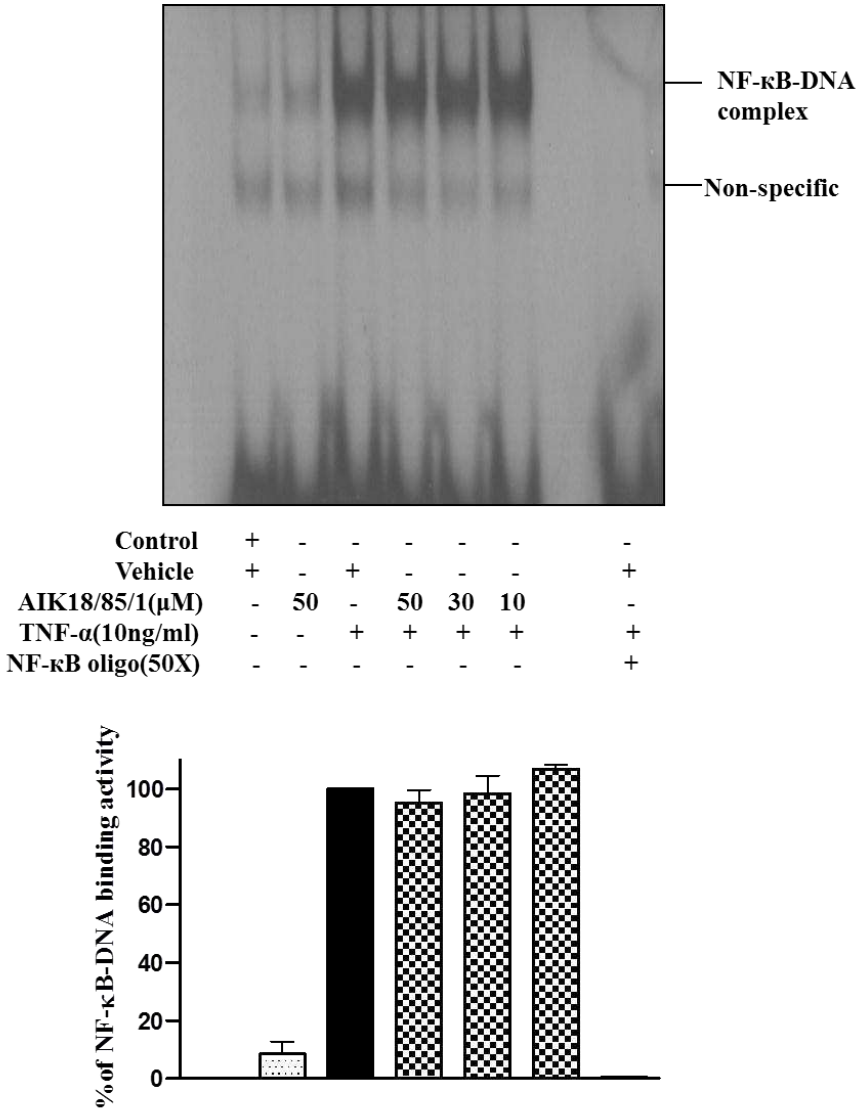


Figure 3.17: Effect of addition of AIK18/85/1 in vitro on TNF-α-stimulated NF-κB-DNA binding in NCTC2544 cells. Cells were stimulated with vehicle or TNF-α (10ng/ml) for 30 min and nuclear extracts prepared. AIK18/85/1 at 10-50μM concentrations or 50x fold excess unlabelled NF-κB oligonucleotide were added to the incubation mixture 30 min prior to initiation of binding reaction. Then this mixture was analysed by EMSA as outlined in Section 2.2.4. NF-κB-DNA binding complex bands were expressed as % of maximum binding activity. Results are representative of three independent experiments and each value represents the mean ± s.e.m.

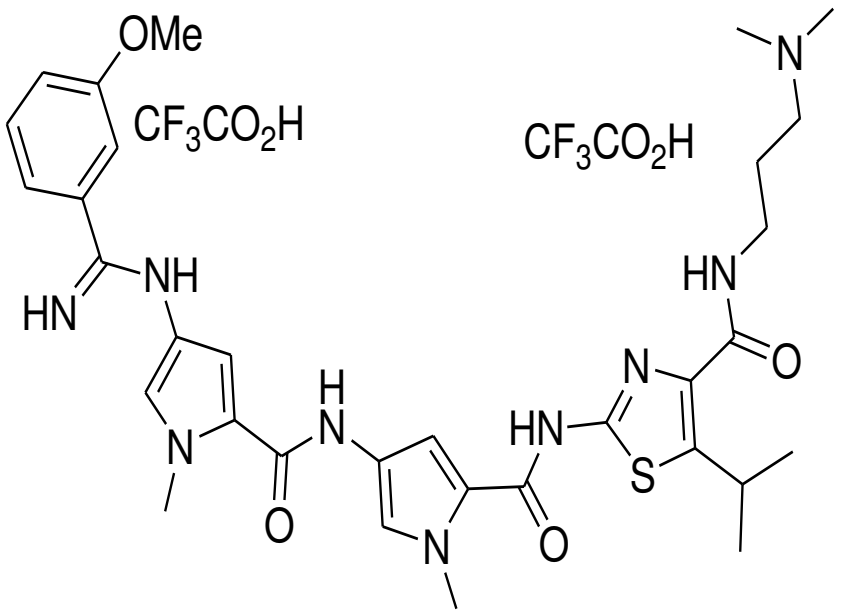
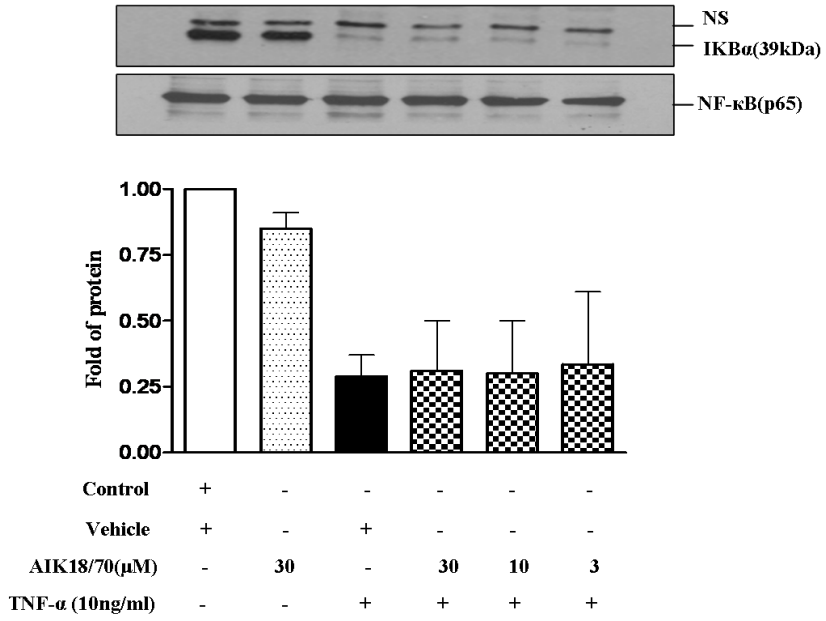


Figure 3.18: Chemical structure and systematic name of AIK18/70. N-[3-(dimethylamino)propyl]-2-{[(4-{[(4-{[imino(3-methoxyphenyl)methyl]amino}-1-methyl-1H-pyrrol-2-yl)carbonyl]amino}-1-methyl-1H-pyrrol-2-yl)carbonyl]amino}-5-isopropyl-1,3-thiazole-4-carboxamide bis(trifluoroacetate)

a)



b)

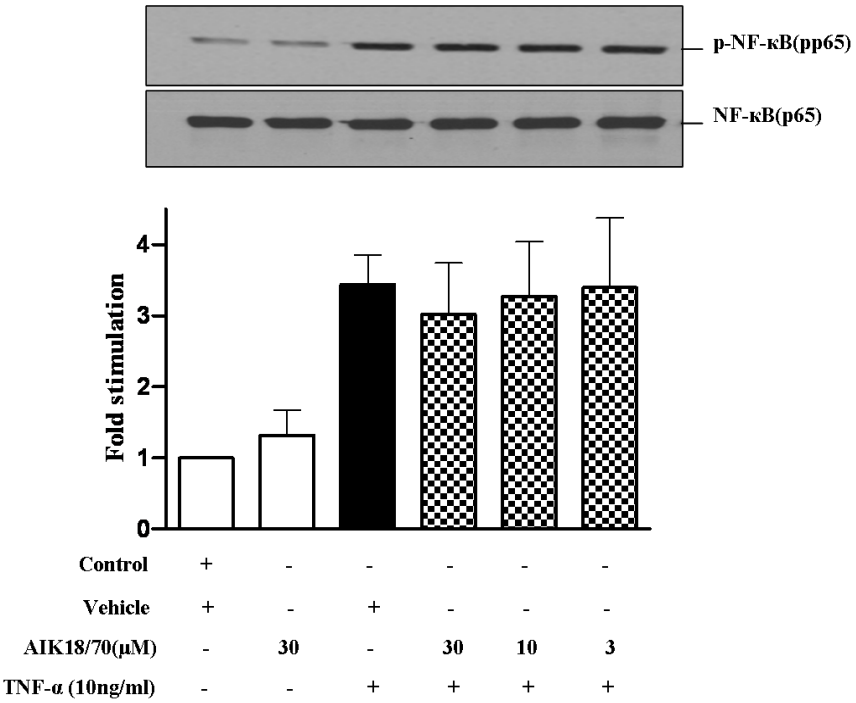


Figure 3.19: Effects of AIK18/70 on TNF-α-induced IκBα degradation and NF-κB (Ser536-p65) phosphorylation in NCTC2544 cells. Cells were treated with 3-30μM AIK18/70 for 30 min prior to stimulation with TNF-α (10ng/ml) for a further 30 min. Whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for IκBα protein (39kDa) and re-probed for total NF-κB (T-p65) in panel **a** and for phospho-NF-κB (p65Ser-536) and re-probed for total NF-κB (T-p65) in panel **b** as outlined in Section 2.2.3. The results are representative of three independent experiments and each value represents the mean ± s.e.m., NS (non-specific).

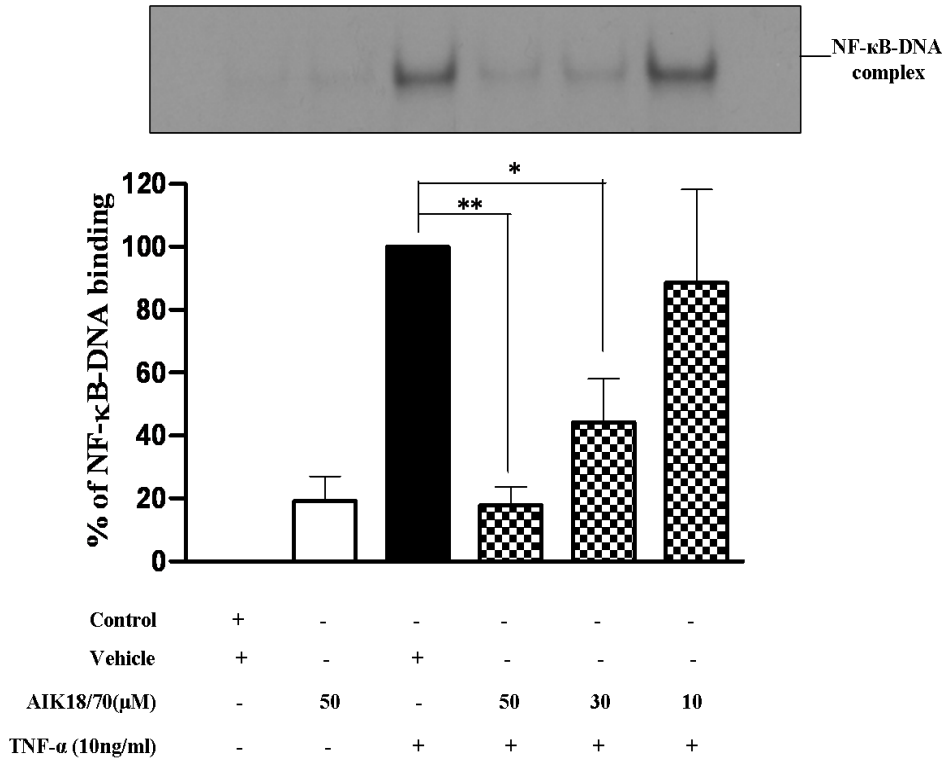


Figure 3.20: Effect of AIK18/70 on TNF-α-induced NF-κB-DNA binding activity in NCTC2544 cells. Cells were grown in 6 well plates as duplicates, serum starved overnight, treated with 10, 30 and 50μM AIK18/70 for 30 min prior to stimulation with TNF-α (10ng/ml) for a further 30 min. Vehicle (DMSO) was added to control untreated wells in parallel to TNF-α. Nuclear extracts were analysed by EMSA as outlined in Section 2.2.4. NF-κB-DNA binding complex bands are expressed as % of maximum binding activity. Results are representative of three independent experiments and each value represents the mean ± s.e.m., * = $p < 0.05$, ** = $p < 0.01$.

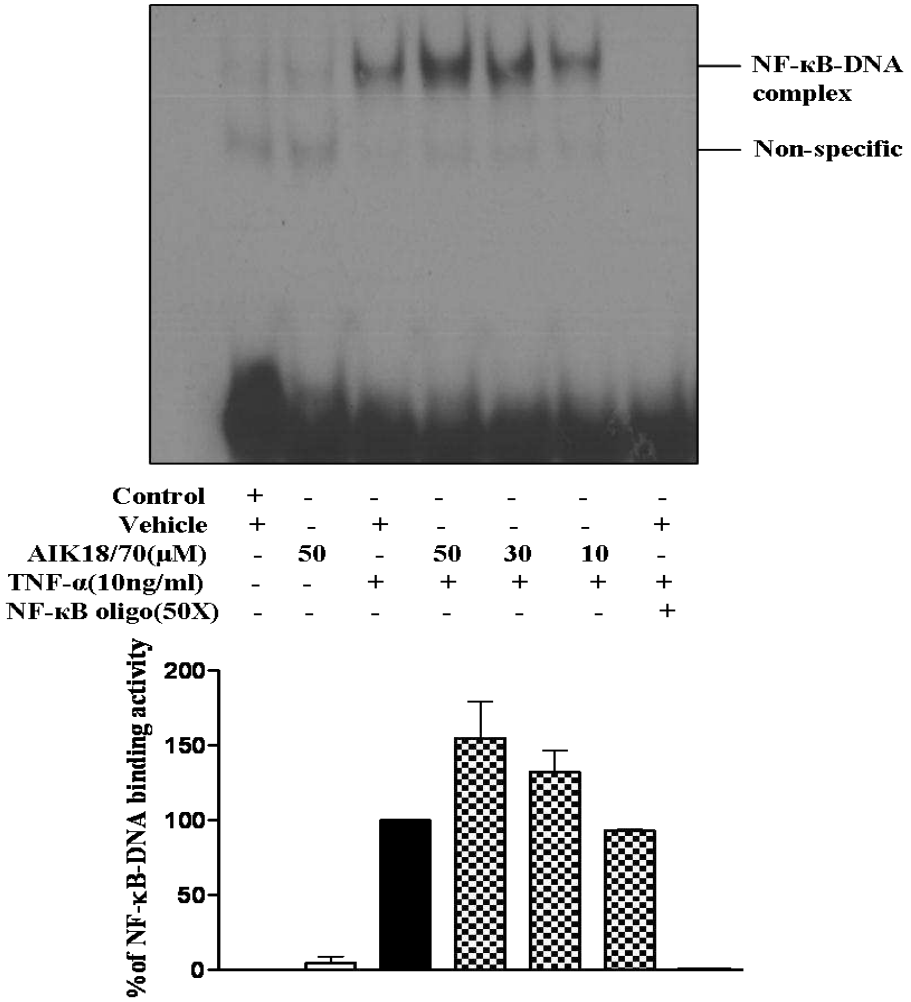


Figure 3.21: Effect of addition of AIK18/70 in vitro on TNF-α-stimulated NF-κB-DNA binding in NCTC2544 cells. Cells were stimulated with vehicle or TNF-α (10ng/ml) for 30 min and nuclear extracts were prepared. AIK18/70 at 10-50μM concentrations or 50x fold excess unlabelled NF-κB oligonucleotide were added to the incubation mixture 30 min prior to initiation of binding reaction. This mixture was analysed by EMSA as outlined in Section 2.2.4. NF-κB-DNA binding complex bands are expressed as % of maximum binding activity. Results are representative of three independent experiments and each value represents the mean ± s.e.m.

3.18 Assessing the effects of AIK18/85/1 on inflammatory mediators production in NCTC2544 cells and RAW 264.7 macrophages.

Having established that AIK18/85/1 was able to inhibit NF-κB activation in NCTC2544 cells the next step was to examine the effects of compound on the release of the inflammatory mediators such as IL-8 which has been assayed previously in the laboratory. However, treatment of NCTC2455 cells with AIK18/85/1 for prolonged periods resulted in marked cytotoxic effects between the 10-30μM range (Figure 3.22a). Therefore, another cell type for AIK18/85/1 assessment was considered. It was decided to use the RAW 264.7 macrophage cell line which has been shown previously to express inflammatory proteins in an NF-κB dependent manner (Suh et al., 1998). Results show that this cell line tolerated AIK18/85/1 much better with significant toxicity observed only at 50μM of the compound and was chosen for subsequent experiments (Figure 3.22b). Alternatively, 1μg/ml LPS (Hatziieremia et al., 2006) used to induce NF-κB dependent iNOS and COX-2 expression, via TLR4/ NF-κB and MAP kinases activation in RAW 264.7 macrophage cell line. This concentration also showed an optimum induction of IκBα degradation according to LPS concentration curve (data not shown).

3.19 Effects of AIK18/85/1 on LPS-induced iNOS protein expression and nitrite production in RAW 264.7 macrophage cells.

Initially the expression of iNOS was examined. A large number of studies have assessed the induction of this enzyme and the subsequent release of ·NO, particularly in the context of examining novel inhibitors and natural products. Firstly, a time course of iNOS induction over 24 h was established in response to LPS (1μg/ml) and the results are shown in Figure 3.23a. Following a delay of 2 h iNOS expression increased rapidly and reached a maximum between 6 and 8 h after exposure to LPS. Expression remained high until 24 h time point after which the signal started to decline. Thus, 6 h was used as a stimulation time to induce iNOS (Suh et al., 1998), AIK18/85/1 was added to cells at a concentration range of 3-30μM for 30 min prior to LPS stimulation. Figure 3.23b shows that AIK18/85/1 strongly inhibited LPS-induced iNOS expression. A concentration of 30μM AIK18/85/1 caused a substantial reduction of almost 90% iNOS expression 11.5% ± 11.0 of stimulation, ($p < 0.001$)

and a significant reduction to 64.27 ± 18.261 of stimulation was also achieved by 10μM of AIK18/85/1 ($p < 0.01$). In order to confirm the inhibitory effect of AIK18/85/1 on iNOS expression, nitrite production induced by LPS stimulation for 24 h was also determined in the presence of similar concentrations of the compound (Chen et al., 2005a). Experiments represented in Figure 3.23c demonstrated a significant and clear reduction of nitrite produced by LPS (1μg/ml), reducing nitrite in stimulated cells from approximately 40μM to 17 μM (39.2 ± 4.1 to 16.96 ± 4.2, $p < 0.05$) at a concentration of 30μM of AIK18/85/1. In contrast to the effects observed on iNOS expression, the compound had no significant effect at 10μM.

3.20 Effect of AIK18/85/1 on LPS-induced COX-2 protein expression in RAW 264.7 macrophage cells.

As a comparator to iNOS, COX-2, another inducible proinflammatory enzyme, was used as a marker to assess the effect of AIK18/85/1, as LPS can induce COX-2 expression through NF-κB signalling. Figure 3.24a revealed an increase in the expression of COX-2 enzyme as early as 4 h following LPS (1μg/ml) treatment. Stimulation was maintained for up to 8 h before returning to almost basal values by 24 h. On this basis, 6 h stimulation time was chosen to assess the effect of the AIK18/85/1 compound (Suh et al., 1998). Under these conditions AIK18/85/1 was found to significantly reduce maximum COX-2 induction stimulated by LPS. Expression fell to as low as 23.6% ± 3.4 ($p < 0.001$) of stimulated values at 30μM and to a lesser extent (80.4% ± 5.5 of stimulation $p < 0.01$) at 10μM (Figure 3.24b).

3.21 Effect of AIK18/85/1 on IFNγ-induced iNOS protein expression in RAW 264.7 macrophage cells.

To explore further whether the inhibitory effect of AIK18/85/1 on iNOS protein expression was stimulant specific, RAW264.7 macrophage cells were activated by IFNγ (100U/ml) (Hatziieremia et al., 2006) through activation of JAK/STAT1 pathway. Firstly, a time course for IFNγ was established over a period of 2-24 h, as shown in Figure 3.25a. Again, following a delay of some 4 h iNOS induction increased rapidly reaching a maximum between 6 and 8 h and was maintained at maximum levels for up to 24 h the longest time point examined. Accordingly, 6 h

was chosen to examine the effect of AIK18/85/1 (3-30μM) on iNOS expression induced by IFNγ stimulation. Figure 3.25b showed that a maximum iNOS expression levels observed following IFNγ stimulation were completely abolished by pretreatment with 30μM of AIK18/85/1 (0.6% ± 0.59 stimulation $p < 0.001$). Furthermore, iNOS expression was significantly reduced by approximately half using 10μM of the compound (55.1% ± 25.3 stimulation $p < 0.01$). Only a small reduction of iNOS protein level was observed at 3μM (86.7% ± 17.3 stimulation).

