Examination of IKKα inhibitors as novel anti- pancreatic cancer drugs

By

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: Date:

Dedication

This Thesis is dedicated to the memory of my father, who passed away before couple of months before the completion of this effort. He was really special person to me, and I thank him for his love and support. No words can heal the pain of losing you. I miss you and always I will

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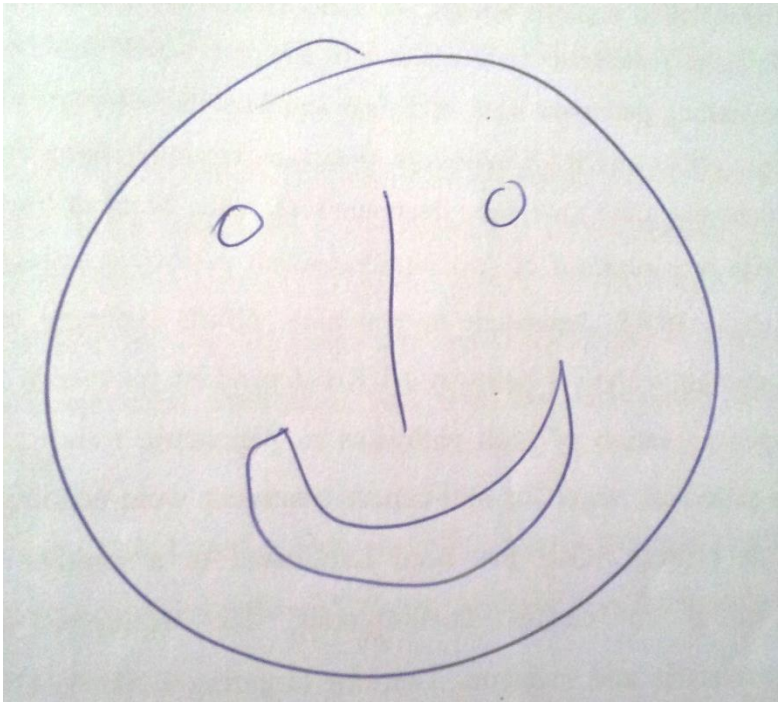

Abstract

Pancreatic cancer has a very poor prognosis, it is the fourth most common cancer worldwide in terms of mortality, and it is expected to be the second most common within a decade. Most patients with pancreatic cancer are either resistante to chemotherapy or become so, thus surgery is the only choice with a considerable chance of tumour re-growth. Therefore an alternative treatment is urgently sought. The Nuclear Factor Kappa B (NFκB) cascade is comprised of two interdependent pathways, recognized as the classical pathway or canonical NFκB pathway, which is IKKβ dependent, and IKKα-dependent non-canonical or alternative NFκB pathway. Studies have linked the hyper-activation of both pathways to pancreatic tumorigenesis. IKKβ inhibitors as a class of potential drugs for anti-cancer treatment have been accompanied by a number of issues regarding toxicity. IKKα has been implicated in a number of biological processes including cancer development, therefore targeting IKKα is a new approach for the development of pancreatic cancer therapies and is examined in this thesis.

In chapter three, both NFκB pathways were characterised using different agonists; LTα1β2, TNFα and FCS. LTα1β2 stimulated the IKKα -dependent non-canonical pathway, inducing phosphorylation of p100 after 4h stimulation, while the maximum activation of p52 formation was between 24 and 48h. TNFα and FCS were without effect. TNFα and LTα1β2 stimulated the canonical NFκB pathway and taken together these studies indicated the presence of a functional non-canonical pathway

In chapter four, a number of novel IKKα inhibitors generated in-house, were also examined against both the non-canonical and canonical NFκB pathways. Three different effects were observed; selective inhibition of IKKα by SU1261, SU1411, SU1349, SU1433, SU1438 and 1434. Inhibition of both IKKα and IKKβ by (SU1087, SU1432, SU1499 and SU1436) and no inhibition of either pathway (SU1392). The effect of selective IKKα inhibitors on cell cycle and growth were also examined and confirmed that IKKα has a role in proliferation of pancreatic cancer cells.

In chapter five, the expression of IKKα- dependent target genes was investigated using the agonist that activates the IKKα-dependent non-canonical NFκB pathway, $LT\alpha_1\beta_2$. The findings confirmed that the expression of genes (BBC3, EZH2, TNFAIP3, VCAM, MAP3K14 and SERPINB6) was likely to be regulated through this pathway. This was confirmed using IKKα selective inhibitors, which resulted in the expression of all gene subsets were reduced.