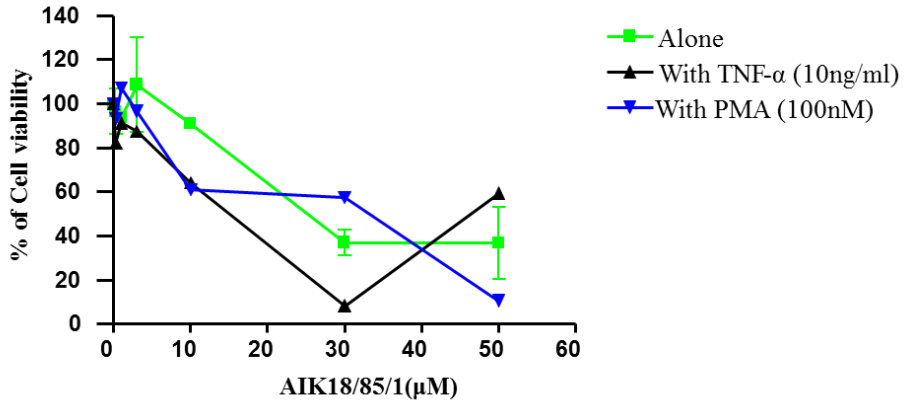
3.22 Effects of AIK18/85/1 on LPS-induced phosphorylation of MAP Kinases and IFNγ-mediated STAT1 phosphorylation and in RAW264.7 macrophage cells.

In order to determine whether the inhibitory effects of AIK18/85/1 on inflammatory mediators COX-2 and iNOS were due to direct inhibition of NF-κB or may be regulated by off-targets. LPS-stimulated MAP kinase signalling was assessed by AIK18/85/1. Furthermore IFNγ-mediated iNOS through JAK/STAT1 signalling was also considered. In order to detect the mechanism of AIK18/85/1 inhibitory effect on IFNγ-mediated iNOS induction (Figure 3.25b), the phosphorylation of STAT1α at Tyr701 by IFNγ was also assessed. As shown in Figure 3.27, stimulation of RAW264.7 macrophages with IFNγ for 30 min resulted in STAT1 phosphorylation (Chen et al., 2005a), which was chosen as an activation time point according to IFNγ (100U/ml) time course (data not shown). A pretreatment of AIK18/85/1 for 30 min prior to IFNγ stimulation resulted in a non-significant reduction of phosphorylated STAT1 at 30μM concentration only. A further experiment is required to confirm this effect.

In Figure 3.26a exposure of RAW 264.7 macrophage cells to LPS (1μg/ml) over a period of up to 2 h resulted in a maximum phosphorylation of ERK1/2, p38 and JNK1/2 within 15 min. These kinases had a distinct pattern of phosphorylation; p38 and JNK1/2 both were phosphorylated at 15 min but ERK1/2 phosphorylated as early as 5 min. In contrast LPS induced phosphorylation of JNK1/2 that ceased beyond 30 min, whilst both ERK1/2 and p38 phosphorylation were sustained over 2 h. The effects of AIK18/85/1 on these cellular events were assessed over a

concentration range (3-30μM). As shown in Figure 3.26b and 3.26c LPS-induced MAP kinases phosphorylation was not altered by the pretreatment with AIK18/85/1.

a)



b)

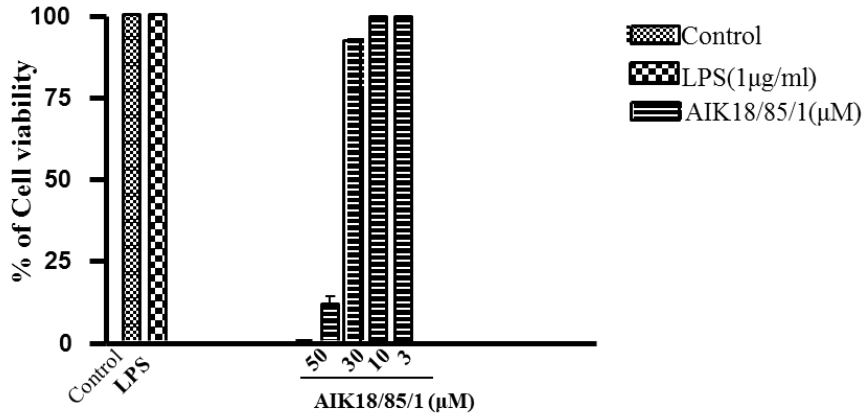
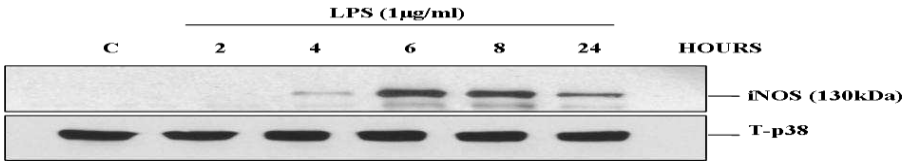
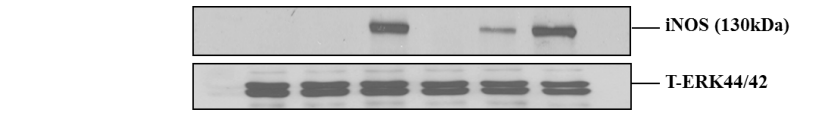


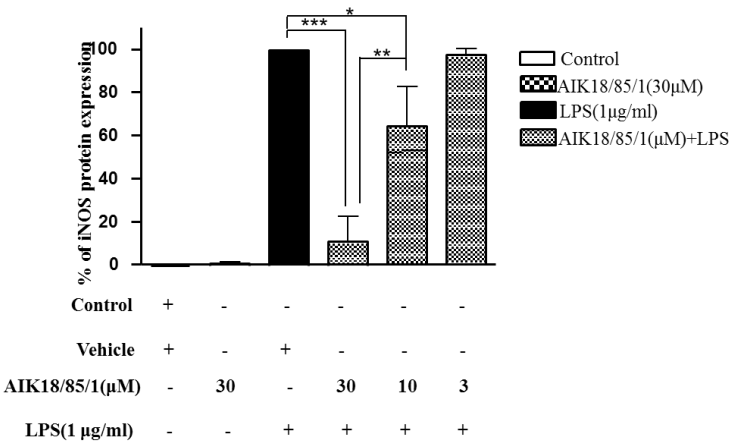
Figure 3.22: Effect of AIK18/85/1 on cell viability. Quiescent NCTC2544 cells in panel **a,** or RAW264.7 macrophage cells in panel **b** were treated with 1, 3, 10, 30, and 50µM AIK18/85/1 for 24 h (alone in panel **b**), in presence or absence of TNF-α (10ng/ml) or PMA (100nM) in panel **a**. Then cell viability was determined as described in the Section 2.2.7. The results are expressed as the % of maximum viable cells. Results are representative of three independent experiments in triplicates and each value represents the mean ± s.e.m.

a)



b)





c)

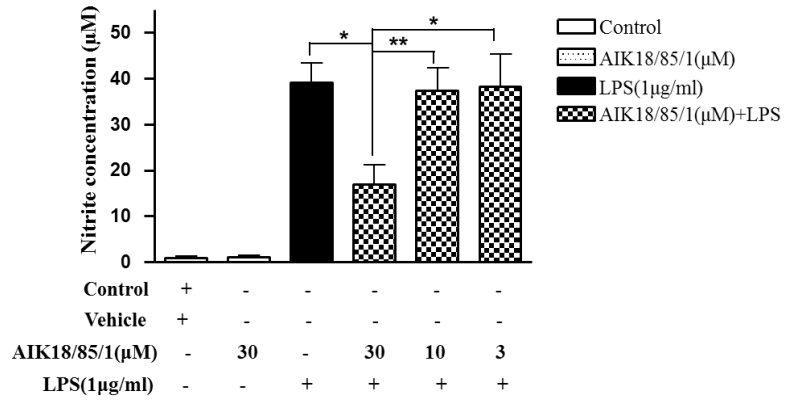
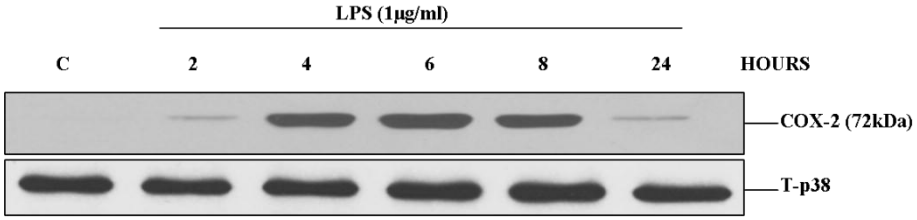


Figure 3.23: Effects of AIK18/85/1 on LPS-induced iNOS expression and nitrite production in RAW 264.7 macrophage cells. Cells were treated with LPS (1µg/ml) over a period of 2-24 h in panel **a** or pretreated for 30 minutes with AIK18/85/1 (3-30µM) prior to LPS stimulation for a further 6 h in panel **b** or for 24 h in panel **c**. In panels **a** and **b** whole cell lysates were prepared, separated by SDS-PAGE and assessed for iNOS (130kDa) expression, blots re-probed for total p38-MAPK, and total-ERK respectively as outlined in Section 2.2.3. Panel **c** shows graphical representation of LPS induced nitrite production (see Section 2.2.8). Results in panel **b** expressed as % of maximum stimulation. Results are representative of three independent experiments and each value represents the mean ± s.e.m., * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to LPS stimulation.

a)



b)

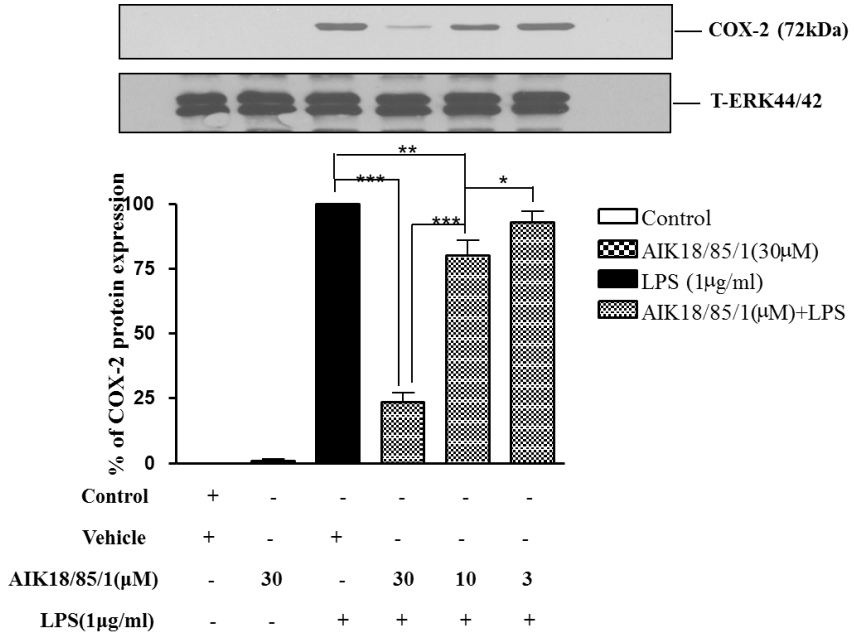
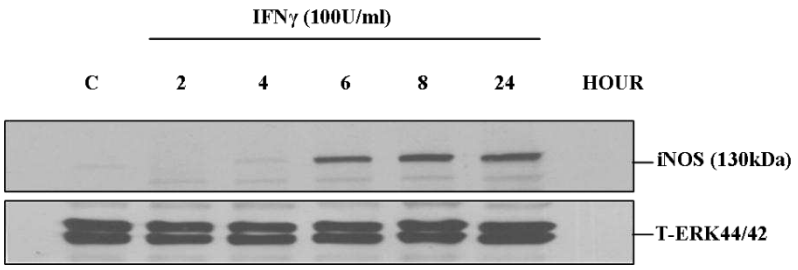


Figure 3.24: Effect of AIK18/85/1 on LPS-induced COX-2 expression in RAW 264.7 macrophage cells. Cells were treated with LPS (1µg/ml) over a period of 2-24 h in panel **a**, or pretreated for 30 min with AIK18/85/1 (3-30µM) prior to LPS stimulation for a further 6 h in panel **b.** In panels **a** and **b** whole cell lysates were prepared, separated by SDS-PAGE and assessed for COX-2 (72kDa) expression, and re-probed for total p38 MAPK, and total-ERK respectively as outlined in Section 2.2.3. Results in panel **b** expressed as % of maximum stimulation. Results are representative of three independent experiments and each value represents the mean ± s.e.m., * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ compared to LPS stimulation.

a)



b)

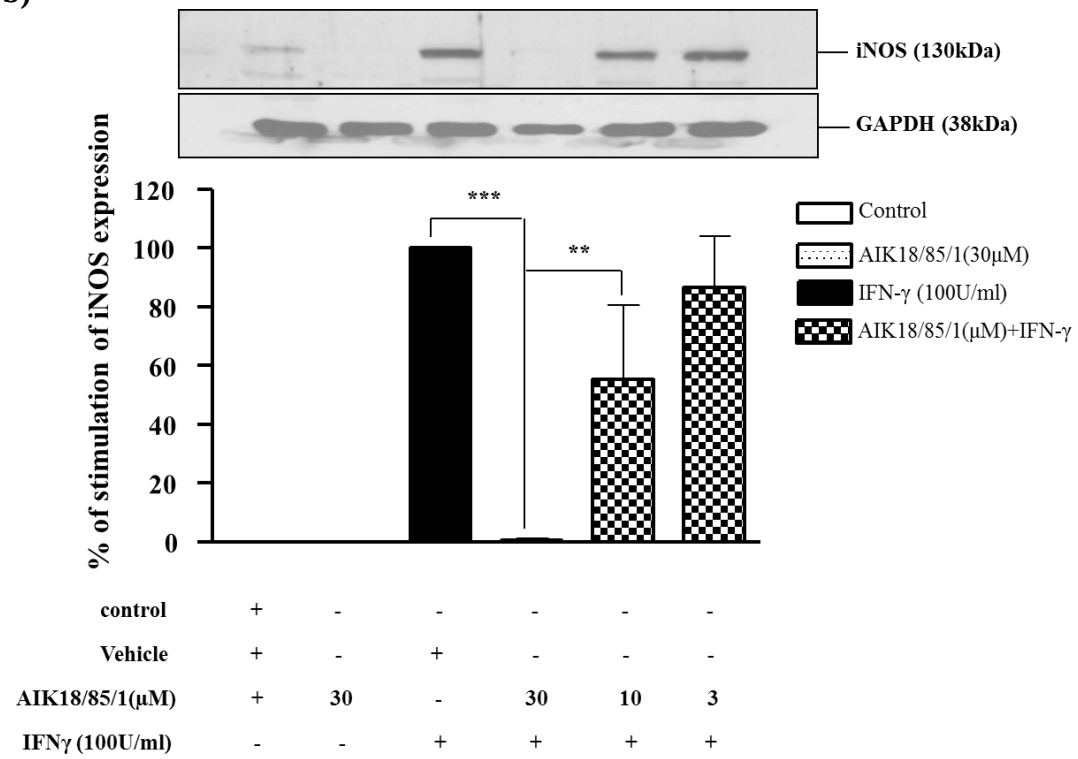


Figure 3.25: Effect of AIK18/85/1 on IFNγ-induced iNOS expression in RAW 264.7 macrophage cells. Cells were treated when confluent with IFNγ (100U/ml) over a period of 2-24 hours as indicated in panel **a**, or pretreated for 30 min with AIK18/85/1 (3-30μM) prior to LPS stimulation for a further 6 h in panel **b**. Whole cell lysates were prepared, separated by SDS-PAGE and blotted for iNOS (130kDa) expression, and re-probed for total-ERK and GAPDH (38kDa) as outlined in Section 2.2.3. Results in panel **b** expressed as % of maximum stimulation. Results are representative of three independent experiments and each value represents the % mean ± s.e.m., ** = $p < 0.01$, *** = $p < 0.001$ compared to treated control.

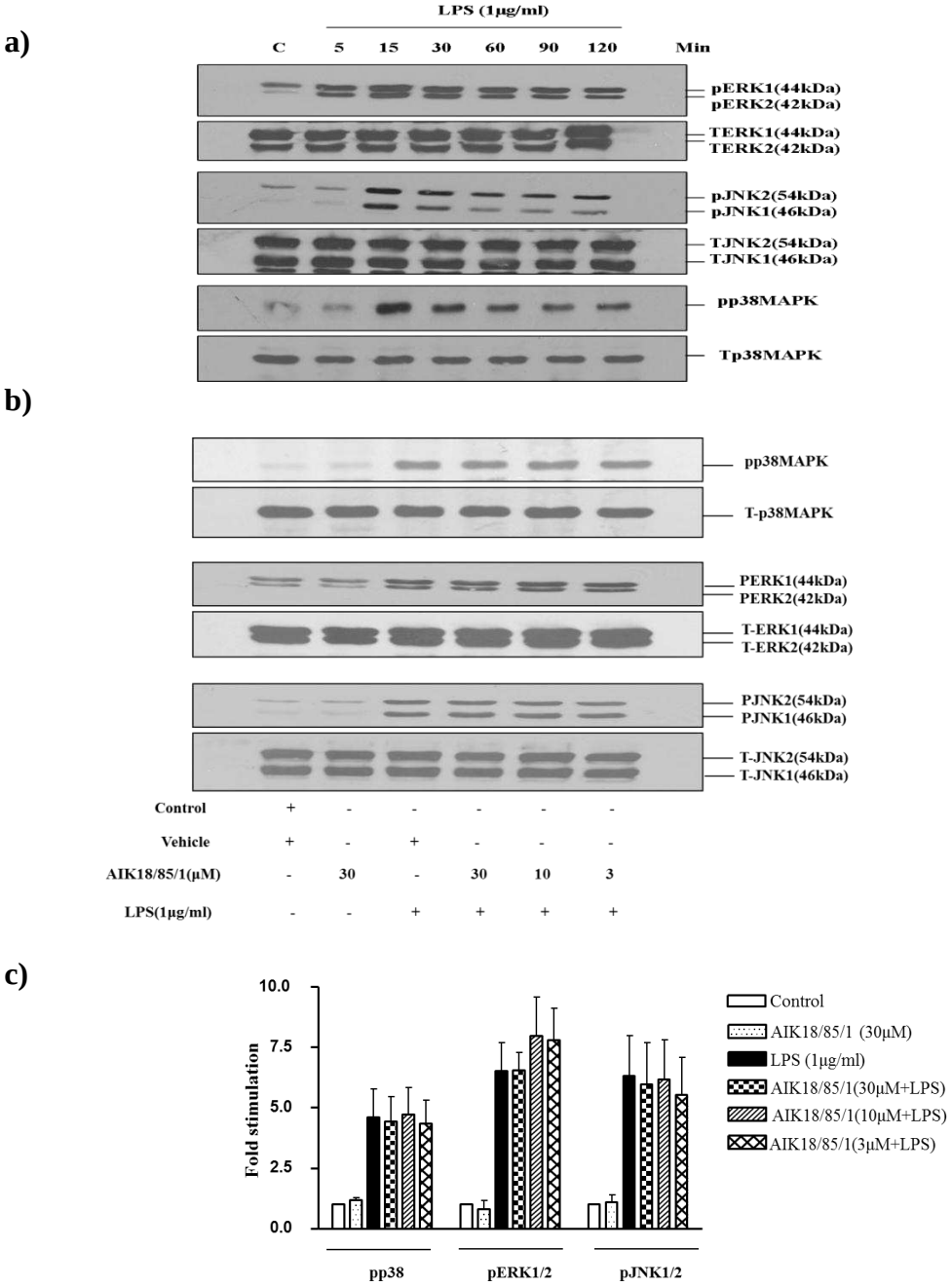


Figure 3.26: Effects of AIK18/85/1 on LPS-induced MAP kinases (ERK1/2, p38-MAP kinase and JNK1/2) phosphorylation in RAW 264.7 macrophage cells. Confluent cells were treated with LPS (1µg/ml) for up to 2 h in panel (**a**) or pretreated for 30 min with AIK18/85/1 prior to further 15 min LPS stimulation in panel **b**. In panels **a** and **b** whole cell lysates were prepared, separated by SDS-PAGE and assessed for; p38-MAPK phosphorylation, pp38 (38kDa) and re-probed for total p38 MAPK, JNK-MAPK phosphorylation (pJNK1/2) (46/54kDa) and total-JNK, and ERK1/2 phosphorylation (pERK42/44) (42/44kDa) and total ERK1/2 as outlined in Section 2.2.3. Panel **c** shows graphical representation of blots in panel **b**. Results are representative of three independent experiments and each value represents the mean ± s.e.m.

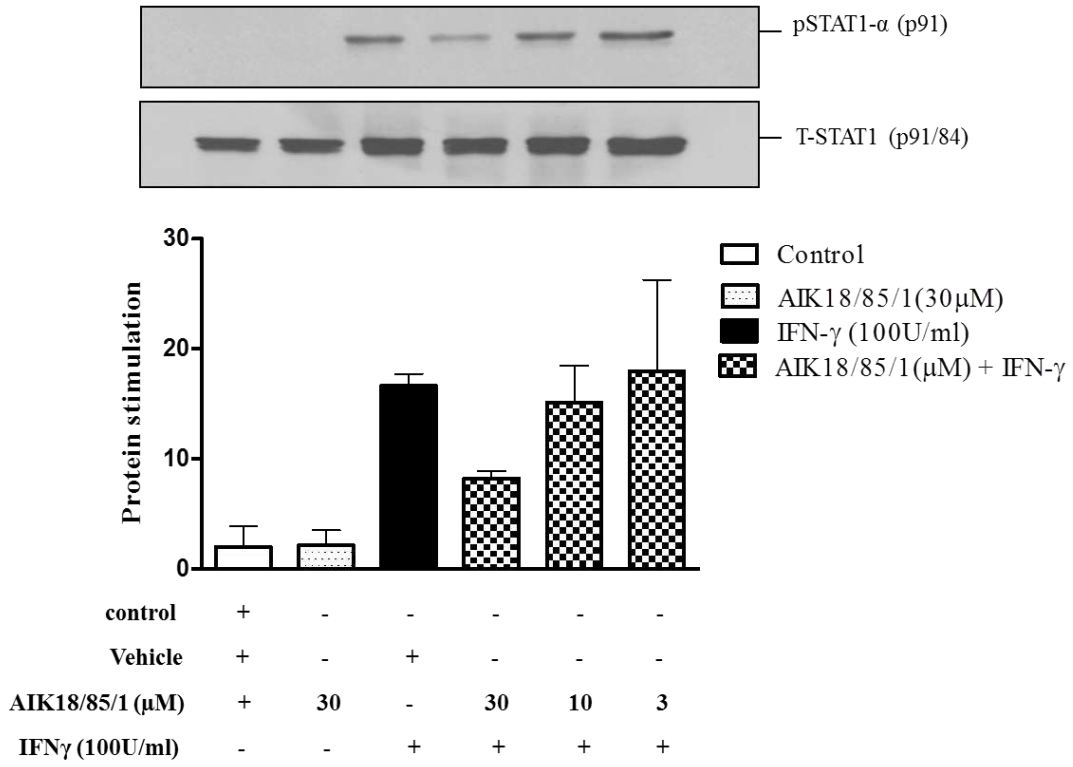


Figure 3.27: Effect of AIK18/85/1 on IFNγ-mediated STAT1 phosphorylation in RAW 264.7 macrophage cells. Confluent cells were pretreated for 30 min with AIK18/85/1 prior to further 30 min stimulation with IFN-γ (100U/ml).Whole cell lysates were prepared, separated by SDS-PAGE and assessed for phosphorylated STAT1α (Tyr701) (p91), and re-probed for total STAT1(p91/84) as outlined in Section 2.2.3. Results are representative of two independent experiments and each value represents the mean ± s.e.m.

Compound	IKK* activity	IκBα loss*	Ser536-p65* phosphorylation	NF-κB* nuclear translocation	NF-κB-DNA* binding activity	NF-κB-linked* luciferase activity	AP-1-linked* luciferase activity	iNOS# expression	COX-2# expression
AIK18/85/1	reduced	no effect	no effect	prevented TNF-α induction	reduced TNF-α and PMA induction, but no effect In vitro	reduced TNF-α and PMA-induced activity	reduced PMA-induced activity	inhibited LPS and IFNγ induction	inhibited LPS induction
AIK18/70	-	no effect	no effect	-	reduced TNF-α induction, but no effect In vitro	reduced TNF-α induced activity	-	-	-

Table 3.1: Summary table illustrates the effects of compounds AIK18/85/1 and AIK18/70 on NF-κB pathway and off-targets.
Abbreviations: *: Assay carried out for compounds in human keratinocytes NCTC2544 cells, #: assay carried out for compounds in murine RAW264.7 macrophages, -: compound not tested.

3.23 Discussion

Inflammatory diseases are common in societies throughout the world. The anti-inflammatory drugs used clinically for example, corticosteroids, NSAIDS and biologics, have multiple disadvantages such as side effects and high cost of treatment. An alternative to these drugs, are natural compounds that offer a great hope in the identification of bioactive lead compounds and their development into drugs for treating inflammatory diseases. In this present study lead compounds synthesised or derived from diverse natural sources and diverse chemical structures were investigated.

Inflammation is caused by a variety of stimuli including microbial invasion, physical damage, ultraviolet irradiation, and immune reactions. A complex inflammatory cascade can lead to development of chronic diseases such as asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and psoriasis. Furthermore, epidemiological studies have identified that inflammation and infections are also major risk factors for various types of cancer (Mantovani and Pierotti, 2008). The transcription factor NF-κB plays a crucial role in the triggering and coordination of both innate and adaptive immune responses within the context of many, if not all, of these conditions. A large number of studies both in vitro and in vivo link cellular responses to these conditions. For example, NF-κB regulates the transcription of various inflammatory cytokines or chemokines such as, IL-1β, IL-2, IL-6, IL-8 and TNF-α, as well as genes encoding cell adhesion molecules, immunoreceptors, and haematopoietic growth factors (Baeuerle and Henkel, 1994). In addition, NF-κB response elements have been demonstrated to be in the promoter regions of iNOS and COX2, which are involved in the inflammatory processes by producing NO and prostaglandins respectively (Karin and Ben-Neriah, 2000). Therefore, NF-κB is currently being pursued as a major molecular target for the screening of anti-inflammatory lead compounds.

Initially, induction of both NF-κB and AP-1 activation have been characterised while induced either by TNF-α or PMA for NF-κB reporters, or just PMA for AP-1 reporter activity. To be able to assess the site and mechanism of inhibition of

potential inhibitory compounds within the NF-κB pathway two agents were used to stimulate the pathway. This approach reflects the increased complexity of the pathway revealed since the discovery of NF-κB itself and more importantly the regulatory IKKs. Activation of reporter cells with TNF-α produced a modest NF-κB transcriptional activity reflecting the activation of the canonical (classical) pathway (TNF-R1/IKK/ IκB-α-NF-κB axis). However, it is recognised that there is at least another NF-κB axis comprising of the noncanonical pathway and involving p100 phosphorylation and processing. TNF-α is also able to activate this alternative pathway which is relevant to keratinocyte function (Bhattacharyya et al., 2010). However, due to time constrains this was not pursued in this work. Herein the assays which included assessment of IκBα loss, phosphorylation and nuclear translocation of p65 and DNA binding using EMSA were directed towards analysis of the canonical pathway. The NF-κB reporter assay utilised a plasmid which containing three (3x) binding sites for NF-κB, derived from the immunoglobulin kappa light chain gene linked with luciferase gene.

It should be noted that even within the canonical pathway there are other key sites of regulation. The magnitude of NF-κB transcriptional activity stimulated by PMA was very strong, indicating the probable involvement of multiple signalling inputs into the canonical IKK/IκB-α-NF-κB axis. Among these signalling inputs PKCs, PI3K/Akt, and calmodulin/calcineurin, kinases or phosphatases are known to contribute to the regulation of the IKKs and the integration of the p50/p65 complex at levels of translocation and transactivation, more specifically in cerebellar granule neurons (Lilienbaum and Israel, 2003). It has also been suggested that PKCα as well as the atypical PKCs (PKCζ, PKCι, and PKCγ) play a critical role in the NF-κB pathway through their binding to the IKKs in vitro and in vivo to specifically stimulate the activity of IKKβ (Lallena et al., 1999). Numerous studies including those carried en-house have shown good expression of PKCα, δ and ε, in the NCTC2544 cell line and primary cell cultures (Goon Goh et al., 2008), (Papp et al., 2003). Previous work has linked Ca^{2+}-dependent PKCα to the activation of the canonical IKK/IκBα/NFκB pathway (Anrather et al., 1999), (Asehnoune et al., 2005). However, Goon Goh et al. 2008 suggested that phosphorylation of p65 can be

dissociated from PKCα -IKK axis at later times and does not require activation of the Ca^{2+}-dependent PKC isoforms, but instead required PKCδ in early and late phases of NF-κB phosphorylation (Goon Goh et al., 2008). This suggests a considerable degree of complexity when considering the site and mechanism of action of NF-κB inhibitory drugs.

Experiments were initially constructed to characterise the activation of the canonical pathway in keratinocytes. These included testing of the proteasome inhibitor MG-132 (Cbz–Leu–Leu–leucinal), which allowed confirmation that the pathway was sensitive to drug intervention. A number of studies have shown that MG-132 prevents the proteasomal degradation of IκBα and subsequent nuclear translocation of p65 in different cell types (Chen et al., 1995), (Spiecker et al., 1998, Sun and Carpenter, 1998). Whilst MG-132 inhibited both IκBα loss and NF-κB reporter activity in response to TNF-α, the concentration required to achieve this was different for each parameter. This suggests the potential of multiple roles for proteasomal degradation in different parts of the pathway, perhaps at the level of DNA binding or at the level of transactivation. While there is no evidence to support this hypothesis, Peiretti, Levine and colleagues have demonstrated that proteasomal inhibition activates the shedding of TNF-α receptors from endothelial and epithelial cells (Levine et al., 2005, Peiretti et al., 2005). They have also demonstrated that the proteasome plays an important role in the regulation of transmembrane components of the TNF-α-TNFR axis (Ortiz-Lazareno et al., 2008).

In recent years, there has been an increasing interest in development of MGBs as human chemotherapeutics to allow targeted delivery of DNA- modifying agents (Baron et al., 2004a). DNA minor groove binding drugs, including the antibiotic distamycin A, constitute a class of drugs that bind AT-rich sequences within the minor groove of DNA in a sequence- and conformation-specific fashion, thereby interfering with transcription factor-DNA binding to AT-rich sequences (Cozzi, 2000, Bell et al., 1997). Initially, AIK18/85/1 a thiazol-peptidomimetic compound, structurally related to distamycin A was examined as this compound displayed activity against IKKs in vitro and may have multiple properties. However

AIK18/85/1 whilst inhibiting NF-κB activity in response to either TNF-α or PMA was not effective against agonist induced IκBα loss or phosphorylation of p65. This immediately suggests that AIK18/85/1 is not an effective inhibitor of IKK, at least in keratinocytes and RAW264.7 macrophages (data not shown). Interestingly, the inhibitory effect of AIK18/85/1 on NF-κB transcriptional activity induced by both TNF-α and PMA seems to be partial and concentration dependent compared to its effect on PMA-induced AP-1 transcriptional activity, which is completely abolished at 30μM concentration. The effective range of the currently available IKKβ inhibitors is between nM and μM ranges either in cell based or in in vivo studies as reviewed recently (Gamble et al., 2012).

The finding that AIK18/85/1 did not affect TNF-α induced NF-κB (p65) subunit phosphorylation at Serine536 by concentrations of either 10 or 30μM, although phosphorylation of Serine536 is clearly linked to the transcriptional activation of NF-κB and further stimulation of Lysine 310 acetylation by enhancing the recruitment of p300 to NF-κB (p65) (Sheppard et al., 1999), (Chen and Greene, 2005). However, other findings raised a conflicting evidence for a role of Ser529/536 phosphorylation on RelA/p65 activation in response to TNF-α (Sizemore et al., 2002, Sakurai et al., 1999, Okazaki et al., 2003).

In addition, accumulating evidence has revealed that Serine 276 is the major phosphorylation site of RelA/p65 induced by TNF-α and also it is critical for transactivation of RelA/p65 at least in response to TNF-α. Moreover, phosphorylation of RelA/p65 at serine 276 facilitates the formation of a stable complex (enhanceosome) with the co-activators CBP/p300, such assembly of a functional complex on the responsive promoters is considered an essential modification for transactivation process (Dong et al., 2008, Jamaluddin et al., 2007).

Surprisingly, AIK18/85/1 prevented TNF-α mediated NF-κB (p65) translocation to the nucleus in NCTC2544 cells. The mechanism by which this effect occurred remains elusive. Nuclear translocation of p65 is regulated by the presence of unmasked NLS (nuclear localisation sequences) within the amino terminus of its

RHD (Miyamoto and Verma, 1995), hence AIK18/85/1 could bind to and mask this sequence. As a MGB one might expect an interaction with the DNA binding domain however, this is unlikely to result in an effect upon the function of NLS. Furthermore AIK18/85/1 might inhibit nuclear translocation by blocking the functions of importin α/β, which are nuclear transport receptors also called (importins and exportins), most of which are members of the β-karyopherin family (Bednenko et al., 2003). It is a specialised system for transporting macromolecules through the nuclear pore complex (NPC) (Stewart, 2007). Usually the binding of protein cargoes to the transport system is mediated by the NLS of protein that is frequently recognised by the adaptor importin α (Gorlich et al., 1996). However NF-κB requires importin family members to cross the nuclear pore, particularly importin α3 and importin α4 are mediating TNF-α-stimulated NF-κB p50/p65 heterodimer translocation into the nucleus (Fagerlund et al., 2005). A recent study has used some peptidomimetic compounds to selectively inhibit nuclear import by importin α/β (Ambrus et al., 2010), likewise it is possible that AIK18/85/1 might interrupt the nuclear import system.

The well-known synthesised inhibitor of NF-κB nuclear translocation SN50 acts by conjugating the NLS of p50 to the amino acid sequence of the cell membrane permeable motif. SN50 is considered to block the intracellular recognition system for the NLS of NF-κB (Lin et al., 1995a, Lin et al., 1995b). It was also reported that the inducible nuclear import of NF-κB, AP-1, NFAT, and STAT1 in Jurkat T cells can be regulated by NLS peptide delivered through targeting members of the importin-α/β NLS receptor complex (Torgerson et al., 1998). A similar mechanism cannot be excluded for AIK18/85/1.

By contrast nuclear translocation induced by PMA was not altered by the effect of AIK18/85/1. Many possible scenarios could be speculated to explain this effect in relation to stimulus and different mechanisms mediating activation of the pathway. Previously it has been shown that activation of PAR-2 in NCTC2544 cells resulted in a strong activation of NF-κB reporter activity but with only very modest loss in IκBα levels (Goon Goh et al., 2008). PAR-2 is coupled to the activation of PKC isoforms

and would have a similar profile of NF-κB activation to that observed for PMA. In addition, inhibition of tyrosine phosphatase activity also results in nuclear translocation of NF-κB in the absence of IκBα loss (Imbert et al., 1996). As these mechanisms do not involve proteolytic degradation of IκBα and presumably exposure of the NLS within p65, there could be another PKC sensitive mechanism that drives nuclear translocation.

For instance TNF-α-induced phosphorylation of RelA/ p65 at Ser276, Ser529 or Ser536 could be essential for its ability to function as an activator of gene expression (Chen and Greene, 2004). In this study only TNF-α-induced phosphorylation of p65 at Ser536 was assessed but not Ser276 or Ser429. Interestingly, previous studies have shown that novel Pim-1 kinase stabilizes SOCS-1(suppressor of cytokine signalling 1) by phosphorylation (Chen et al., 2002). In addition, recent study showed that Pim-1 is also responsible for phosphorylating RelA/p65 at Ser276 to prevent it from SOCS-1-mediated ubiquitination upon TNF-α stimulation. However, as Pim-1 is present both in the nucleus and the cytoplasm whereas SOCS-1 is localised exclusively in the cytoplasm, it is thus conceivable that TNF-α-induced nuclear targeting of RelA/p65 can hardly enable SOCS-1 to its ubiquitination and subsequent degradation (Nihira et al., 2010). Thus, AIK18/85/1 may prompt p65 ubiquitination and degradation through modulating Pim-1 kinase.

Accordingly, the amount of NF-κB-DNA binding complex induced by TNF-α observed in gel shift mobility assay was significantly reduced by pretreatment with AIK18/85/1 in a concentration dependent manner (Figure 3.15). In addition, AIK18/85/1 also reduced NF-κB-DNA binding activity in response to PMA. This would be expected of a minor groove binder which has the ability to interrupt the binding of transcription factors to specific DNA sequences (Baraldi et al., 2004). It is unlikely that the characteristics of NF-κB-DNA binding induced by TNF-α or PMA would be different. However, as translocation of NF-κB in response to TNF-α was prevented, this additional effect was not observed for this stimulus.

Studies showed that MGBs inhibited the nuclear factor binding to DNA by direct steric interference, because in fact, most proteins which bind specifically to DNA AT-rich sequences have major contacts within the minor groove (Turner and Denny, 2000). This model did not apply to the action of AIK18/85/1 and the other novel compound AIK18/70, as both compounds failed to disrupt the DNA binding when added immediately prior to the binding reaction. This suggests another mechanism of action within the nucleus which prevents DNA binding. This is similar to results from a study conducted by Hatziieremia et al., in which cardamonin inhibited DNA binding but did not prevent nuclear translocation of NF-κB (Hatziieremia et al., 2006). These data may indicate that these compounds accumulate within the nucleus to have their effects.

Having established that AIK18/85/1 had an effect on the NF-κB pathway at the level of DNA binding and NF-κB translocation, the action of the compound in relation to the expression of inflammatory mediators was assessed. A large number of studies have suggested a role of NF-κB in the regulation of COX-2 and iNOS expression in several cell types including RAW264.7 macrophages (Paul et al., 1999), (Gomez et al., 2005), (Hatziieremia et al., 2006). AIK18/85/1 was found to inhibit the induction of both enzymes over the concentration range used to inhibit NF-κB reporter activity in NCTC2544 cells. It would have been more accurate to generate the equivalent reporter assay in RAW264.7 macrophages, as numerous studies have shown this (Liang and Gardner, 1999), (Lim et al., 2004, Khan et al., 2011). However, this would exceed the scope of thesis.

The results shown here support the interpretation that inhibition of NF-κB by AIK18/85/1 is responsible for the reduction in iNOS and COX-2 expression. The iNOS promoter has a number of NF-κB binding sites (Xie and Nathan, 1993), (Xie et al., 1994), (Eberhardt et al., 1996). In addition, substantial numbers of studies have demonstrated that inhibition of iNOS expression correlates with effects on the NF-κB pathway. For example, parthenolide a sesquiterpene lactone natural product, has been shown to inhibit activation of NF-κB in Jurkat T leukemia cells (Hehner et al., 1998), NF-κB translocation and iNOS transcription in LPS/IFN-γ-stimulated rat aortic

smooth muscle cells (Wong and Menendez, 1999) and share with artemisinin the inhibition of NO production in human astrocytoma T67 cells (Aldieri et al., 2003). Furthermore RAW264.7 macrophages express TLR4 which has been previously demonstrated to link to the IKK/IκB/NF-κB axis via MYD88 (Chen et al., 2005b), (Kawai and Akira, 2006). If time had allowed, the effect of AIK18/85/1 on intermediates of the NF-κB pathway would have been assessed, particularly effects on LPS-stimulated p65 translocation.

The results presented cannot exclude an effect on other signalling pathways and transcription factors that regulate iNOS and COX-2 expression. Both iNOS and COX-2 can be regulated at the transcriptional, translational and post-translational levels through modulation of protein stability, dimerization, phosphorylation, cofactor binding and availability of substrates (Aktan, 2004). Therefore, definition of the exact site and mechanism of action of AIK18/85/1 remain unclear. AIK18/85/1 was without effect on LPS-induced MAP kinase activation thus excluding a site of action upstream in this cascade. As this pathway plays a key role in the induction of iNOS and COX-2 through effects on AP-1, a number of novel inhibitors have been targeted this pathway (Pasparakis et al., 2002, Rajapakse et al., 2008), (Hsieh et al., 2008, Hsieh et al., 2011). However, AIK18/85/1 completely abolished PMA-stimulated AP-1 reporter activity in NCTC2544 cells. Assuming that AIK18/85/1 was without effect on MAP kinases in this cell type, this would suggest a direct effect upon AP-1 mediated transcription or the transcription factors that make up the complex including Fos, Jun or ATF (Karin et al., 1997). This is a similar profile of inhibition to that observed with cardamonin (Hatziieremia et al., 2006) and because AP-1 is essential for iNOS and COX-2 transcriptions (Chen et al., 1999) could also explain the effect of AIK18/85/1. In fact AIK18/85/1 was more potent against COX-2 expression than iNOS possibly reflecting the different contributions that NF-κB and AP-1 make to the inductions of these proteins.

A number of studies have looked more closely at the assembly of the transcription factor complexes as a site of drug interaction. It could be suggested the involvement of an architectural transcription factor like the high mobility group (HMG)-I(Y)

protein (recently known as HMGA1) contributes to iNOS gene transactivation by inflammatory mediators (Pellacani et al., 2001). HMGA1 does not drive transcription itself, but it facilitates the assembly of a functional nucleoprotein complex (enhanceosomes). Hence, it was reported that binding of both HMGA1 and NF-κB subunits in the downstream 5`-flanking sequence of the iNOS gene was essential for the most potent activation of the promoter (Perrella et al., 1999). Moreover, it has also been demonstrated that HMGA1 itself was up-regulated (both at the mRNA and protein levels) by inflammatory cytokines (Pellacani et al., 1999). Since HMGA1 bind exclusively to AT-rich DNA in the minor groove (Bewley et al., 1998, Huth et al., 1997) the interference of MGBs with HMGA1-DNA binding was investigated in vitro (Wegner and Grummt, 1990, Radic et al., 1992) and in vivo (Baron et al., 2004b). For example distamycin A conferred a significant survival benefit following intraperitoneal injection of LPS and attenuated the hypotensive response during murine endotoxemia. This beneficial effect in vivo correlated with attenuation of iNOS induction in tissues and in murine macrophages. Furthermore MGBs interfered specifically with transcription factors-DNA binding in a selective fashion to a distinct AT-rich region of the iNOS enhancer (Baron et al., 2004b). Moreover, this kind of interaction was also confirmed by distamycin A-mediated inhibition of HMGA1-Binding to the P-Selectin Promoter, which resulted in attenuation of lung and liver inflammation during murine endotoxemia (Baron et al., 2010).

It has been established that NO production is enhanced by IFNγ (Nathan, 1992), (Lorsbach et al., 1993). IFNγ, through the JAK/STAT1 pathway, mediates the induction of iNOS (Delgado, 2003). Thus, in order to assess the specificity of AIK18/85/1, the effect on iNOS induction in response to IFNγ was also examined in RAW264.7 macrophages. Although the effect of AIK18/85/1 on upstream phosphorylation of the cytoplasmic transcription factor STAT1α was not extensively investigated, the primary experiment showed only slight reduction of phosphorylation. Unexpectedly it was found that the compound was also highly effective against IFNγ-induced iNOS expression. This result may be attributed to the interference of the compound with different key sites within the pathway; including the formation of STAT1 dimerisation and/or their eventual translocation into the

nucleus to bind to the distinct IFNγ responsive promoter region known as γ-activating sequences (GAS) (Stark et al., 1998), which in turn directly activate transcription of several genes, including interferon regulatory factor-1 (IRF1) (Subramaniam et al., 2001), (Ramana et al., 2002). This in turn activates transcription of secondary responsive genes, through the binding to interferon-stimulated response element (ISRE) sites (Stoffels et al., 2007). Further investigation is required to determine the exact site of action of AIK18/85/1 upon IFNγ-stimulated iNOS expression.