Taken together these data indicate that IKKα plays a key role in the regulation of the non-canonical NFκB pathway in pancreatic cancer cells and that selective inhibition IKKα may be a new strategy for developing anti-cancer drugs.

Poster and oral Presentation

Oral Presentation

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Abbreviations

ADEX	Aberrantly Differentiated Endocrine Exocrine
AIB1	Amplified in breast cancer 1
AJCC	American Joint Committee on Cancer
ANOVA	Analysis of variance
APS	Ammonium Persulphate
BAFF	B cell activation factor
BBC3	BCL2 Binding Component 3
BLC	B lymphocyte chemoattractant
BSA	Bovine Serum Albumin
CGH	Comparative genomic hybridization
c-IAP	Cellular inhibitor of apoptosis protein
DMEM	Dulbecco's Modified Eagle Medium
DN	Dominant Negative
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ERK	Extracellular Signal-regulated Kinase
ESPAC-1	The European Study Group of Pancreatic Cancer 1
EZH2	Enhancer Zeste homolog2
GSK3	Glycogen synthase kinase 3
HLH	Helix-loop-helix
HNPCC	Hereditary non-polyposis colorectal cancer
HRT	Hormone replacement therapy
ICAM-1	Intercellular Adhesion Molecule-1

IGF-1	Insulin–Like Growth Factor 1
IKK	inhibitory kappa kinase B
IL6	Interleukin-6
IL-8	Interleukin-8
KRAS	Kirsten rat sarcoma viral oncogene homolog
KSHV	Kaposi's Sarcoma Herpes Virus
LTβR	Lymphotoxin β receptor
MAP3K	Mitogen-activated protein/FRK kinase 3 family
MAP3K14	Mitogen activated protein kinase kinase kinsae 14
MIP-2	Macrophage inflammatory protein 2
NCBI	National centre for Biotechnology Information
NFAT	Nuclear Factor Activated T-cell
NFκB	Nuclear Factor Kappa B
NIK	NFκB-inducing Kinase
NRF2	Nuclear Factor like-2
PCR	Polymerase chain reaction
PDACs	Pancreatic ductal adenocarcinoma
PI	Propidium Iodide
PI3 Kinase	Phosphoinositide 3-kinase
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction amplification
RHD	Rel homology domain
SDF1	stromal cell- derived factors-1α
SDS	Sodium Dodecyl Sulphate
SERPINB6	Serpin family B member 6
siRNA	Small interfering RNA

SLC	Secondary lymphoid tissue chemokine
TEMED	N,N,N’,N’ tetramethendiaamine
TLRs	Toll like Receptors
TNFAIP3	TNF alpha induced protein 3
TNFα	Tumour Necrosis Factor-alpha
TRAF2	TNFα receptor-associated factor 2
TRAF3	TNFα receptor-associated factor 3
TSP-1	Thrombospondin type 1
UPA	U-Plasminogen Activator
UV	Ultraviolet Radiation
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
IκB	Inhibitory kappa B
β-ME	β-mercaptoethanol
ES	Estrogen receptor
CDK9	Cyclin-depended kinase 9

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Chapter One

Introduction

1.1 Introduction

Cancer is defined as the uncontrolled proliferation of cells which can occur in any organ of the body. In this condition, differentiation is compromised and cell growth takes over resulting in tumour formation, which in turn leads to spread to other organs (metastasis) (Hanahan and Weinberg, 2011). Cancer can spread to different parts of the body by two main pathways, the lymphatic system or the bloodstream. Today cancer is considered to be the biggest health problem globally (Hanahan and Weinberg, 2011) with around 12.8 million people affected by different cancers each year it causes 13% of all deaths worldwide.

1.1.1 External factors linked to cancers

There are about two hundred types of cancer identified. There are many organs in the body which can be affected by cancer and it is hard to determine the causative agent of cancer (Sasco et al., 2004). However, there are many studies which have identified external factors, called carcinogens, associated with certain types of cancer. Classification of these factors have identified three main groups; biological carcinogens, physical carcinogens and chemical carcinogens (Sasco et al., 2004).