A large number of NF-κB inhibitors have been developed (Gilmore and Herscovitch, 2006). They can be broadly classified as either upstream or downstream inhibitors of NF-κB activity. The upstream inhibitors are largely focused on the specific inhibition of IKKβ and despite activity in vitro against this kinase AIK18/85/1 was ineffective within this pathway at the level of IKKβ-mediated IκBα phosphorylation and degradation in a cellular setting. Downstream inhibitors comprise compounds with the ability to modulate NF-κB nuclear translocation, DNA binding, and transactivation. Targeting these events specifically is likely to be much more difficult but could represent an interesting alternative to the existing small molecule inhibitors. However, far more studies are required. The potential inhibitory effects of AIK18/85/1 in nuclear functions remain to be determined in order to help identify clearly the mechanism of action.

CHAPTER 4

EFFECTS OF ALKALOID COMPOUNDS ON NF-ĸB SIGNALLING IN NCTC2544 CELLS AND RAW264.7 MACROPHAGES

4.1 Introduction

In this chapter three alkaloid compounds generated from an in-house screening program (SU-compounds) have been examined for their effects on the NF-κB pathway in similar way to that carried out in chapter three. One of these compounds, SU182 (canthine-6-one): 6H-indolo [3,2,1-de][1,5]naphthyridin-6-one (Figure 4.1a); is a β-carboline alkaloid previously extracted from different plant families. Canthine-6-one isolated from the plant Zanthoxylum chiloperone (Rutaceae) (Thouvenel et al., 2003), possesses a broad spectrum of antifungal and leishmanicidal activities (Ferreira et al., 2002). Furthermore, it has been recently indicated that canthin-6-one exhibits trypanocidal activity in vivo in a mouse model of acute or chronic infection (Ferreira et al., 2007, Ferreira et al., 2011). Beside several β-carboline alkaloids the canthin-6-one alkaloid and its derivatives, isolated from Eurycoma longifolia, exhibit cytotoxic activities against a panel of human cancer cell types including; breast, colon, fibrosarcoma, lung, melanoma, KB, KB-V1 and murine lymphocytic leukemia (Cao et al., 2007).

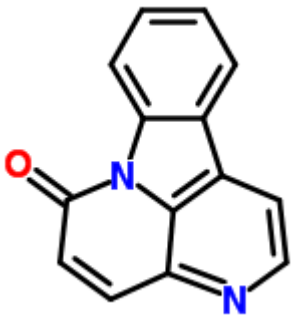
Despite the broad action of canthin-6-one, its underlying mechanism of action is unknown. Recently, it has been reported that Quassidines, (ex: quassidine E)-a bis-β-carboline alkaloid in which a canthin-6-one moiety and a β-carboline moiety are connected together by a single carbon–carbon bond, showed potent inhibitory activity on the production of NO, TNF-α, or IL-6 in RAW264.7 macrophages stimulated by LPS (Jiao et al., 2011). Thus, here in this study canthin-6-one was investigated for its anti-inflammatory activity specifically through examining its effect on the NF-κB pathway.

In addition, two further alkaloid SU-compounds SU331: 2-amino-3-bromo-5-(pyridin-4-yl) benzamide (Figure 4.1b) and the β-carboline SU432: 6-bromo-9H-pyrido[3,4-b] indole also known as (eudistomin N) (Figure 4.1c) were investigated for their possible effects on the IKK/NF-κB pathway, using the approaches outlined in chapter three. These include measuring cellular loss of IκBα, phosphorylation of NF-κB (p65-Ser536), NF-κB-DNA binding activity, and transactivation activities

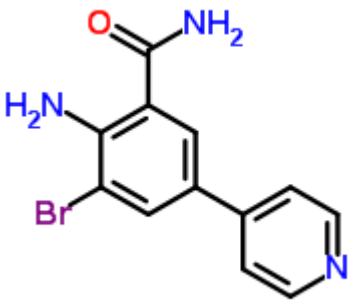
and the expression of inflammmtory proteins such as iNOS and COX-2, known to be regulated by this pathway.

Assays of these parameters and the direct assessment of IKK activity in vitro, allowed the successful development of specific IKKβ inhibitors at Millennium Pharmaceuticals Inc. (Hideshima et al., 2002, Castro et al., 2003). The ability of a selective IKK inhibitor to block the activation of NF-κB in whole cells, exemplified by PS-1145 (Castro et al., 2003), is a key aspect of developing new drugs (Yemelyanov et al., 2006). Nevertheless the three compounds under study were originally found to possess inhibitory effects on IKKβ examined as part of an "in-house" screening program and represent potentially routes to novel drugs. Assays carried out in this Chapter to assess the effects of compounds are similar to those investigated in Chapter 3. Experiments were performed under similar conditions including stimulation times as well as concentrations of stimuli and compounds tested.

a)



b) **c)**



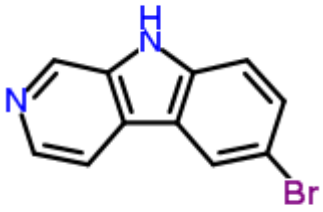


Figure 4.1: Chemical structure of alkaloid SU-compounds. a) SU182: (Canthin-6-one). 6H-indolo [3,2,1-de][1,5] naphthyridin-6-one. b) SU331: 2-amino-3-bromo-5-(pyridin-4-yl) benzamide. c) SU432 (eudistomin N): 6-bromo-9H-pyrido [3,4-b] indole.

4.2 Effects of SU182 on TNF-α-, PMA-induced NF-κB- and PMA-induced AP-1-linked luciferase reporter activities in NCTC2544 cells.

NCTC2544 cells were pretreated with increasing concentrations of SU182 over a range of 0.3-30μM for 30 min prior to stimulation with (10ng/ml) TNF-α or (100nM) PMA for 6 h, and were evaluated for transcriptional activity by luciferase reporter assay as described in Section 2.2.2. As shown in Figure 4.2a, TNF-α stimulation resulted in an increase of NF-κB-linked luciferase reporter activity of approximately 8 fold above basal (8.3 ± 3.6 fold). Pretreatment with SU182 produced a concentration dependent reduction in TNF-α induced NF-κB reporter activity. The maximum reporter activity was reduced by approximately 80% (1.9 ± 0.4 fold of stimulation) at 30μM, about 60% (3.6 ± 1.2 fold) at 10μM and by around 30% (5.9 ± 2.2 fold) at 3μM. Despite the clear concentration dependent effect of SU182 on TNF-α induced activity inhibition was non-significant statistically.

Figure 4.2b shows that NF-κB reporter activity increased in response to PMA stimulation by about 102.9 ± 21.4 fold above basal values. SU182 caused significant inhibition of NF-κB reporter activity at all concentrations applied. It reduced reporter activity to approximately 30% of maximum stimulation at 30μM (35.6 ± 2.7 fold, $p < 0.001$), and to exactly 35.4 ± 4.1 fold ($p < 0.01$) at 10μM. In contrast to the effect of SU182 on TNF-α-induced NF-κB reporter activity, PMA activation was significantly inhibited by SU182 in a concentration dependent manner which was even achieved at the lowest concentration used, 0.3μM ($p < 0.05$).

In addition, the effect of SU182 was evaluated against PMA-induced AP-1-linked luciferase reporter activity in NCTC2544 cells. PMA caused stimulation of AP-1 reporter activity giving a maximum stimulation of 5.4 ± 0.4 fold. Pretreatment of AP-1 reporter cells by SU182 led to reduction in the PMA-induced AP-1 reporter activity in a concentration dependent manner. A significant reduction of stimulation, approximately half, was achieved at 30μM (2.6 ± 0.5 fold, $p < 0.001$) as illustrated in Figure 4.2c. However no significant effect was observed at lower concentrations.

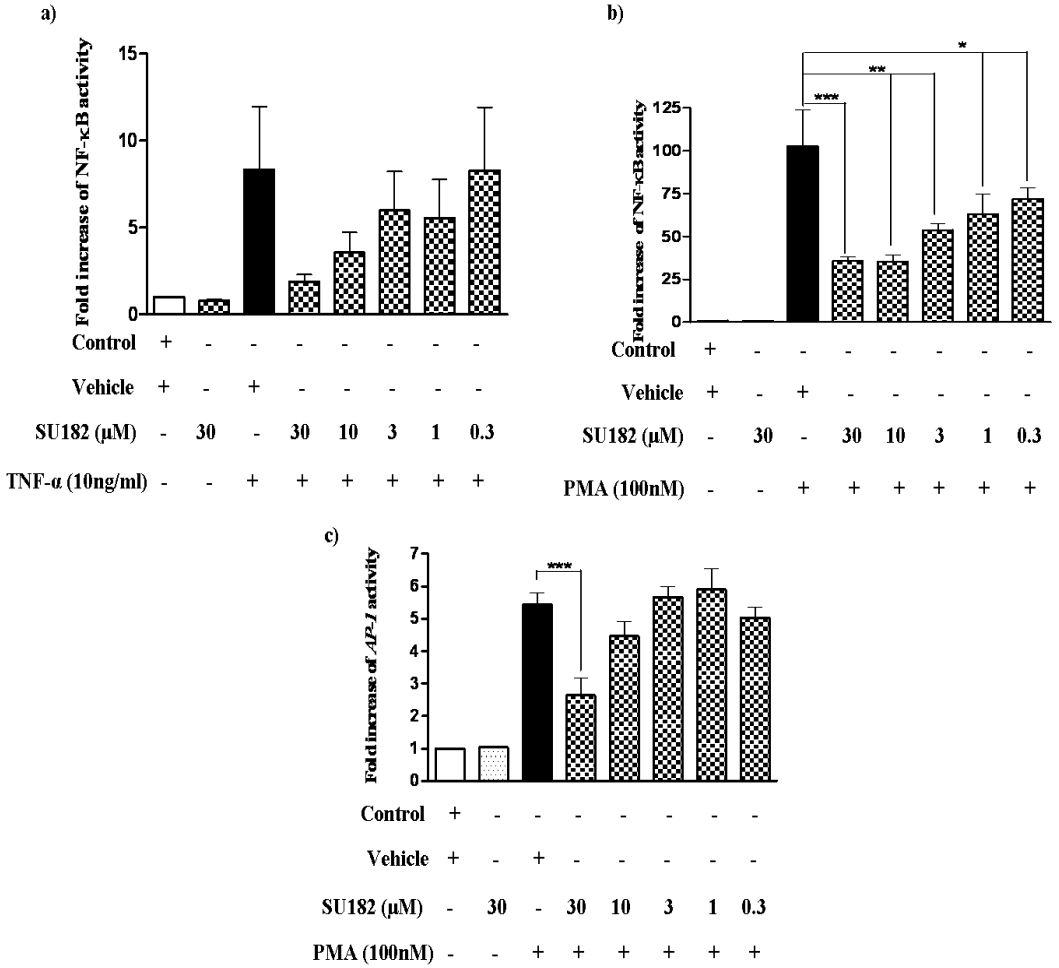


Figure 4.2: Effects of SU182 on NF-κB- and AP-1-linked luciferase reporter activities in NCTC2544 cells. Reporter cells were serum starved overnight then treated with SU182 (0.3-30μM) for 30 min prior to 6 h stimulation with TNF-α (10ng/ml) in panel **a**, and with PMA (100nM) in panels **b** and **c**. Lysed cells were then assayed for NF-κB transcriptional activity in **a**, **b** and for AP-1 transcriptional activity in **c** as outlined in Section 2.2.2. Each value represents the mean ± s.e.m., of three independent experiments, * = $p < 0.05$, ** = $p < 0.01$and *** = $p < 0.001$ compared with stimulated control group.

4.3 Effects of SU331 on TNF-α-, PMA-induced NF-κB- and PMA-induced AP-1-linked luciferase reporter activities in NCTC2544 cells.

SU331 was tested on NF-κB reporter NCTC2544 cells, as shown in Figure 4.3a. TNF-α caused induction of NF-κB reporter activity to 14.3 ± 3.1 fold above basal values. This maximum activation was inhibited in a concentration dependent manner following pretreatment with SU331. A marked, significant inhibition of approximately 85% reduction of NF-κB reporter activity was achieved at 30μM (2.1 ± 0.6 fold, $p < 0.01$) with roughly 60% inhibition at 10μM (5.4 ± 1.2 fold, $p < 0.05$). However, the maximum NF-κB transcriptional activity induced by PMA was only significantly inhibited by SU331 (from 151.8 ± 31.9 fold to 36.1 ± 16.2 fold, $p < 0.05$) at 30μM as shown in Figure 4.3b. The observed inhibition that was achieved at 10μM was not significant (76.1 ± 19.5 fold). In contrast, AP-1 reporter transcriptional activation by PMA was weakly affected by SU331 as illustrated in Figure 4.3c. A marked but non-significant inhibition was only observed at 30μM, fold stimulation was reduced by approximately half (reduced from 6.6 ± 1.5 fold to 3.7 ± 0.5 fold).

4.4 Effects of SU432 on TNF-α-, PMA-induced NF-κB- and PMA-induced AP-1-linked luciferase reporter activities in NCTC2544 cells.

The effect of SU432 was evaluated in NF-κB-linked luciferase reporter NCTC2544 cells, as can be seen in Figure 4.4a. Generally speaking, SU432 produced a concentration dependent inhibition of TNF-α induced transcriptional activity. This was apparent at 30μM of the compound when maximum activity declined from 14.3 ± 3.1 fold to 3.0 ± 0.5 fold of stimulation ($p < 0.05$). Inhibition was also observed at 10μM, maximum activity fell to half of control stimulated values (6.9 ± 1.7 fold). However, the effect of SU432 on PMA-mediated NF-κB transcriptional activity was weak compared to its effect against TNF-α. As shown in Figure 4.4b, no significant difference in the inhibition observed following pretreatment of cells with 30μM SU432 (from 151.8 ± 31.9 fold to 70.2 ± 35.2 fold). In addition, AP-1-linked luciferase reporter activity induced by PMA in NCTC2544 cells was not significantly affected by any of applied concentrations of SU432 compound as shown in Figure 4.4c.

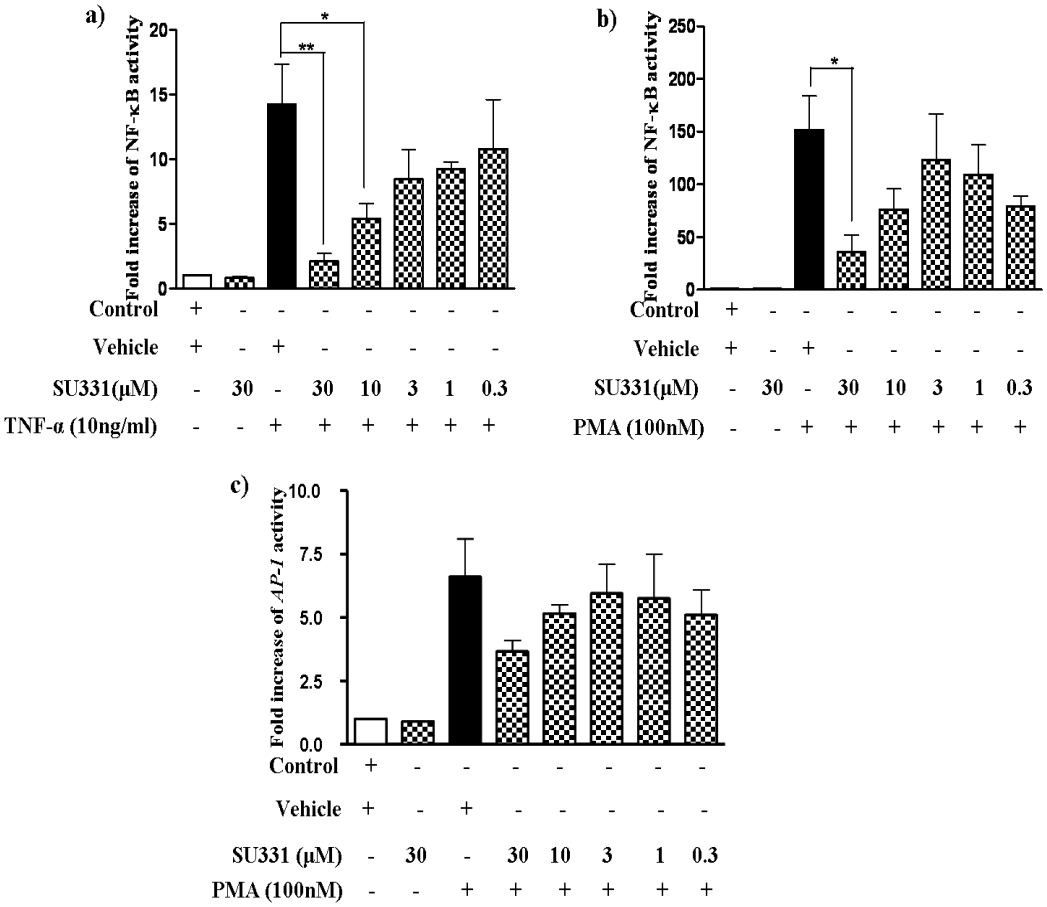


Figure 4.3: Effects of SU331 on NF-κB- and AP-1-linked luciferase reporter activities in NCTC2544 cells. Reporter cells were serum starved overnight then treated with SU331 (0.3-30μM) for 30 min prior to 6 h stimulation with TNF-α (10ng/ml) in panel **a**, and with PMA (100nM) in panels **b** and **c**. Lysed cells were then assayed for NF-κB transcriptional activity in **a**, **b** and for AP-1 transcriptional activity in **c** as outlined in Section 2.2.2. Each value represents the mean ± s.e.m., of three independent experiments, * = $p < 0.05$ and ** = $p < 0.01$ compared with stimulated control group.

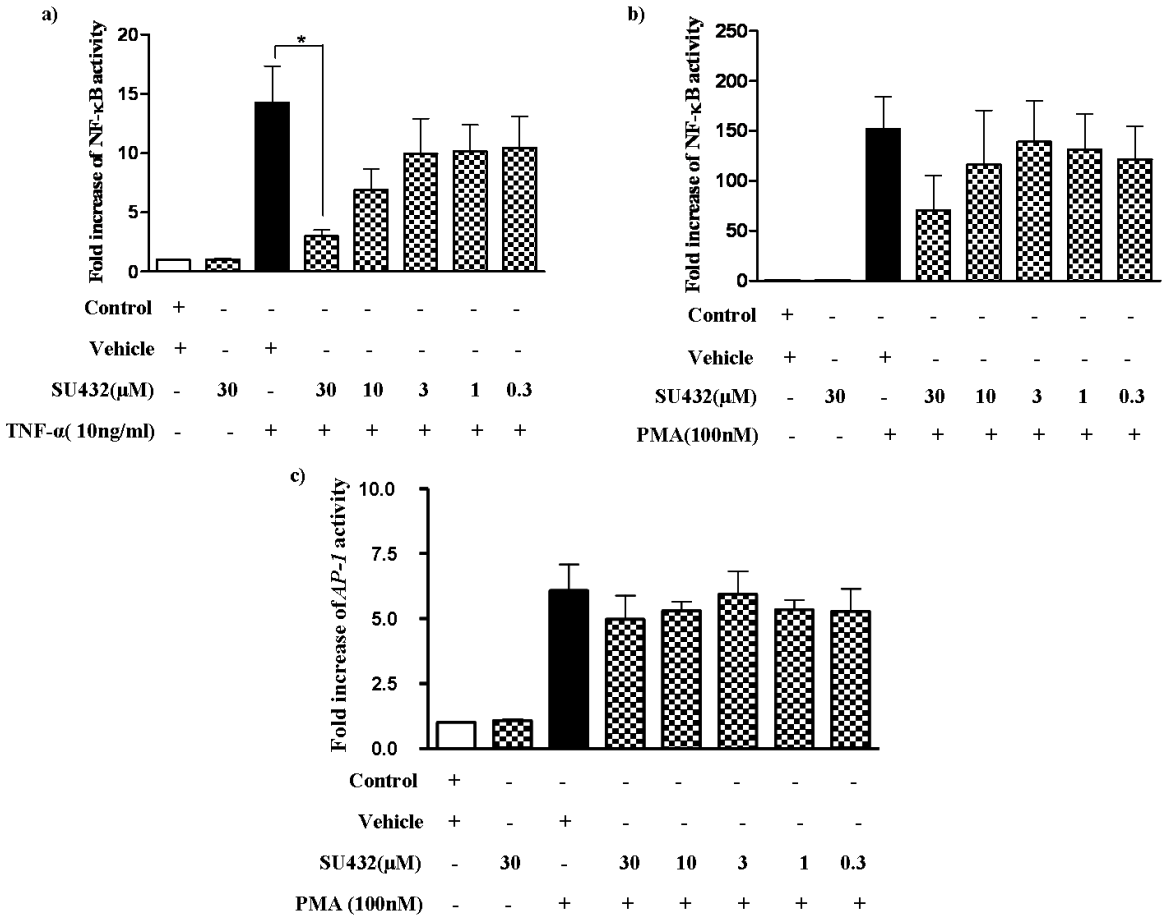


Figure 4.4: Effects of SU432 on NF-κB- and AP-1-linked luciferase reporter activities in NCTC2544 cells. Reporter cells were serum starved overnight then treated with SU432 (0.3-30μM) for 30 min prior to 6 h stimulation with TNF-α (10ng/ml) in panel **a**, and with PMA (100nM) in panels **b** and **c**. Lysed cell were then assayed for NF-κB transcriptional activity in **a,** and **b** and for AP-1 transcriptional activity in **c** as outlined in Section 2.2.2. Each value represents the mean ± s.e.m., of three independent experiments, * = $p < 0.05$ and compared with stimulated control group.

4.5 Effects of SU182, SU331 and SU432 on TNF-α-induced IκBα degradation in NCTC2544 cells.

Having established that TNF-α produced IκBα protein degradation in NCTC2544 cells at 30 min as shown in the previous chapter (Figure 3.5a), a range of concentrations (3-30μM) of SU182, SU331 and SU432 compounds were added to cells 30 min prior to TNF-α stimulation for further 30 min. Cell lysates were then assessed for IκBα protein by Western Blot analysis as outlined in Section 2.2.3.