There are many infectious agents including parasites, viruses, and bacteria which have potential to promote cancer development. One noted example is Kaposi's Sarcoma which is a skin cancer associated with cutaneous lesions caused by Kaposi's Sarcoma Herpes Virus (KSHV) (Schulz, 2000). Many studies have shown a correlation between some species of bacteria and cancer development, for example, *Chlamydiae pneumonie* with lung tumours, *Bartonella species* with vascular tumours and *Streptococcus bovis* has been shown to be implicated in colon cancer (Ellmerich et al., 2000, Marshall and Windsor, 2005, Littman et al., 2005, Dehio, 2005).

Physical carcinogens are defined as environmental factors which can help to cause tumour formation, for example, exposure of the foetus to high levels of ionising radiation has been linked to increasing the risk of developing childhood leukaemia (Stewart et al., 1958). Another example is Ultraviolet (UV) radiation, UV is an electromagnetic radiation with a range of wavelengths between 10nm - 400nm and is a component of sunlight and electric products. Studies have shown that 95% of skin cancers (melanomas) are associated with long term exposure to UV (Andrade et al., 2012).

Finally, chemical carcinogens encompass any chemical agent which causes cancer, for instance, water contaminants (arsenic) or tobacco smoke components. There are about four thousand chemicals in tobacco smoke, 93% of all lung tumours have been demonstrated to be related to smoking (Sasco et al., 2004, Villeneuve and Mao, 1994). Another example is an Aflatoxin B toxin, this toxin produced by two specific fungi, called *Aspergillus flavus* and *Aspergillus parasiticus*. This toxin is linked to hepatocellular tumours in Africa (Alpert et al., 1968). Bennett and Klich found increased levels of this toxin induced tumour development in animal models (Bennett and Klich, 2003). Another example is formaldehyde, a chemical used in industry and also found in some types of fruits or other foods. Formaldehyde is associated with a number of human cancers, for example leukaemia and nasopharyngeal cancer, hematopoietic and lung cancers (Rager, et al 2011). Research needs to continue to study chemical carcinogenesis as it likely to have an increasing influence on cancer development.

1.1.2 Cellular factors related to cancer development

Whilst a number of external factors are linked to cancer development the predominant focus is on cellular factors, particularly hormones, growth factors, intracellular pathways and the resultant effect upon cellular proliferation. At this point it should be noted that the risk factors outlined in the paragraphs above results in either direct or indirect genetic mutations within the cell, called somatic mutations that lead to cancer.

A number of studies have suggested the involvement of hormones and growth factors in the development of cancers, as at the cellular level they play a role in the regulation of cell growth, cell proliferation and apoptosis. A number of cancers such as breast, uterine, cervical and prostate cancer, are driven by levels of estrogen, androgen and progesterone (Lukanova and Kaaks, 2005). Furthermore, in the past 30 years, hormones have been considered a risk factor for cancer development through their induction of cell proliferation. Many studies have demonstrated that increasing the level of estrogen and progesterone, such as via hormone replacement therapy (HRT) increases the risk of breast cancer. A study found that more than 160,000 women, who used HRT, were diagnosed with breast cancer and it was linked with duration of HRT intake, the risk increased 2.3% each year of use (Lancet, 1997). Another example is the hormone androgen, Chuu and co-workers reported that approximately 80% of prostate cancer proliferation was due to the hyper production of androgen, therefore, they suggested reducing the production of androgen as a potential therapeutic strategy for treatment of prostate cancer (Chuu et al., 2011).

Insulin-Like Growth Factor 1 (IGF-1) has an anti-apoptotic role in many types of cell (Yu and Rohan, 2000). Excess concentrations of IGF-1 can lead to the reduction of

some pro-apoptotic proteins which have a role in tumour formation in the prostate and breast (Kaaks and Lukanova, 2001). Elevating the concertation of insulin increases binding to IGF-1 receptors and leads to the activation of signalling pathways which enhance cancer development (Moore et al., 1998). In addition, insulin has also been implicated in metabolism and synthesis of some pro-cancerous hormones including the androgens (Fairfield et al., 2002, Kaaks and Lukanova, 2001).