Results illustrated in Figure 4.5 indicated that SU182 did not cause any reversal of IκBα degradation induced by TNF-α (0.29 ± 0.11 fold) at any concentrations of the compound utilised. In contrast to SU182, as shown in Figure 4.6, the inhibitory effect of SU331 on TNF-α-stimulated IκBα protein loss was marked. In particular at 30μM, TNF-α induced IκBα protein degradation was reduced by approximately 50% (from 0.25 ± 0.06 fold to 0.60 ± 0.04 fold, $p < 0.01$).

Similar to SU331, SU432 interfered with the degradation of IκBα, the upstream inhibitory signalling component of NF-κB pathway that binds to NF-κB isoforms to render them inactive. Nevertheless the reversal effect was clear but non-significant on TNF-α induced IκBα protein loss at 30μM. This inhibitory effect of SU432 illustrated in Figure 4.7 as protein levels increased by approximately 50% (from 0.21 ± 0.01 fold to 0.43 ± 0.11 fold).

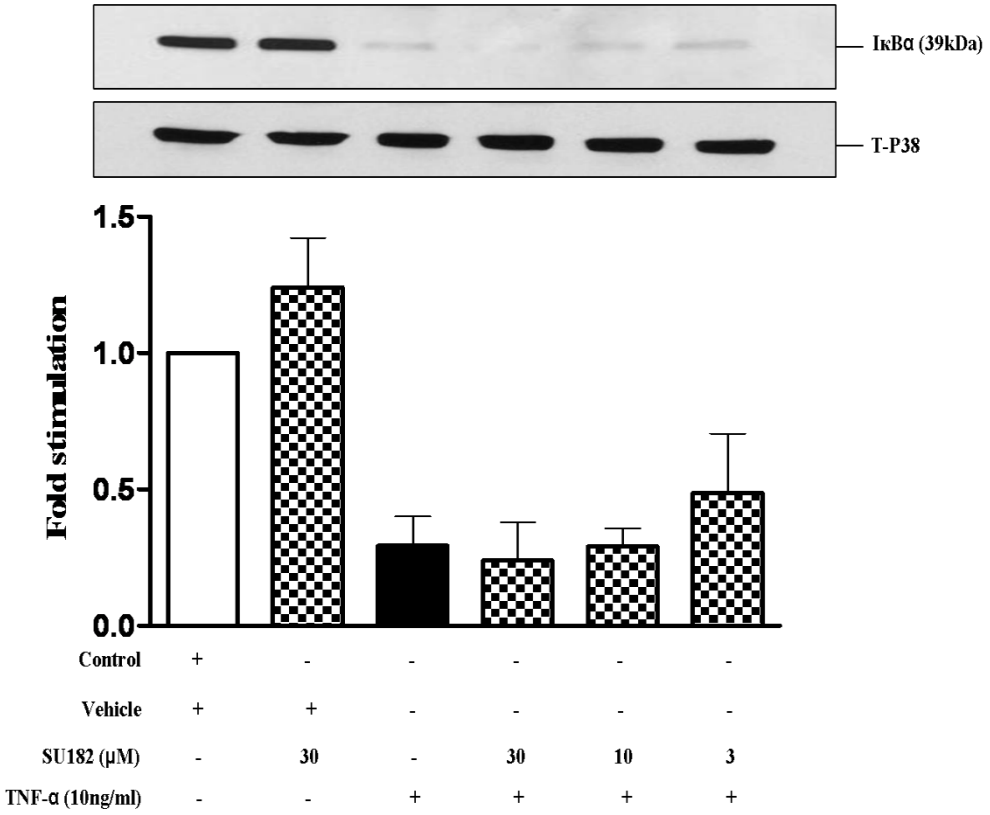


Figure 4.5: Effect of SU182 on TNF-α-induced IκBα degradation in NCTC2544 cells. Cells were treated with SU182 (3-30μM) for 30 min prior to stimulation with TNF-α (10ng/ml) for a further 30 min. Whole cell lysates were prepared, separated by SDS-PAGE, then assessed for IκBα protein (39kDa) and re-probed for total p38-MAP kinase as outlined in Section 2.2.3. The results are representative for three independent experiments and each value represents the mean ± s.e.m.

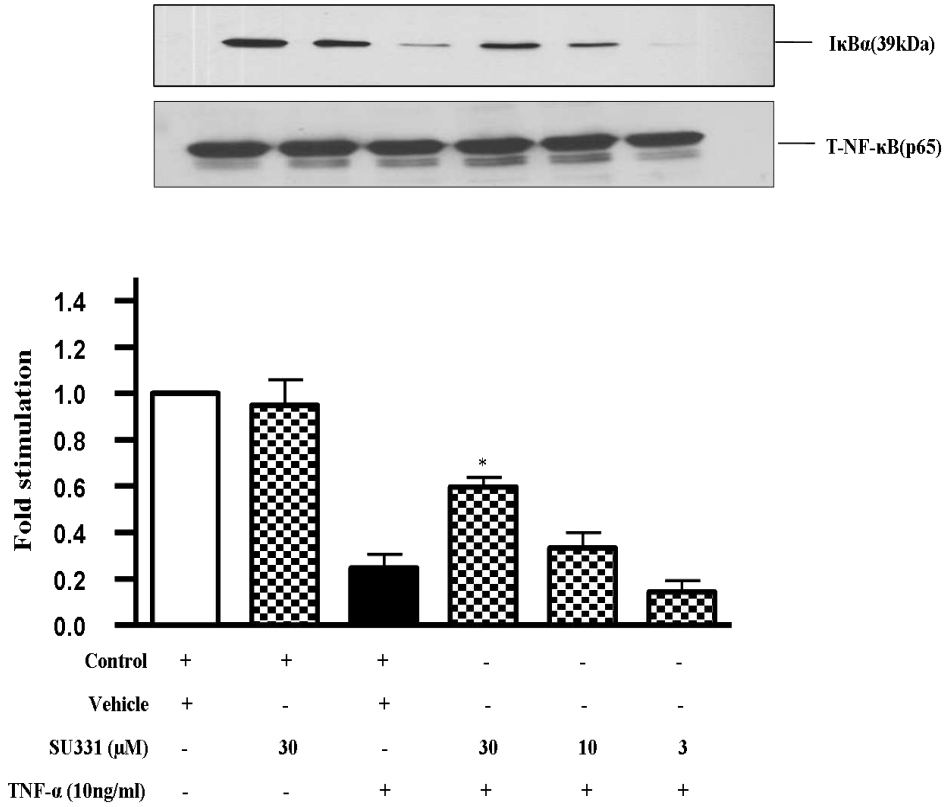


Figure 4.6: Effect of SU331 on TNF-α-induced IκBα degradation in NCTC2544 cells. Cells were treated with SU331 (3-30μM) for 30 min prior to stimulation with TNF-α (10ng/ml) for a further 30 min. Whole cell lysates were prepared, separated by SDS-PAGE, assessed for IκBα protein (39kDa) and then re-probed for total NF-κB (T-p65) as outlined in Section 2.2.3. The results are representative for three independent experiments and each value represents the mean ± s.e.m., * = $p < 0.05$.

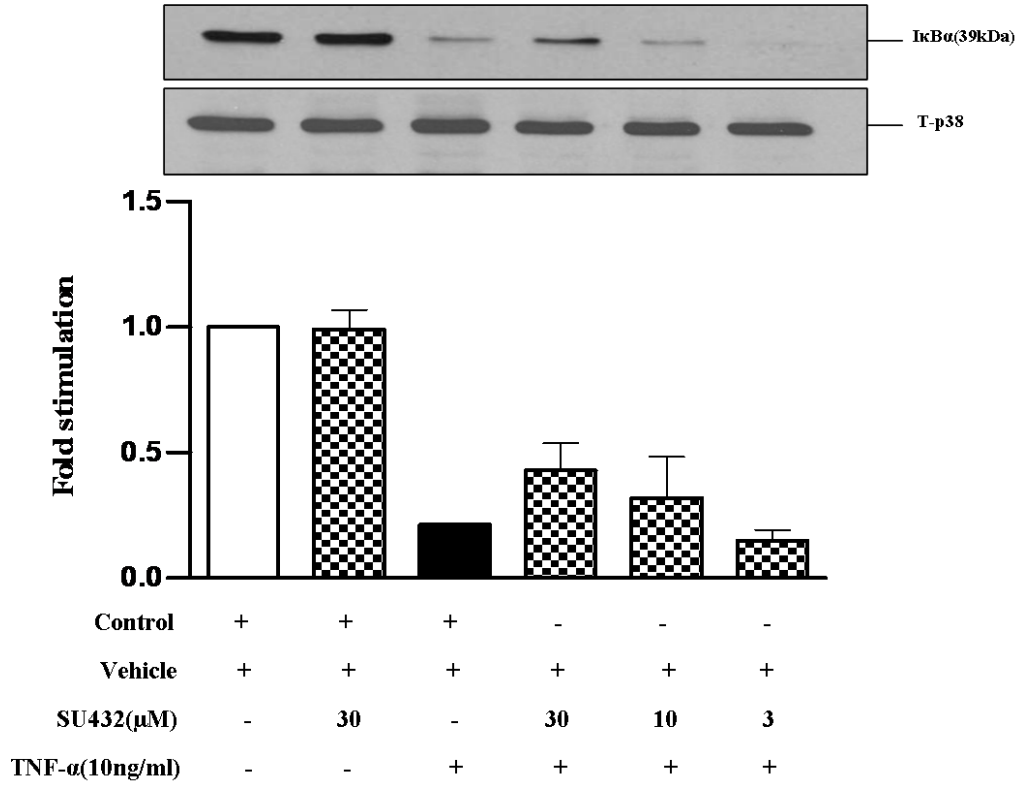


Figure 4.7: Effect of SU432 on TNF-α-induced IκBα degradation in NCTC2544 cells. Cells were treated with SU432 (3-30μM) for 30 min prior to stimulation with TNF-α (10ng/ml) for a further 30 min. Whole cell lysates were prepared, separated by SDS-PAGE, assessed for IκBα protein (39kDa) and then re-probed for total p38 MAP kinase as outlined in Section 2.2.3. The results are representative for three independent experiments and each value represents the mean ± s.e.m.

4.6 Effects of SU182, SU331 and SU432 on TNF-α-induced phosphoylation of NF-κB (Ser536-p65) in NCTC2544 cells.

Compounds were examined against a further upstream signalling component of the NF-κB pathway, which is the phosphorylation of the RelA (Ser536-p65) NF-κB subunit that can be induced by TNF-α, as shown in the previous Chapter (Figure 3.9).

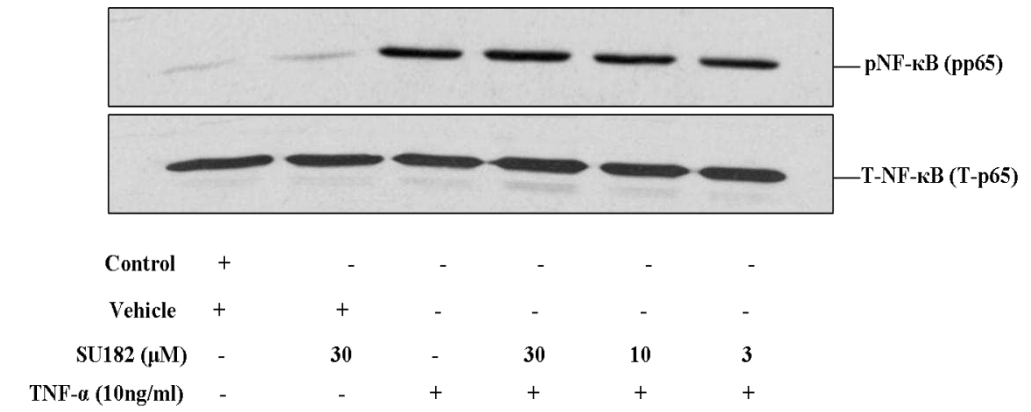
The effect of SU182 on p65 phosphorylation was evaluated as can be seen in Figure 4.8a. Again none of the concentrations of SU182 utilised caused any changes in the level of TNF-α-induced phosphorylation of p65 protein. Although SU331 and SU432 compounds were both able to interfere with IκBα degradation, unexpectedly neither affected TNF-α induced p65 phosphorylation nor the expression of p65 as shown in Figures 4.8b and 4.8c.

4.7 Effects of SU182, SU331 and SU432 on TNF-α-mediated NF-κB (p65) translocation into the nucleus in NCTC2544 cells.

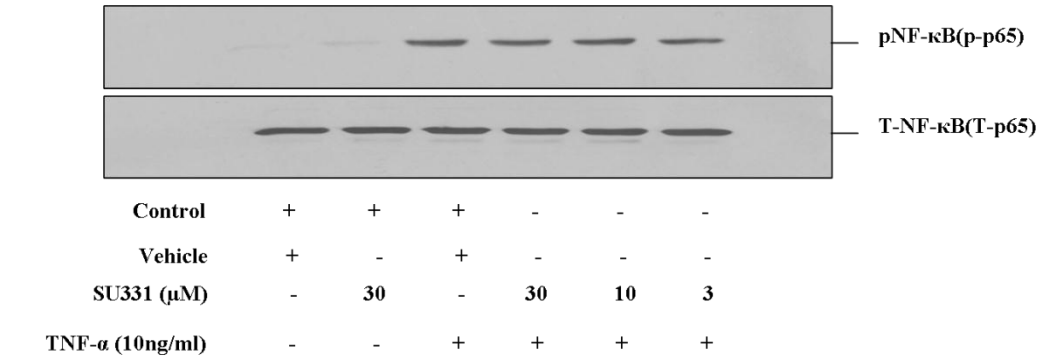
It has been demonstrated previously that TNF-α can induce NF-κB (p65) subunit translocation to the nucleus within 30 min of activation in NCTC2544 cells (see Section 3.11). This translocation is a crucial step for NF-κB subunits to bind to specific κB binding sites within the DNA of gene promoters. Figure 4.9 shows that pretreatment of cells with different concentrations of SU182 had no effect upon TNF-α-stimulated p65 translocation.

Consistently, TNF-α produced clear nuclear translocation of p65 detected by immunofluorescence at 30 min stimulation as shown in Figure 4.10. In contrast, pretreatment of cells with SU331 resulted in an inhibition of TNF-α-stimulated translocation of NF-κB, which was complete at 30μM. However, at 10μM only a proportion of the cells, approximately 50% showed nuclear localisation and no inhibitory effect was observed at 3μM. The TNF-α-induced nuclear localisation of p65 was also reduced following pretreatment with 30μM of SU432, as can be observed from images in Figure 4.11. No effect was observed at 10 and 3μM concentrations of the compound.

a)



b)



c)

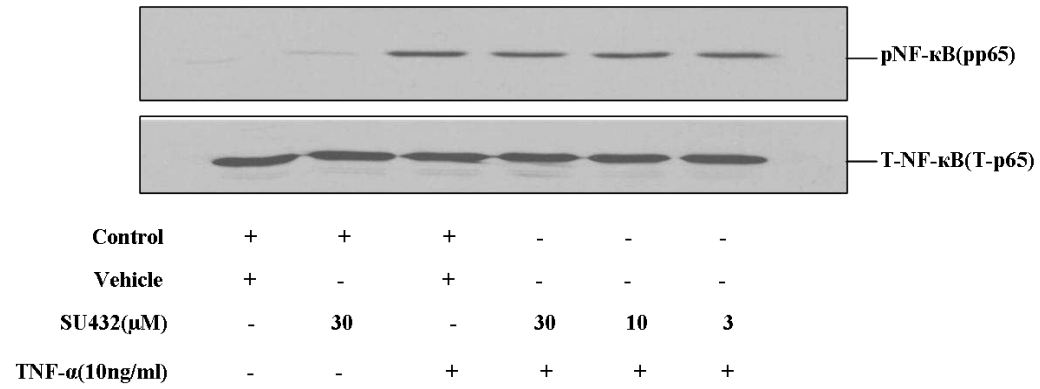


Figure 4.8: Effects of SU182, SU331 and SU432 on TNF-α-induced NF-κB (Ser536-p65) phosphorylation in NCTC2544 cells. Cells were treated with 3-30μM of SU182 panel **a**, SU331 panel **b** and SU432 in panel **c** for 30 min prior to stimulation with TNF-α (10ng/ml) for a further 15 min. Whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for phospho-NF-κB (Ser536-p65) or total NF-κB (T-p65) as outlined in Section 2.2.3. The results are representative of three independent experiments and each value represents the mean ± s.e.m.

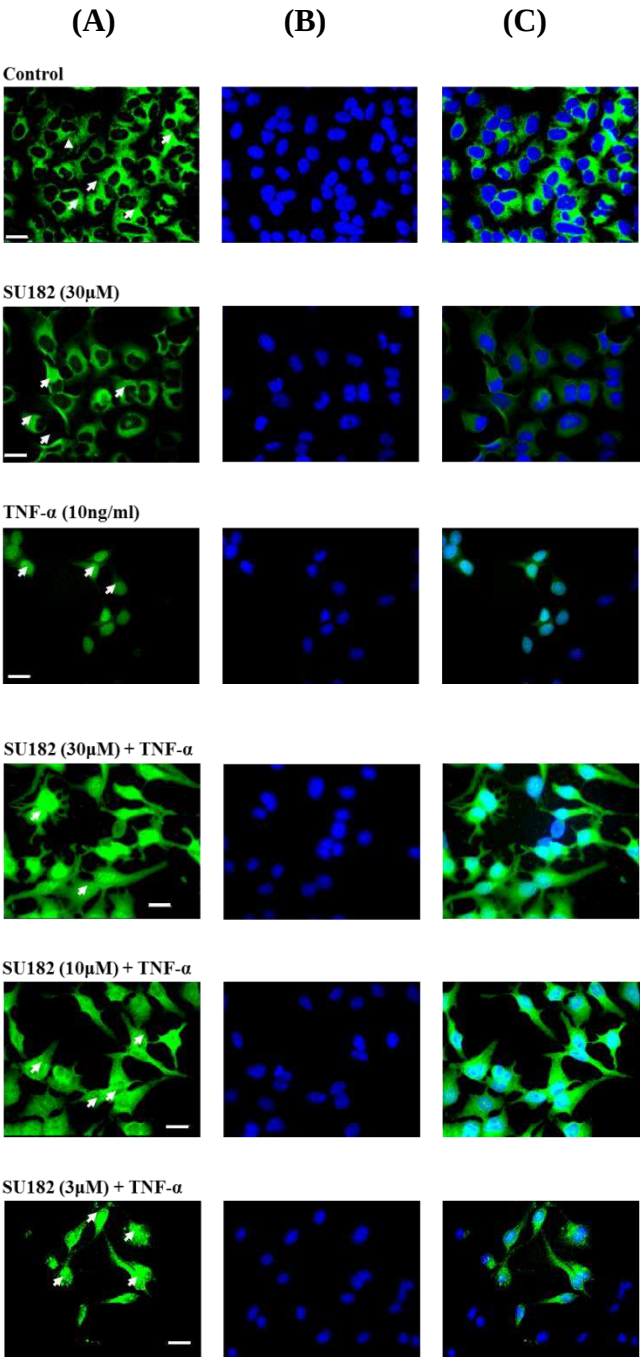


Figure 4.9: Effect of SU182 on TNF-α-induced NF-κB (p65) translocation into the nucleus in NCTC2544 cells. Quiescent cells grown on cover slips up to 70% confluence were treated with SU182 at concentrations (3-30µM) for 30 min prior to TNF-α stimulation for a further 30 min then evaluated by indirect immunofluorescence **(A)** NF-κB localisation was visualised using an anti-NF-κB (p65) antibody and a FITC-linked secondary antibody (green). **(B)** Nuclei were visualised by DAPI counterstaining (blue). **(C)** Merge of the two fluorescence images (green and blue). White arrows indicate the areas of p65 localisation. Images were acquired using fluorescence microscopy at 40x magnification (scale bar = 25µm). The results are representative of three independent experiments.

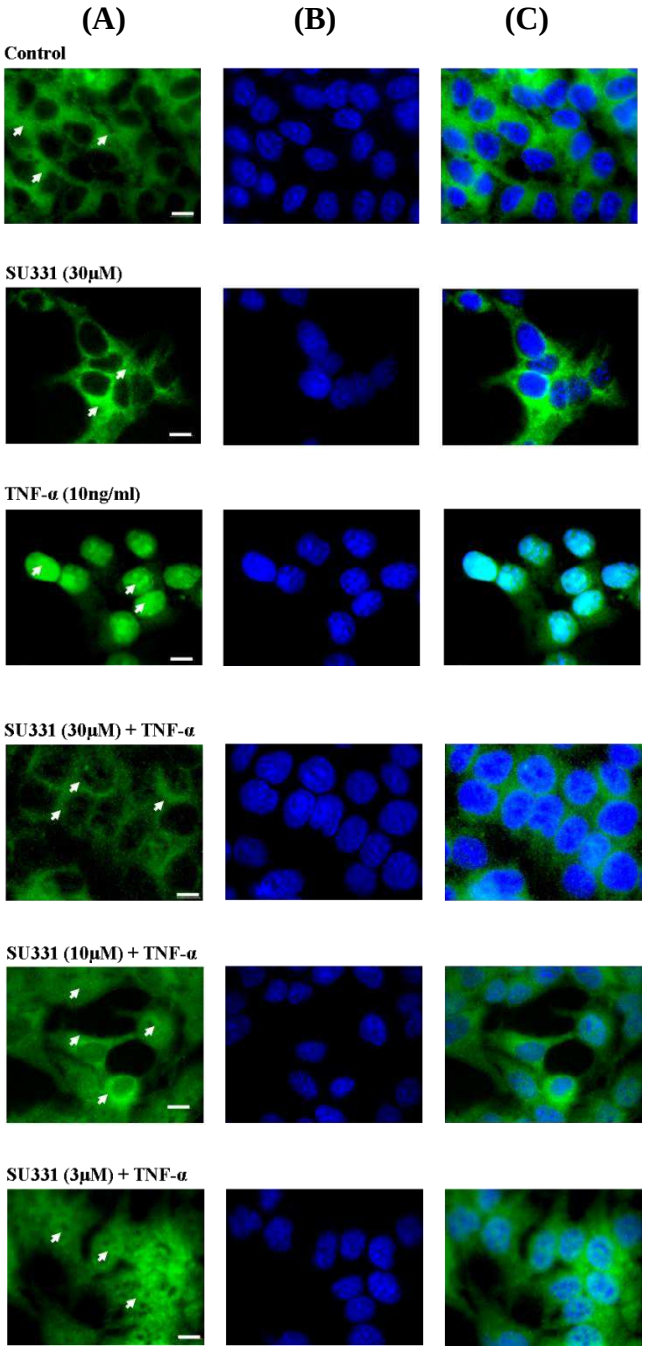


Figure 4.10: Effect of SU331 on TNF-α induced NF-κB (p65) translocation into the nucleus in NCTC2544 cells. Quiescent cells grown on cover slips up to 70% confluence were treated with SU331 at concentrations (3-30µM) for 30 min prior to TNF-α stimulation for a further 30 min then evaluated by indirect immunofluorescence **(A)** NF-κB localisation was visualised using an anti-NF-κB (p65) antibody and FITC-linked secondary antibody (green). **(B)** Nuclei were visualised by DAPI counterstaining (blue). **(C)** Merge of the two fluorescence images (green and blue). White arrows indicate the areas of p65 localisation. Images were acquired using fluorescence microscopy at 100x magnification (scale bar = 10µm). The results are representative of three independent experiments.

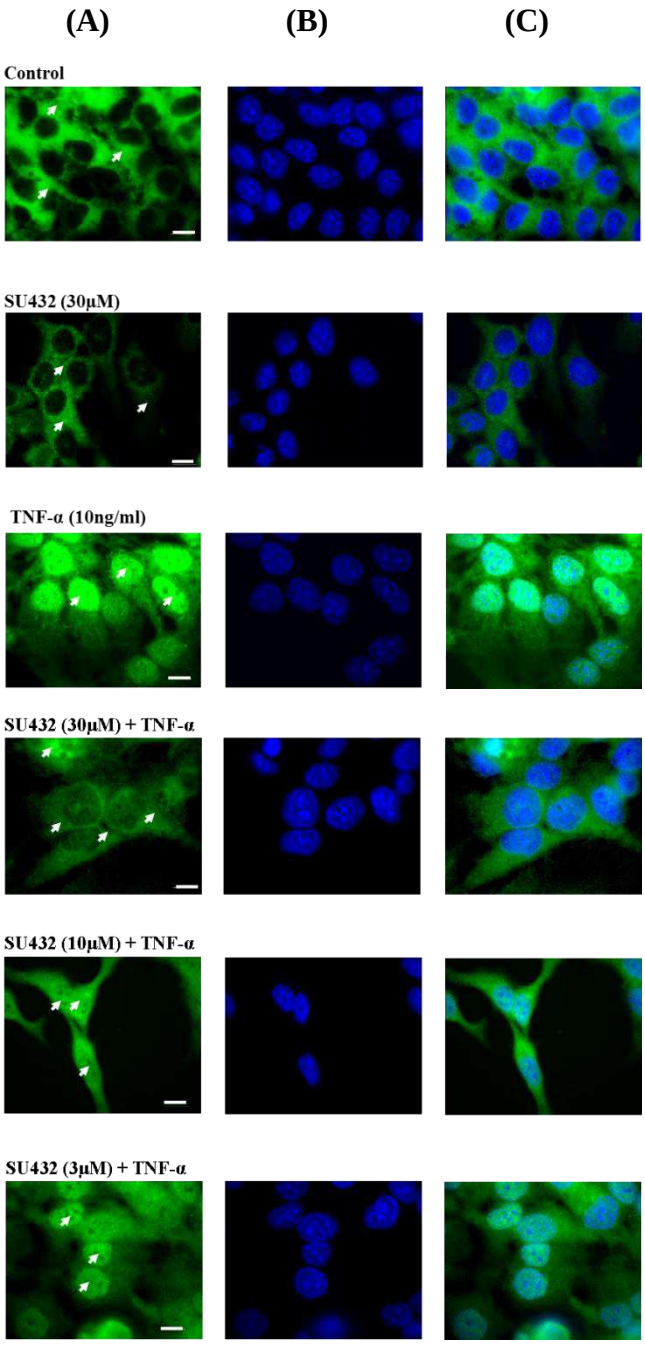


Figure 4.11: Effect of SU432 on TNF-α-induced NF-κB (p65) translocation into the nucleus in NCTC2544 cells. Quiescent cells grown on cover slips up to 70% confluence were treated with SU432 at concentrations (3-30µM) for 30 min prior to TNF-α stimulation for a further 30 min then evaluated by indirect immunofluorescence **(A)** NF-κB localisation was visualised using an anti-NF-κB (p65) antibody and a FITC-linked secondary antibody (green). **(B)** Nuclei were visualised by DAPI counterstaining (blue). **(C)** Merge of the two fluorescence images (green and blue). White arrows indicate the areas of p65 localisation. Images were acquired using fluorescence microscopy at 100x magnification (scale bar =10 µm). The results are representative of three independent experiments.

4.8 Effects of SU182, SU331 and SU432 on TNF-α-induced NF-κB-DNA binding activity in NCTC2455 cells.

The effect of SU alkaloid compounds on NF-κB binding with DNA in NCTC2544 keratinocytes stimulated with TNF-α was also investigated. Similar to SU52 that was studied in chapter 3, concentrations of compounds applied were 10, 30, and 50μM. Nuclear extracts were prepared from duplicate samples, and then analysed for NF-κB-DNA complex formation by EMSA.

Indeed, whilst TNF-α enhanced DNA binding of NF-κB, as shown in Figure 4.12 SU182 did not inhibit TNF-α-induced NF-κB-DNA binding activity, but rather values were increased non-significantly by almost 2 fold. This result was compatible with the lack of effect of SU182 on the upstream proteins, IκBα and p65.

As the other SU alkaloids SU331 and SU432 were effective specifically in attenuation of TNF-α-mediated IκBα protein degradation, it was likely that a reduction would be observed in nuclear translocation. Thus, accordingly, nuclear NF-κB-DNA binding activity was also reduced. As shown in Figure 4.13 maximum induction of NF-κB-DNA complex formation by TNF-α activation was reduced to approximately half (53.3 % ± 19.2 of binding activity) following pretreatment with 50μM SU331. In addition, binding activity was reduced by about 20% of maximum levels (80.3 % ± 17.3) and (87.6 % ± 27.1) at 30 and 10μM respectively.

In addition to the inhibitory effect of SU432 observed on upstream proteins, a marked decline in TNF-α-induced NF-κB-DNA binding activity to 46.1% ± 13.9 at 50μM and 55.1% ± 9.5 at 30 μM was also attained as shown in Figure 4.14.

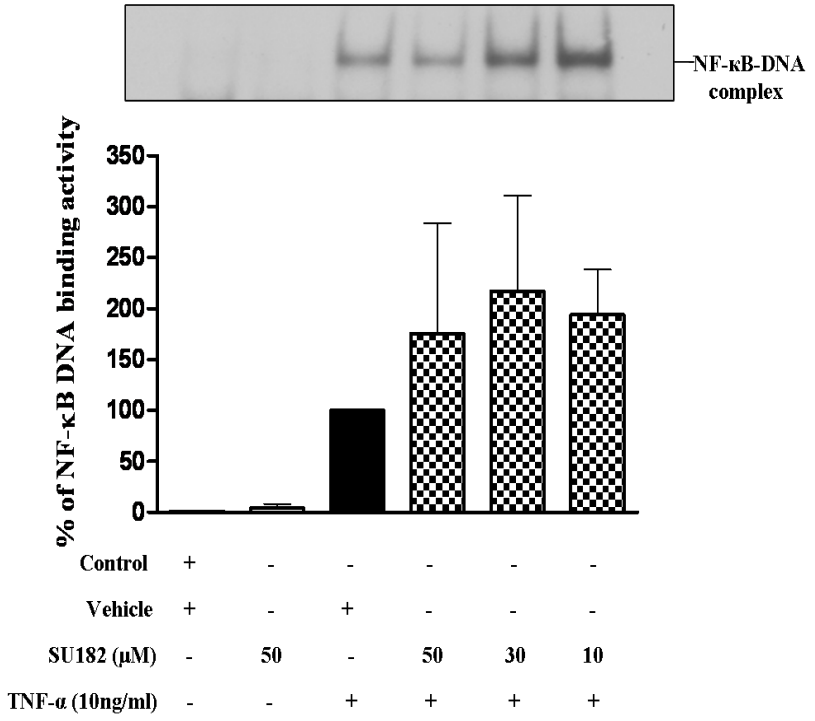


Figure 4.12: Effect of SU182 on TNF-α-induced NF-κB-DNA binding activity in NCTC2544 cells. Cells were grown in 6 well plates as duplicates, serum starved overnight, treated with 10, 30 and 50μM SU182 for 30 min prior to stimulation with TNF-α (10ng/ml) for a further 30 min. Vehicle (DMSO) was added to control untreated wells in parallel to TNF-α. Nuclear extracts were analysed by EMSA as outlined in Section 2.2.4. NF-κB-DNA binding complex bands are expressed as % of maximum binding activity. Results are representative of three independent experiments and each value represents the mean ± s.e.m.

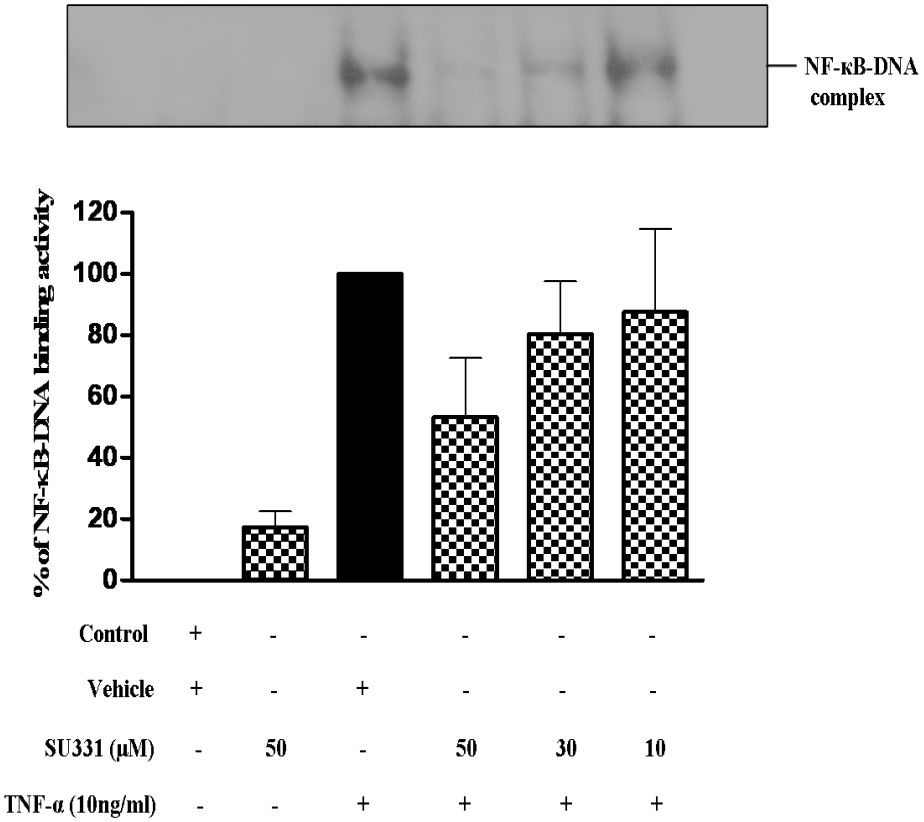


Figure 4.13: Effect of SU331 on TNF-α-induced NF-κB-DNA binding activity in NCTC2544 cells. Cells were grown in 6 well plates as duplicates, serum starved overnight, treated with 10, 30 and 50μM SU331 for 30 min prior to stimulation with TNF-α (10ng/ml) for a further 30 min. Vehicle (DMSO) was added to control untreated wells in parallel to TNF-α. Nuclear extracts were analysed by EMSA as outlined in Section 2.2.4. NF-κB-DNA binding complex bands are expressed as % of maximum binding activity. Results are representative of three independent experiments and each value represents the mean ± s.e.m.

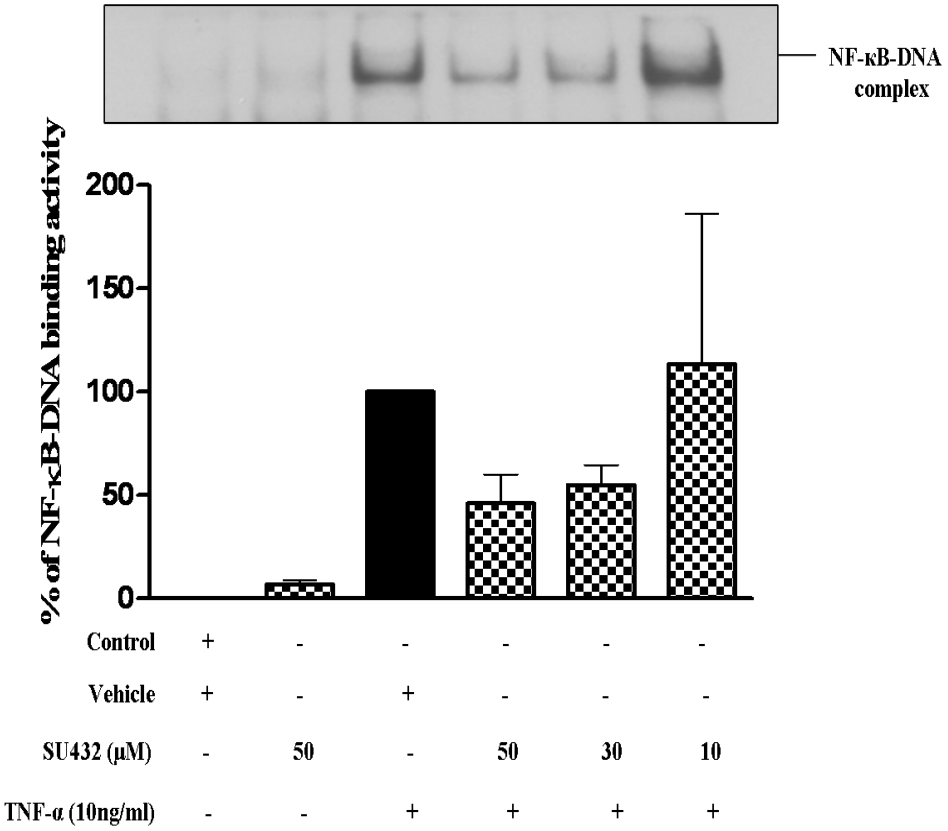
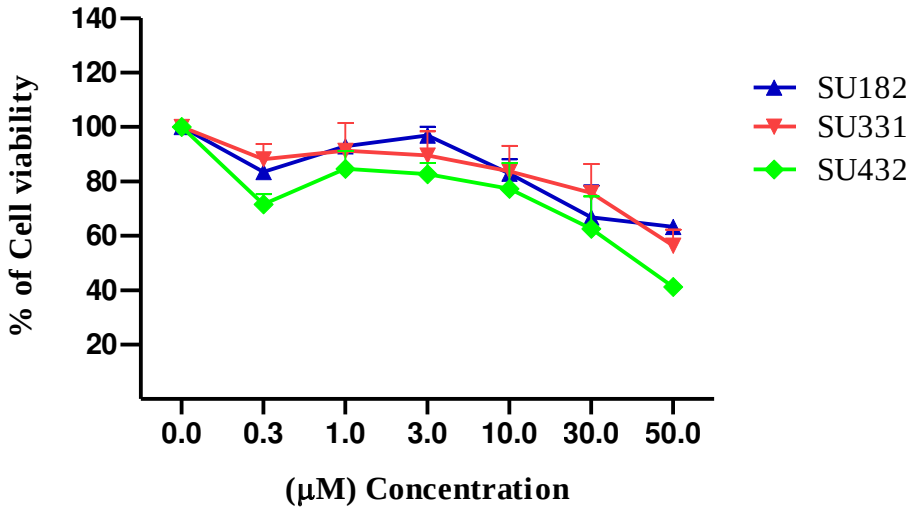


Figure 4.14: Effect of SU432 on TNF-α-induced NF-κB-DNA binding activity in NCTC2544 cells. Cells were grown in 6 well plates as duplicates, serum starved overnight, treated with 10, 30 and 50μM SU432 for 30 min prior to stimulation with TNF-α (10ng/ml) for a further 30 min. Vehicle (DMSO) was added to control untreated wells in parallel to TNF-α. Nuclear extracts were analysed by EMSA as outlined in Section 2.2.4. NF-κB-DNA binding complex bands are expressed as % of maximum binding activity. Results are representative of three independent experiments and each value represents the mean ± s.e.m.

4.9 Assessing the effects of SU182, SU331 and SU432 on the production of inflammatory mediators in NCTC2544 cells and RAW 246.7 macrophage cells.

Similar to AIK18/85/1 that was investigated in Chapter 3, SU-alkaloid compounds were assessed for their effects on iNOS and COX-2 expression in RAW264.7 macrophage cells. Whilst each compound was able to inhibit NF-κB activation in NCTC2544 cells a cytotoxic effect was observed following prolonged periods of treatment of cells with the compounds. Around a 20-60% reduction in cell viability was observed between the range of 10-50μM concentrations (Figure 4.15a). However, treatment of RAW264.7 macrophage cells with SU182 and SU331 resulted in non-significant minor cytotoxic effects (10-15% loss in cell viability) between the range of 3-50μM concentrations (Figure 4.15b). Whereas SU432 did not exhibit any toxic effect at concentrations used. Thereby these compounds were assessed for their effects on LPS-induced iNOS and COX-2 expression in the RAW264.7 macrophage cell line.

a)



b)

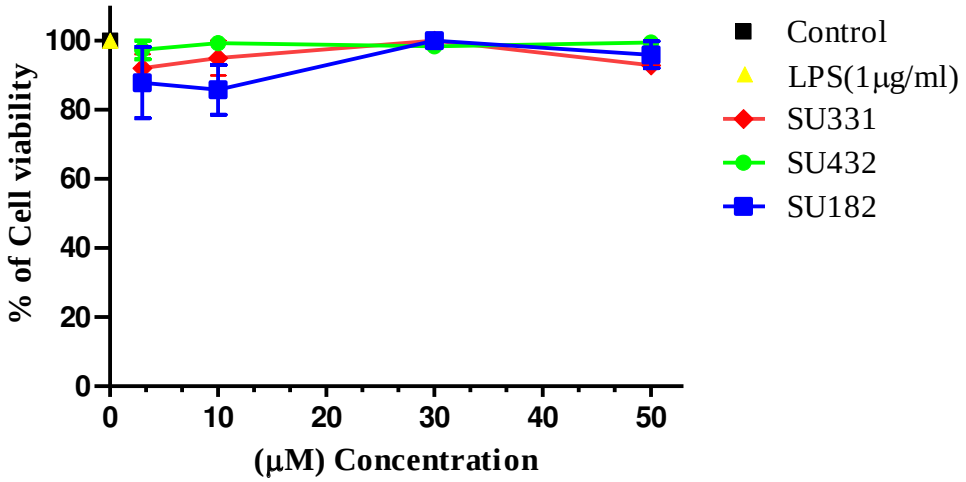


Figure 4.15: Effects of SU182, SU331 and SU432 on cell viability. Quiescent NCTC2544 cells in panel **a,** or RAW264.7 macrophage cells in panel **b** were treated with compounds over the concentration ranges of 1-50µM in **a**, 3-50µM or LPS (1µg/ml) in **b** for 24 h. Then cell viability was determined as described in the Section 2.2.7. The results are expressed as the % of maximum viable cells. Results are representative of three independent experiments in triplicates and each value represents the mean ± s.e.m.

4.10 Effects of SU182, SU331 and SU432 alkaloids on LPS-induced iNOS protein expression and nitrite production in RAW 264.7 macrophage cells.

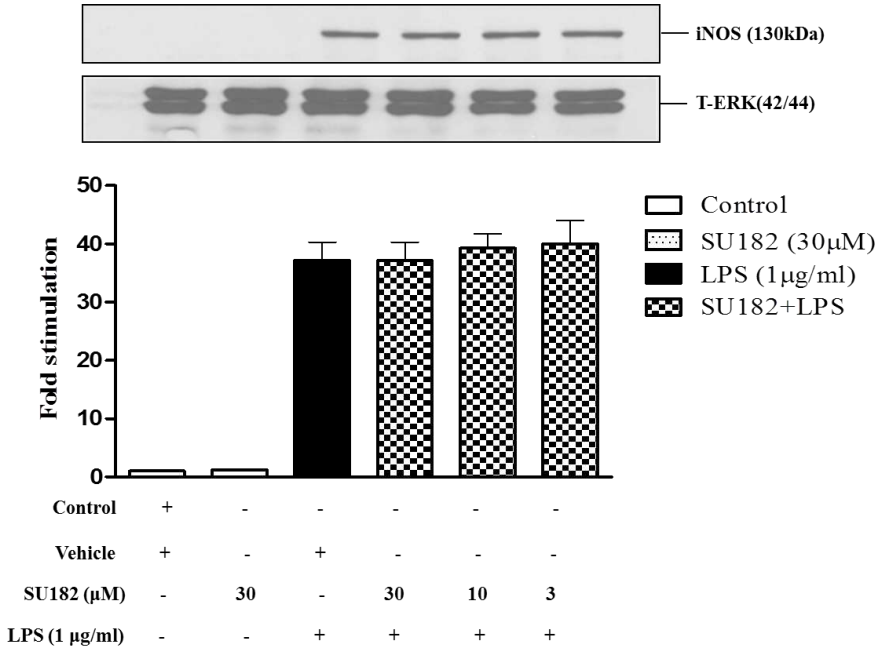
As outlined in Section 1.10 the expression of several inflammatory mediators are regulated by NF-κB induction. Our candidate compounds were assessed for their effects on LPS induced iNOS expression in the RAW 264.7 macrophage cells, as outlined in Chapter 3. In addition their effects on nitrite production following iNOS expression were also investigated.

Figure 4.16a shows that the maximum iNOS expression achieved by LPS stimulation (37.1 ± 3.2 fold) was not affected by even the highest concentration of SU182 (30µM). Figure 4.16b demonstrates that SU182 behaved similarly when assessed for nitrite production induced by LPS over 24 h, hence the effect of SU182 on maximum fold of nitrite produced was minor and not significant (reduced from 35.9 % ± 4.3 to 28.5 % ± 0.2).

The effect of SU331 on LPS induced iNOS expression is shown in Figure 4.17a. The maximum iNOS expression was significantly reduced by over 95% (reduced to 2.8% ± 1.4 of maximum stimulation, $p < 0.001$) by pretreatment of cells with 30µM and by around 60% (42.1 % ± 17.0, $p < 0.001$) at 10µM. However, this great inhibitory effect of SU331 on iNOS expression did not reflect its effect upon nitrite production (Figure 4.17b), which only reduced the LPS induced nitrite fold to approximately half (from 39.2 ± 4.1 fold to 15.7 ± 0.7 fold, $p < 0.05$).

Again, significant inhibition of LPS-stimulated iNOS expression was achieved by pretreatment of cells with SU432, as shown in Figure 4.18a. Maximum induction of iNOS expression in response to LPS decreased by approximately 70% (29.5 % ± 4.6 of maximum stimulation, $p < 0.001$) at 30µM and by approximately 20% at 10µM (83.2 % ± 3.8). The same pattern of inhibition by SU432 was also obtained in nitrite production as shown in Figure 4.18b, as levels were reduced from 35.9 ± 4.3 fold to 8.1 ± 5.3 fold at at 30µM.

a)



b)

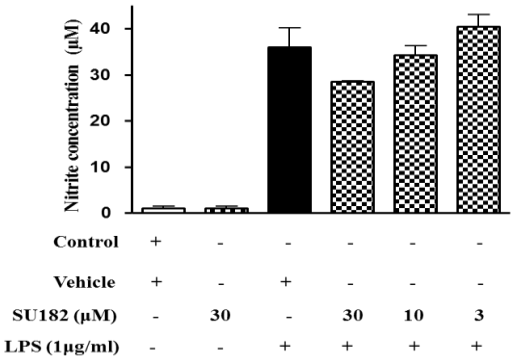
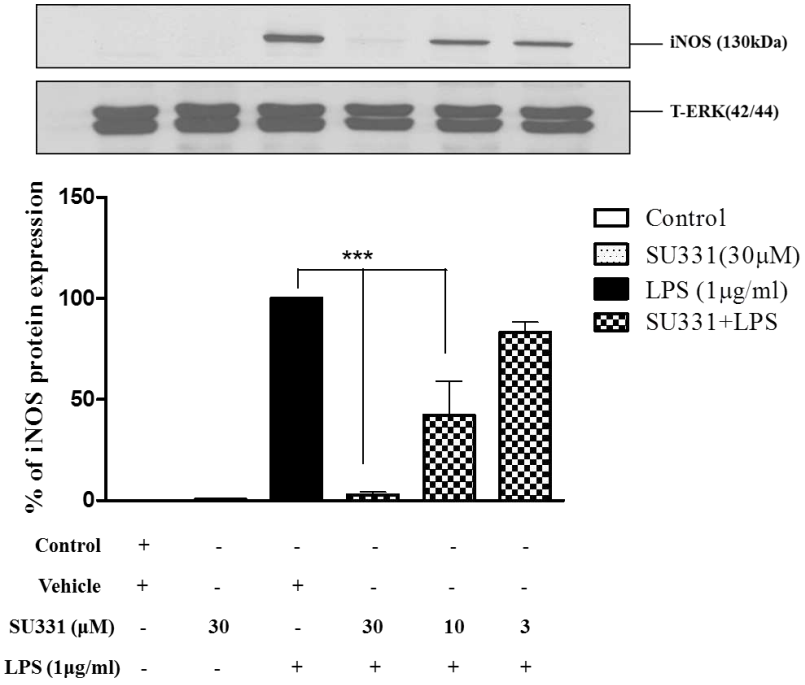


Figure 4.16: Effects of SU182 on LPS-induced iNOS expression and nitrite production in RAW 264.7 macrophage cells. Cells were pretreated with SU182 (3-30μM) for 30 min prior to LPS stimulation for a further 6 h in panel **a,** or for a further 24 h in panel **b**. In panels **a** whole cell lysates were prepared, separated by SDS-PAGE, assessed for iNOS (130kDa) expression, and re-probed for total-ERK (42/44kDa) as outlined in Section 2.2.3. Panel **b** shows graphical representation of LPS induced nitrite production (see Section 2.2.8). Results are representative of at least three independent experiments and each value represents the mean ± s.e.m.

a)



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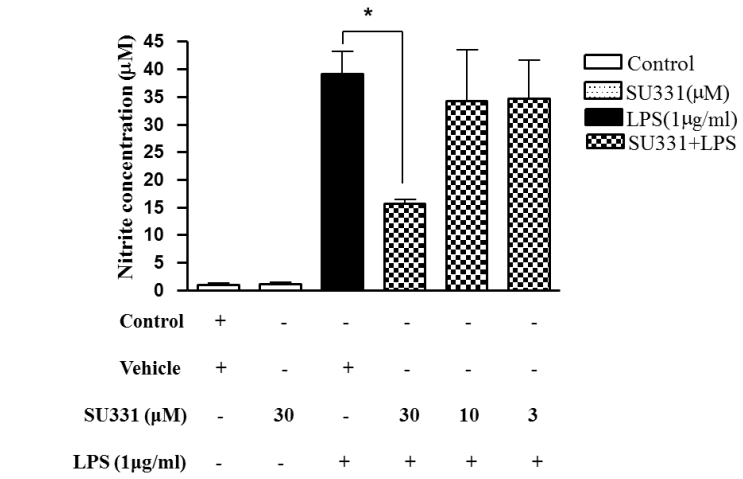
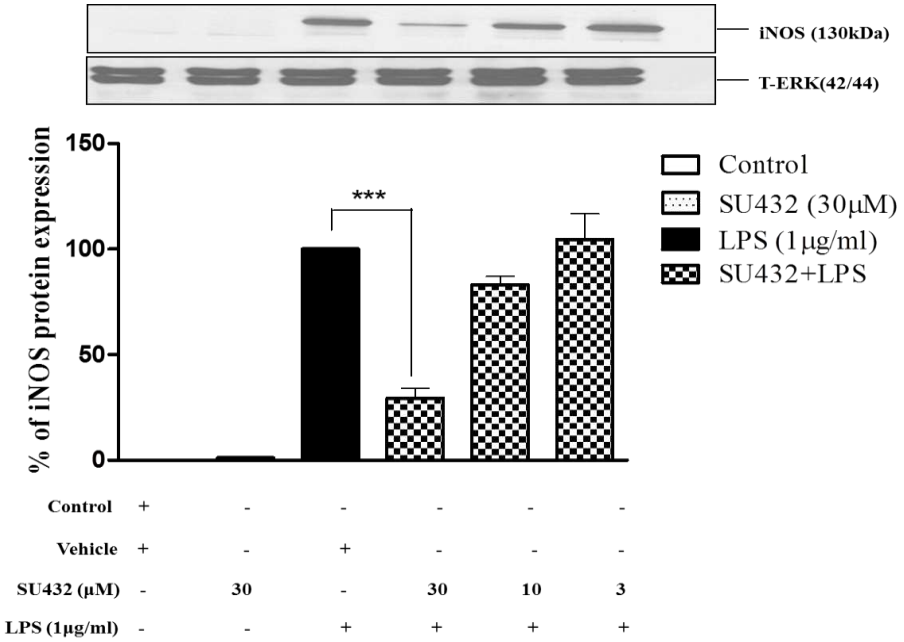


Figure 4.17: Effects of SU331 on LPS-induced iNOS expression and nitrite production in RAW 264.7 macrophage cells. Cells were pretreated with SU331 (3-30μM) for 30 min prior to LPS stimulation for a further 6 h in panel **a** or for a further 24 h in panel **b**. In panels **a** whole cell lysates were prepared, separated by SDS-PAGE, assessed for iNOS (130kDa) expression, and re-probed for total-ERK (42/44kDa) as outlined in Section 2.2.3. Results in panel **a** expressed as % of maximum stimulation and panel **b** shows graphical representation of LPS induced nitrite production (see Section 2.2.8). Results are representative of at least three independent experiments and each value represents the mean ± s.e.m., * = $p < 0.05$, *** = $p < 0.001$ compared to LPS stimulation.

a)



b)

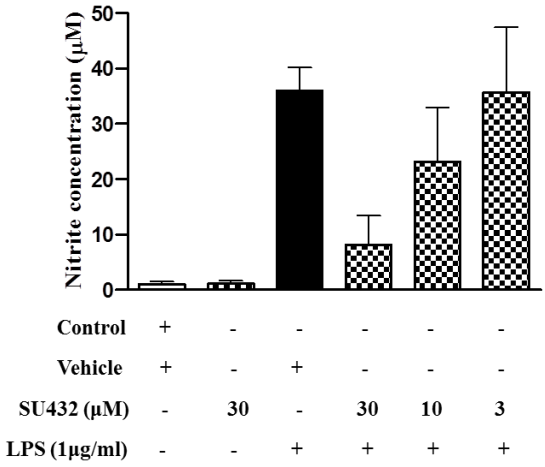


Figure 4.18: Effects of SU432 on LPS-induced iNOS expression and nitrite production in RAW 264.7 macrophage cells. Cells were pretreated with SU432 (3-30μM) for 30 min prior to LPS stimulation for a further 6 h in panel (**a**) or for a further 24 h in panel **b**. In panels **a** whole cell lysates were prepared, separated by SDS-PAGE, assessed for iNOS (130kDa) expression and re-probed for total-ERK (42/44kDa) as outlined in Section 2.2.3. Results in panel **a** expressed as % of maximum stimulation and panel **b** shows graphical representation of LPS induced nitrite production (see Section 2.2.8). Results are representative of at least three independent experiments and each value represents the mean ± s.e.m., *** = $p < 0.001$ compared to LPS stimulation.

4.11 Effects of SU182, SU331 and SU432 alkaloids on LPS-induced COX-2 protein expression in RAW 264.7 macrophage cells.

Having established LPS-induced COX-2 expression in the previous Chapter, which reached a maximum at 6 h stimulation (Figure 3.24a), the effect of SU alkaloids on COX-2 expression was investigated to assess their effect as anti-inflammatory compounds.

As shown in Figure 4.19 treatment of macrophage cells with LPS (1µg/ml) for 6 h resulted in COX-2 expression (53.2 ± 24.3 fold). This maximum expression of COX-2 was not altered by the pretreatment of cells with SU182 at the concentrations (3-30µM) used. Thus, SU182 did not affect COX-2 expression.

However, in contrast, SU331 was strongly effective in inhibiting COX-2 expression induced by LPS, as demonstrated in Figure 4.20. Expression was reduced substantially to approximately 40% of stimulated values (41.4 % ± 4.4, $p < 0.001$) at 30µM and significantly reduced (74.4 % ± 11.3, $p < 0.05$) at 10µM.

Furthermore, SU432 was assessed for its effect on LPS-induced COX-2 expression. It was found that SU432 strongly inhibited LPS-stimulated COX-2 expression as shown in Figure 4.21. Expression fell substantially to as low as 40% of stimulated values (36.1 % ± 9.2 of stimulation, $p < 0.001$) following pretreatment of cells with 30µM and to approximately 70% (71.4 % ± 4.6, $p < 0.01$) at 10µM.

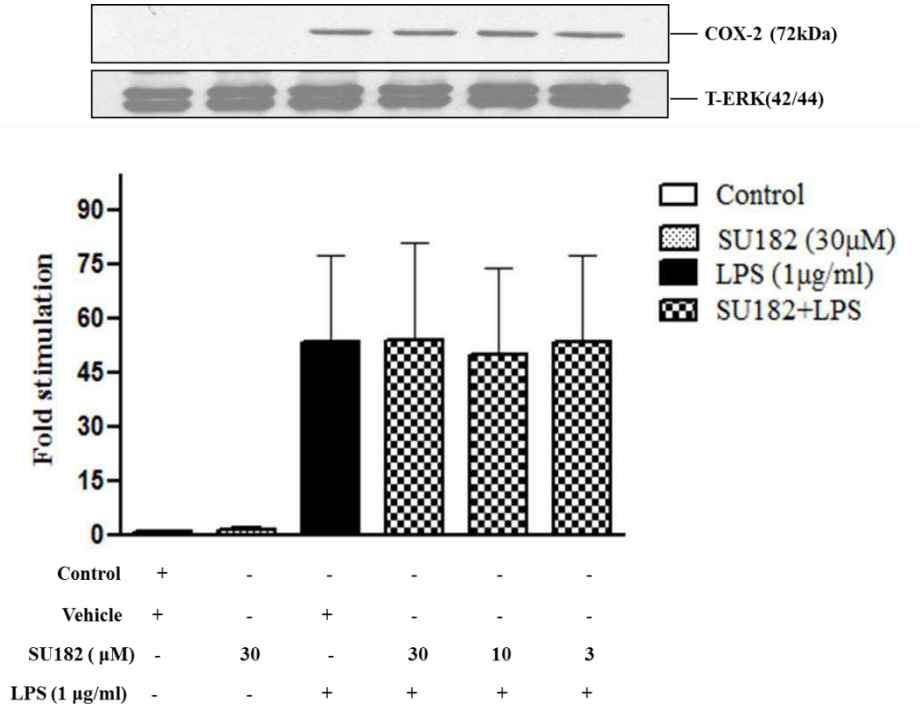


Figure 4.19: Effect of SU182 on LPS-induced COX-2 expression in RAW 264.7 macrophage cells. Cells were pretreated with SU182 (3-30μM) for 30 min prior to LPS (1μg/ml) stimulation for a further 6 h**.** Whole cell lysates were prepared, separated by SDS-PAGE, assessed for COX-2 (72kDa) expression and re-probed for total-ERK (42/44kDa) as outlined in Section 2.2.3. Results are representative of three independent experiments and each value represents the mean ± s.e.m.

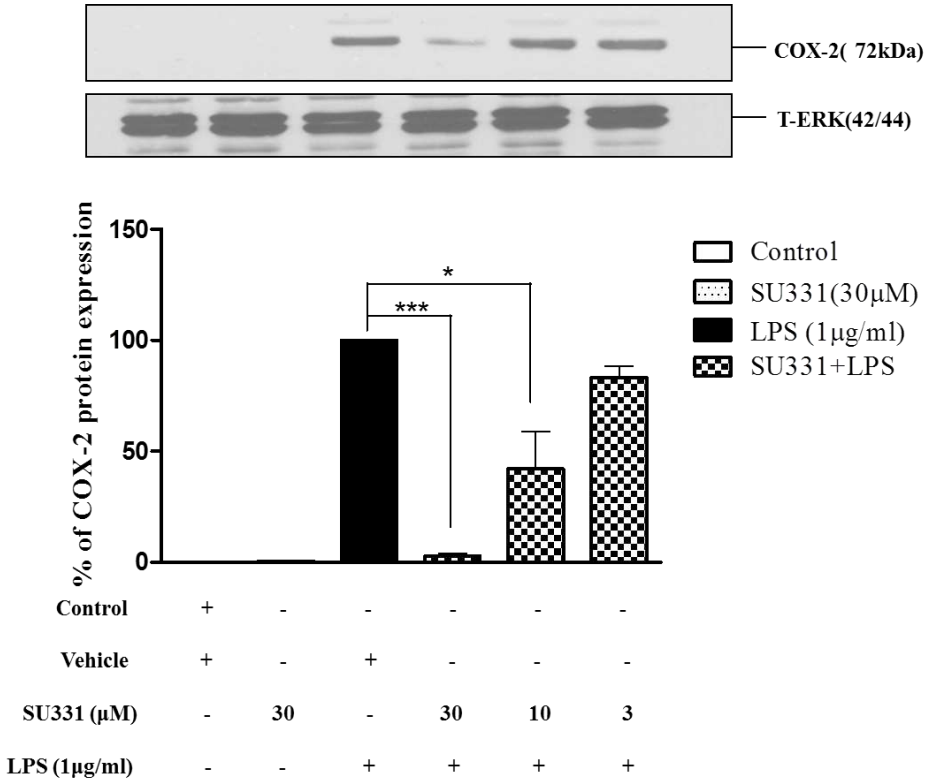


Figure 4.20: Effect of SU331 on LPS-induced COX-2 expression in RAW 264.7 macrophage cells. Cells were pretreated with SU331 (3-30μM) for 30 min prior to LPS (1μg/ml) stimulation for a further 6 h**.** Whole cell lysates were prepared, separated by SDS-PAGE, assessed for COX-2 (72kDa) expression and re-probed for total-ERK (42/44kDa) as outlined in Section 2.2.3. Results are expressed as % of maximum stimulation, representative of three independent experiments and each value represents the mean ± s.e.m., * = $p<0.05$, *** = $p<0.001$ compared to LPS stimulation.

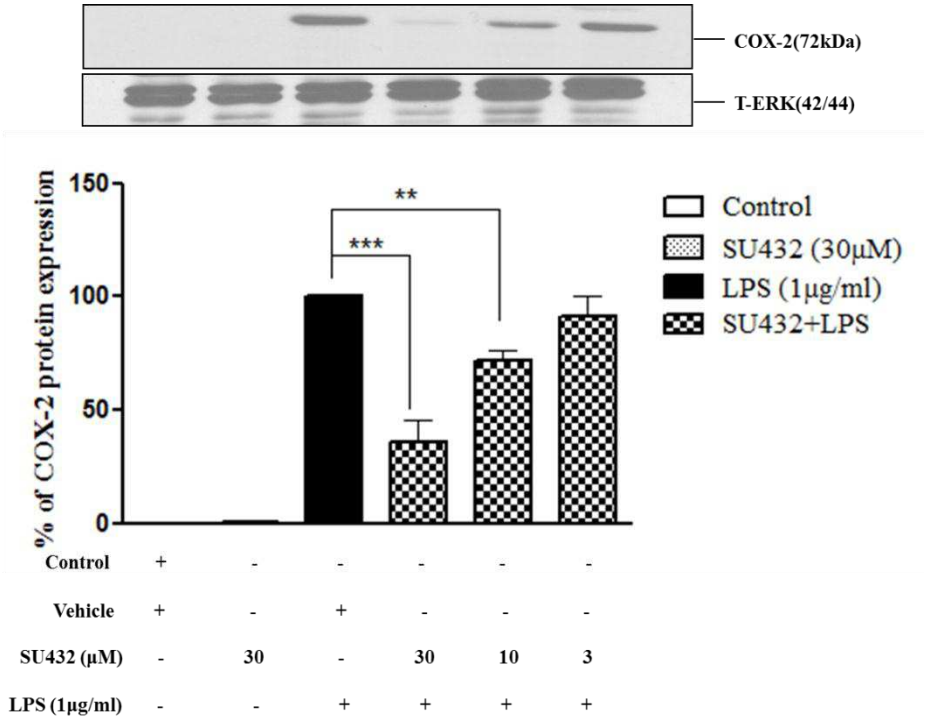


Figure 4.21: Effect of SU432 on LPS-induced COX-2 expression in RAW 264.7 macrophage cells. Cells were pretreated with SU432 (3-30µM) for 30 min prior to LPS (1µg/ml) stimulation for a further 6 h**.** Whole cell lysates were prepared, separated by SDS-PAGE, assessed for COX-2 (72kDa) expression and re-probed for total-ERK (42/44kDa) as outlined in Section 2.2.3. Results are expressed as % of maximum stimulation, representative of three independent experiments and each value represents the mean ± s.e.m., ** = $p<0.01$, *** = $p<0.001$ compared to LPS stimulation.

Compound	IKK activity	IκBα loss	Ser536-p65 phosphorylation	NF-κB nuclear translocation	NF-κB-DNA binding activity	NF-κB-linked luciferase activity	AP-1-linked luciferase activity	iNOS expression	COX-2 expression
SU182	Reduced	no effect	no effect	no effect	no effect	reduced TNF-α and PMA-induced activity	reduced PMA-induced activity	no effect	no effect
SU331	Reduced	Reversed	no effect	reduced TNF-α induction	reduced TNF-α induction	reduced TNF-α and PMA-induced activity	reduced PMA-induced activity	inhibited LPS induction	inhibited LPS induction
SU432	Reduced	Reversed	no effect	reduced TNF-α induction	reduced TNF-α induction	reduced TNF-α and PMA-induced activity	no effect	inhibited LPS induction	inhibited LPS induction

Table 4.1: Summary table illustrates the effects of compounds SU182, SU331 and SU432 on NF-κB pathway and off-targets.
Abbreviations: *: Assay carried out for compounds in human keratinocytes NCTC2544 cells, #: assay carried out for compounds in murine RAW264.7 macrophages.

4.12 Discussion

Previous studies have shown that many plant-derived alkaloid compounds possess a remarkable range of pharmacological activities including anti-inflammatory actions. Among these alkaloids, rutacarpine, sinominenine, tetrandrine and berberine have been identified as promising anti-inflammatory agents acting on various molecular targets that mediated the inflammatory responses. This includes intermediates of NF-κB pathway (Gautam and Jachak, 2009). For instance berberine was found to exhibit suppressive effects on receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis by inhibiting NF-κB activation through inhibition of phosphorylation at the activation loop of IKKβ, phosphorylation and degradation of IκBα, and NF-κB (p65) nuclear translocation (Hu et al., 2008). This anti-inflammatory activity of berberine was also observed in vivo against serotonin-induced hind paw oedema and acetic acid-induced increase in vascular permeability (Kupeli et al., 2002). In addition, the berberine derivatives, 13-methyl or 13-ethylberberine both exhibited an inhibitory effect on iNOS, TNF-α, and COX-2 expression in RAW 264.7 cells (Lee et al., 2003). These and other studies exemplify the potential usefulness of these plants in the discovery of new inhibitors.

In the current study three compounds were assessed for their anti-inflammatory effects. Two of these SU182 and SU432 belong to a class of compounds derived from plants, the β-carboline alkaloids. The β-carbolines have been isolated from several medicinal plants and display a variety of actions on the central nervous, muscular, and cardiovascular systems. It was discovered that β-carboline derivatives might function as anti-tumour agents through multiple mechanisms, such as targeting DNA (Guan et al., 2006), suppression of the activity of topoisomerase (Funayama et al., 1996), CDK (Song et al., 2004) and also IKK (Castro et al., 2003). Such inhibition of the IKK complex in NF-κB signalling pathway suggests the potential of anti-inflammatory effects. Indeed, it has been reported by Castro and colleagues that the β-carboline analogue PS-1145 is able to inhibit the phosphorylation of IκBα and subsequent activation of NF-κB in whole cells with an IC_{50} value in the nanomolar range and block TNF-α release in LPS-challenged mice (Castro et al., 2003).

In contrast, SU331 is a pyridinyl benzamide derivative of a structure which resembles the novel IKKβ inhibitor IMD-0354 (N-(3,5-Bis-trifluoromethyl–phenyl)5-chloro-2-hydroxy-benzamide. A recent report has demonstrated that IMD-0354 possesses anti-inflammatory anti-proliferative effects in mice injured arteries and as a result may effectively prevent restenosis after coronary intervention and other cardiovascular diseases (Hamaya et al., 2012).

In the current study SU182, known as canthin-6-one has been investigated for anti-inflammatory effects based on its broad antifungal and leishmanicidal activities (Ferreira et al., 2002). Also it has been recently indicated that canthin-6-one exhibits trypanocidal activity in vivo in a mouse model of acute or chronic infection (Ferreira et al., 2007, Ferreira et al., 2011). The mechanism of action of canthin-6-one is entirely unknown, but it has been hypothesised that its antifungal and trypanocidal activities could indicate, the inhibition of sterol C-14α demethylase in intracellular Trypanosoma cruzi amastigotes, a mechanism of action similar to that proposed for antifungal triazoles (Urbina et al., 2003). In addition, an anti-ulcerogenic effect of canthin-6-one in ethanol-induced ulcer model is mediated in part through an increase in the production of protective endogenous ·NO. However, canthin-6-one has no effect on the mucosal membrane levels of PGE_2, indicating that the gastroprotective effect of these agents is independent of PGE_2 modulation (de Souza Almeida et al., 2011), suggesting another mechanism of action.

In preliminary experiment SU182 was found to have an inhibitory effect on IKK activity in vitro suggesting the potential to be a useful initial lead compound. However, although SU182 produced an attenuation of NF-κB and AP-1 transcriptional activities, induced by TNF-α or PMA, neither upstream IκBα and NF-κB (p65) phosphorylation nor downstream NF-κB nuclear translocation and binding to DNA were altered by the compound. This suggests that an inhibitory effect on IKK activity in vitro does not necessarily accord to a reduction in the downstream pathways in whole cells. Furthermore, since neither of the LPS-stimulated NF-κB dependent inflammatory proteins examined in RAW264.7 cells, iNOS and COX-2, were inhibited, SU182 does not appear to interfere with the pathways linked to the

induction of these inflammatory enzymes. It would have been useful to examine a NF-κB dependent marker in NCTC2544 cells for example IL-8, which has previously been identified as NF-κB dependent (Goh et al., 2009), however it was found that long term treatment of cells compromised survival.

Several studies have shown the potential for β-carbolines to have effects on other signalling pathways which may be relevant to their effects on transcription. For instance, recently the psychoactive β-carboline alkaloid, harmine has been found to act as a regulator of the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) expression and to suppress TNF-α release in a diabetic mouse model (Waki et al., 2007). A more recent study has reported the potential anti-inflammatory effect of harmine, which strongly suppresses TNF-α and NO production, to be mediated by a unique mechanism independent of NF-κB translocation/DNA binding or MAP kinases (Yamazaki and Kawano, 2011). Thus, involvement of another mechanism other than direct NF-κB inhibition could be hypothesised, such as disruption of protein-protein interactions or an activation of another key transcription factor like PPARs, which known to interfere with NF-κB, AP-1 and STAT activities (Ricote et al., 1998), probably by competing for limiting amounts of general co-activators (Li et al., 2000), or by direct protein-protein interaction (Delerive et al., 1999).

In contrast a substantial inhibitory effect of SU331 on NF-κB activation in NCTC2544 cells was observed over sequential steps of TNF-α induced NF-κB activation. The effect was initially observed as a strong reversal of IκBα loss in the absence of any effect on NF-κB (p65) phosphorylation. This was reflected in attenuated NF-κB (p65) translocation to the nucleus and reduced NF-κB-DNA binding activity. These effects were further translated to an inhibition of NF-κB transcriptional activity. It is noteworthy that PMA induced NF-κB-DNA binding activity (data not shown) and NF-κB transcriptional activity were also attenuated by SU331, suggesting a common site and mechanism of inhibition. This is unlikely to be a direct effect on PKC isoforms which are strongly activated by PMA as it is accepted that conventional PKCs do not usually play a role in the activation of the

NF-κB pathway in response to TNF-α (Lallena et al., 1999). At this point it is unclear if the PKC pathway can regulate intermediates upstream in the TNF-α cascade.

Additional experiments did however show an inhibitory effect on PMA induced AP-1 activity suggesting a potential off-target effect. However, no effect was observed on activated MAP kinases (p38, ERK1/2 and JNK1/2) (data not shown) in spite of the reduced AP-1 activity. This suggests that the site of inhibition is downstream of these pathways potentially within the nucleus. In addition, SU331, despite being able to reverse the IκBα loss was unable to affect TNF-α induced NF-κB (p65) phosphorylation at the Ser536 residue. This may suggest p65 phosphorylation to be IKK independent, and could be attributed to interference with kinases other than IKKs which possibly target the phosphorylation sites such as Ser529 induced by CK2 (Wang et al., 2000), or Ser311 induced by PKCζ (Duran et al., 2003). Taken together these data suggest the potential of additional effects of SU331 in addition to IKK inhibition.

Although SU331 has potential inhibitory effect on NF-κB pathway, it clearly needs to be optimised further. It compares poorly with PS-1145 a β-carboline based IKK inhibitor which was found to inhibit the phosphorylation of IκBα and subsequent activation of NF-κB in whole cells, as well as blocking TNF-α release in LPS-challenged mice in nanomolar concentrations (Castro et al, 2003). Alternatively, the potency of SU331 could be linked with a structure activity relation-ship (SAR) for many benzamide derivatives recently referred as potent anti-inflammatory agents. For example, the NF-κB inhibitor IMD-0354 (Hamaya et al., 2012) or the MS-275 which is specific for class I HDACs and preferentially inhibits HDAC1 versus HDAC3 and has no inhibitory activity towards HDAC8 (Blanchard and Chipoy, 2005). MS-275 in particular was reported recently to have anti-inflammatory potential in vivo which effectively ameliorated collagen induced arthritis, strongly attenuated paw swelling, bone erosion and resorption, and decreased serum IL-6 and IL-1β levels (Lin et al., 2007). In addition, it exhibited strong immunomodulatory activities on dendritic cells in vitro (Nencioni et al., 2007). Furthermore, recent study have shown that MS-275 could effectively suppress inflammation in experimental

autoimmune neuritis, through suppressing inflammatory T cells, macrophages and cytokines, and inducing anti-inflammatory immune cells and molecules (Zhang et al., 2010).

Interestingly, SU432 displayed marked effects on TNF-α induced NF-κB signalling, which included an incomplete reversal of cellular IκBα loss, unaffected phosphorylation of the NF-κB (p65) subunit, but remarkable inhibition of NF-κB-DNA binding and subsequent reduction of NF-κB transcriptional activity. These results observed in NCTC2544 cells were in accordance with the inhibitory effects of SU432 on LPS-induced expression of both COX-2 and iNOS proteins in RAW264.7 macrophage cells and reflected the potency of inhibition of TNF-α-induced both NF-κB-DNA binding and NF-κB transcriptional activity but not the modest inhibition of IκBα. Therefore, SU432 might in part display these effects through direct interaction with DNA, or some other effect within the nucleus in addition to detected IKK inhibition.

The IKKβ inhibitory effect of SU432 can be compared with that obtained by structurally related β-carboline IKKβ inhibitors, PS-1145 and ML120B, in cell based and in vivo models (Castro et al., 2003, Schopf et al., 2006). In agreement with the observation above many of the β-carboline compounds have been shown to intercalate into DNA, to inhibit CDK, Topisomerase, and monoamine oxidase, and to interact with benzodiazepine receptors and serotonin receptors. However, discovery of β-carboline metabolites as potent antiviral agents have encouraged the synthetic and pharmacological studies of β-carboline derivatives in the area of infection rather than inflammation. It was reported that different eudistomine derivatives including eudistomin N (SU432) have antimicrobial activities against Bacillus subtilis bacteria and antiviral activities against herpes simplex virus-1 (HSV-1) (Cao et al., 2007).

In a preliminary experiment carried in-house, SU432 inhibited TNF-α-stimulated p38 MAP kinase phosphorylation in NCTC2544 cells but not in RAW264.7 macrophages (data not shown), pinpointing a cell specific effect. This again may explain the relative effectiveness of SU432 at the level of IκBα loss versus effects upon

transcriptional activation. For example, a role has been suggested for transcriptional activators as targets for the p38 MAP kinase pathway in LPS-mediated regulation of IL-6 gene expression (Beyaert et al., 1996). Furthermore, the p38 MAP kinase pathway can positively regulate NF-κB activity indirectly involving chromatin remodelling through Ser10 phosphorylation of histone H3 at NF-κB-dependent promoters such as IL-8 and MCP-1 (Saccani et al., 2002).

In the context of NF-κB inhibition certain plant-derived β-carboline alkaloids are considered as important sources of antitumor lead compounds. For example, the canthin-6-one isolated from Eurycoma longifolia in addition to harmine and harmane alkaloids, were found to exhibit cytotoxicities against cancer including breast, colon, fibrosarcoma, lung, melanoma, KB, KB-V1 and murine lymphocytic leukemia P-388 (Cao et al., 2007). Additionally it was reported that 1-methoxy-canthin-6-one induces JNK-dependent apoptosis and synergises with tumor necrosis factor-related apoptosis-inducing ligand activity in human neoplastic cells of hematopoietic or endodermal origin (Ammirante et al., 2006). At present it is unclear whether these actions are linked to inhibition of NF-κB which plays a key role in cell survival. Further studies of the compounds examined in this chapter in relation to anti-proliferative effects are clearly required.

The results in this Chapter demonstrate the potential of novel IKK inhibitory compounds to be useful in reducing the activation of the classical NF-κB pathway. However, considerable development of the compounds using structure activity studies is required to reduce off-target effect and to increase the potency of the compounds. Nevertheless, a drug which can inhibit this cascade and prevent the expression of multiple inflammatory mediators is likely to be useful. For example, a previous study has shown that NO directly increases COXs activity and leads to a remarkable 7-fold increase in PGE_2 formation (Vassalle et al., 2003); further study suggested that there is a considerable cross talk between NO and PGs biosynthetic pathways (Sibilia et al., 2008). Therefore, a compound with the dual inhibitory effect on iNOS and COX-2 expression would provide potential in advancing the treatment of inflammatory or chronic immune disorders.

CHAPTER 5

GENERAL DISCUSSION

Current anti-inflammatory therapies such as steroids, non-steroidal anti-inflammatory drugs and anti-histamines are largely based on inhibiting the synthesis or action of inflammatory mediators (Holgate and Polosa, 2006). However, all these established therapies have their drawbacks and side effects (Sandborn et al., 2005). Although these clinically available anti-inflammatory drugs do not specifically target NF-κB, at least some of the effects are due to the inhibition of NF-κB activation (Yamamoto and Gaynor, 2001). Given the limitations of existing small molecule and biopharmaceuticals, there remains a clear need for identification and validation of new anti-inflammatory drugs and targets.

In the last few years great advances in discovery of kinase inhibitors for the treatment of inflammation and autoimmune disorders have been produced. A large number of kinase inhibitors are designed to inhibit the enzyme by binding at or near the ATP-binding site. Therefore, an inhibitor of one kinase is often found to inhibit other structurally related or unrelated kinases (Karaman et al., 2008). A number of drugs targeting the kinases p38 MAPK, JNK, MEK, IKKβ and JAK3 are currently undergoing clinical trials for the treatment of diseases related to inflammation and autoimmunity (Bhagwat, 2009). Moreover, several small-molecule IKK inhibitors and other strategies targeting IKK and IKK-related kinases have shown significant results in preclinical studies and some are generating promising preliminary results in clinical trials. The hope is that several of these small-molecule drugs and/or targeted gene therapies will enter future clinical trials to help provide a complete evaluation of the potential for targeting IKK and IKK-related kinases as a therapeutic intervention for cancer (Lee and Hung, 2008).

This project sought to investigate the effects of several compounds prepared and synthesised by the chemistry department in SIPBS at University of Strathclyde as potential novel anti-inflammatory compounds by screening them against NF-κB signalling. I have assessed the effect of these compounds at different levels of the pathway as same as many pharmaceutical companies have programmes to develop selective inhibitors of NF-κB signalling and the IKKs, the key kinase in this cascade (Verma, 2004). By assessing whether compounds can target stimuli-induced IκBα

protein degradation, NF-κB (p65) protein phosphorylation, blocking the induced nuclear translocation of NF-κB (p65) subunit, subsequent NF-κB-DNA binding activity, NF-κB transcriptional activity and expression of NF-κB dependent inflammatory mediators, a number of small-molecules were characterised for inhibitory effects on NF-κB activity.

In Chapter 3 characterisation of the TNF-α-induced NF-κB signalling was carried out in NCTC2544 cells and LPS-induced iNOS, COX-2 expression and MAP kinase phosphorylation in RAW264.7 macrophage cells. This was followed by an assessment of the effects of the AIK18/85/1 compound on these characterised signalling events. A novel effect of the peptidomimetic AIK18/85/1 was observed as inhibition of TNF-α induced NF-κB translocation to the nucleus, NF-κB-DNA binding and transcriptional reporter activities. Although the effect on nuclear translocation was specific to TNF-α, with no effect upon PMA-mediated induction, AIK18/85/1 still inhibited PMA-induced NF-κB-DNA binding activity as well as transcriptional activity. In addition, its effectiveness was marked in abolishing LPS-induced iNOS and COX-2 expression. However, the results revealed that the site of action of AIK18/85/1 and the related compound AIK18/70 were unrelated to the direct targeting of NF-κB-DNA binding as expected from such minor groove binders such as distamycin A and polyamides (Raskatov et al., 2012). This opposes the concept, that the minor groove binders are able to induce alteration of DNA structure which can modify the binding of transcription factors to AT-rich regulatory elements (Broggini et al., 1989) in particular to displace NF-κB from its DNA binding motif (Chenoweth et al., 2007).

Nevertheless the results above suggest AIK18/85/1 mediated its inhibitory effects by targeting multiple sites in the NF-κB cascade. However, it was observed that in the context of TNF-α-stimulated signalling AIK18/85/1 inhibited NF-κB (p65) translocation specifically, as this was not observed for PMA treatment. This may identify an agonist-specific effect for AIK18/85/1. In a similar manner, research has clearly demonstrated that DHMEQ, a derivative of the antibiotic epoxyquinomicin C, which was studied as an inhibitor of NF-κB (Matsumoto et al., 2000), does not

directly disrupt NF-κB interactions, rather it inhibits NF-κB activation by eliminating the TNF-α-induced nuclear translocation of NF-κB (Cardile et al., 2010). However, this scenario was not applicable to PMA-induced NF-κB (p65) translocation despite evidence of abolished NF-κB-DNA binding and NF-κB reporter activities, providing an alternative mechanism probably related to stimulus-induced NF-κB signalling specificity (Nishizuka, 1984). As such PMA-induced signalling includes the involvement of a subset of PKC isoforms. Thus, modulation of the PKC isoforms by AIK18/85/1 cannot be precluded.

In addition to the NF-κB, MAP kinases were also reported to mediate iNOS and COX-2 expression induced by LPS (Chen et al., 1999, Paul et al., 1999). Whilst the LPS activated iNOS and COX-2 expressions were strongly inhibited by AIK18/85/1, which correlated directly with inhibition of NF-κB activity, LPS-induced MAP kinase phosphorylation (ERK1/2, JNK1/2 and p38 MAP kinase) was not affected by pretreatment with AIK18/85/1. This result indicated that inhibition was by mechanism of action independent of MAP kinases, nonetheless this could not exclude a role of downstream mediation of AP-1 further to NF-κB. Therefore, since the activation of AP-1 and NF-κB precede clinical arthritis and metalloproteinase gene expression, AIK18/85/1 may represent a promising candidate 'lead' compound for anti-inflammatory effects (Han et al., 1998). Furthermore, it has also been revealed that AIK18/85/1 inhibited iNOS induction mediated by IFNγ stimulation through inhibition of JAK/STAT1 signalling. This may reflect the involvement of multiple transcription factors in mediating a mechanism of induction and in the ability of AIK18/85/1 to mediate JAK/STAT1 inhibition (Ivanenkov et al., 2011). Given that AIK18/85/1 is also being considered as a potential small-molecule drug candidate against different inflammatory diseases.

Recently an extensive investigation of many natural alkaloids, for example evodiamine and rutacarpine have revealed different biological activities related to inflammation including immune modulation, NO inhibition (Yarosh et al., 2006, Chiou et al., 2002) and inhibition of viral infection related inflammatory disorders (Chiou et al., 2011). In this study SU alkaloid compounds have produced diverse

effects on NF-κB and AP-1 reporter activities. The lack of inhibitory effect of SU182 (canthin-6-one) on either LPS-induced MAP kinase signalling or the expression of iNOS and COX-2 in RAW264.7 macrophages may reflect a possible involvement of an indirect mechanism which interacts with NF-κB, such as PPAR which have been reported to interfere with the activity of transcription factors, such as AP-1, NF-κB or STAT1 at the transcriptional level (Ricote et al., 1998). This conflicting experimental data for SU182 may in part be mediated by activation of PPARs. Since they were consistent with inhibitory effect of PPAR agonists on NF-κB activity demonstrating agonists amplify cytokine-elicited ·NO generation in mesangial cells, potentiating iNOS protein expression rather suppression (Cernuda-Morollon et al., 2002). In support of this the antifungal effect of canthin-6-one due to its accumulation in lipid droplets and affect fatty acid metabolism in Saccharomyces cerevisiae suggesting a stimulation of alkyl chain desaturase enzyme systems (Lagoutte et al., 2008). Given that possible link between increased lipid metabolism and PPARs activation could be considered (Wang et al., 2003).

In contrast, two other SU alkaloids, SU331 and SU432 displayed inhibition of TNF-α-induced NF-κB signalling; inhibition of IκBα degradation, NF-κB-DNA binding and reporter activity, however, the phosphorylation of NF-κB (Ser536-p65) was not affected. In turn, the LPS-induced iNOS or COX-2 protein expression in RAW264.7 macrophages were inhibited by both compounds. This was consistent with the effects of β-carboline alkaloid compounds derived from Melia azedarach which suppress the de novo synthesis of these two enzymes (Lee et al., 2000). Recently, harmine a β-carboline alkaloid found to exhibit the ability to strongly suppress TNF-α and ·NO production via a unique mechanism, suggesting the involvement of a nuclear target molecule (Yamazaki and Kawano, 2011). Taken together, these results and those of this thesis may suggest that these compounds target multiple sites within the NF-κB cascade.

Collectively, all compounds investigated have modulated the NF-κB signalling; in particular AIK18/85/1 has manipulated the NF-κB pathway by a distinctive mechanism. On the other hand, SU331 and SU432 both inhibited the upstream NF-

κB signalling functioning as IKK inhibitors. These results provide important data in identifying promising anti-inflammatory 'lead' compounds. In addition, further studies are required to uncover the possible promiscuity and potential cross-talk mechanisms of these compounds, perhaps targeting PKC isoforms and PI3K/Akt, which may provide additional targets for yet to be discovered inhibitors of NF-κB.

Generally, many further experiments are required in order to pinpoint the exact mechanism of action. For example, examination of the effects of compounds on the phosphorylation of p65 subunits at serine residues other than serine 536. Moreover, the effects of these compounds in vivo and in different cell types can also be investigated in future work.

Further studies are required using labelled compounds or capillary gel electrophoresis may help to elucidate the mechanism of action of these compounds at the level of DNA binding and transactivation. Further EMSA experiments, using consensus sequences of different transcription factors such as AP-1, SP-1 and GAS could be carried out in order to determine nucleotide sequence selectivity of compounds. In addition, much information about drugs can be obtained from the use of different novel techniques. For example, novel technologies like nuclear-magnetic resonance (NMR) can provide details about binding location of protein, while affinity chromatography may provide information regarding drug absorption, distribution, excretion and receptor activation. In addition, the use of surface plasmon resonance (SPR) would be helpful in characterising the interaction of drug candidates with macromolecular targets, identifying the binding partners and quantifying complexes formed. Moreover, DNA microarray can be applied in drug screening in order to detect the effects of these compounds on different genes associated with human diseases, which then opens new and exciting opportunities for drug discovery (Liu et al., 2004).

CHAPTER 6

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