1.1.3. Genetic mutations related to cancer development

A number of cancers can be the result of mutations passed down through generations. Kastrinos and co-workers found a number of germline mismatch repair gene mutations related to colon cancer including: MLH1, MSH1, MSH6 and PMS2 (Kastrinos, et al, 2011). In addition hereditary non-polyposis colorectal cancer (HNPCC) also called Lynch's syndrome, is a genetically heterogeneous disorder responsible for approximately 2-10% of all colorectal cancer cases (Rajender, et al 2010). Some of the same genes, plus others, have been shown to be involved such as:MSH2, MSH6, MLH1, MLH3, PMS2, PMS1 and TGFBR2 (Fishel, et al 1993, Worthley, et al, 2005, Papadopoulos, et al 1994, Miyaki, et al 1997 Nicolaides, et al, 1994, Lu, et al 1998, Ou, et al 2009).

There are a number of hereditary mutations that have been linked to familial pancreatic cancer such as PRSS1 and CLDN. In addition, Whitcomb and co-workers found these mutations to be associated with hereditary disorders of the pancreas (Howes et al., 2004, Whitcomb et al., 1996). A previous study found that around 10% of pancreatic cancers were related to BRCA2 germline mutations as opposed to mutations in BRCA1 (Goldgar, et al, 1995). Both these mutations are associated with

ovarian and breast tumours (Friedman et al., 1994). Other examples include; mutations in KRAS2 and N-RAS genes which are associated with lung, pancreatic and colorectal tumours (Futreal et al., 2004). Furthermore, Chen and co-workers have found mutations within the PTEN gene in 70% of patients with prostate cancer (Chen et al., 2005) and this gene mutation is related to many types of tumours such as uterus, thyroid and breast (Pilarski and Eng, 2004).

In some cases the mutated gene can be both inheritable and the result of somatic mutation due to exposure to risk factors, again the BRCA1 gene is an example. Overall, reduced exposure to both internal and external factors can decrease development of tumours irrespective of whether a person already carries mutation or not.

There are two types of genes which are found in normal cells which influence the potential of development of tumours (see Table 1.1). The first type are proto-oncogenes; these genes express proteins which are responsible for many functions within the cell which are usually critical to growth and proliferation and include *abl, K-sam* and *ras* genes (Doolittle et al., 1983). The second type of mutations is linked to tumour suppressor genes that have the ability to regulate cell division and cell death, for example, p53, BRCA1 and BRCA2. Mutations in these genes whether inherited or acquired, lead to tumour development instead of working to regulate cell growth under normal conditions (Todd and Wong, 1999).

Table 1.1 Common oncogenes and suppresser genes involve of in human cancers.

From (Weinberg, 1996)

Oncogenes	
KRAS	Involved in ovarian, lung, pancreatic and colon cancer This gene has an intracellular function in ERK-MAP signalling.
MDM2	Involved in sarcoma and other cancer, this gene codes a negative regulator of tumour suppresser protein (p53).
BLC2	Involved in follicular B cell lymphoma, codes for a proteins that prevents cell suicide.
CTNB1	Involved in the liver cancer, codes for beta catenin.
NRAS	Involved in leukaemia, has a role in the regulating of cell division.
EGFR	Involved in breast and brain cancer, encodes the receptor for epidermal growth factor.
PRAD1	Involved in neck, breast and pancreatic cancer. Also named CCNDA, encodes cyclin D1.
L-MYC	Involve in lung cancer, This gene has intracellular function in ERK-MAP signalling. This gene codes for multi nuclear phosphoproteins (De Greve, et al 1988).
Tumour suppressor genes.	
BRCA1 and BRCA2	Involved in breast cancer, have a role in DNA repair damage.
RB1	Involved in bladder, bone, breast, retinoblastoma and small cell lung cancer.
APC	Involved in stomach and colon cancers, encodes APC protein which play a role in many cellular processes.
MTS1	Involved in many cancers, codes for p16 protein that had a role in the cell cycle.
Development signalling pathway	
GLI1 SOX3 CREBBP	Involved in pancreatic cancer, has a role in the hedgehog signalling pathway.
p21 TCF4	Involved in pancreatic cancer, has a role in cell death crosstalk with the NFκB signalling pathway.
MYC WNT9A	Involved in pancreatic cancer, has a role in Wnt signalling pathway.

1.2 Tumorigenesis

There are multiple routes that promote the transformation of a normal cell to a cancer cell, depending on the nature of mutation, this process is called tumorigenesis or carcinogenesis. Tumorigenisis includes three stages; initiation, promotion and progression (see Figure 1.1) (Pitot, 1993, Barrett, 1993, Frank, 2007, Lapidot et al., 1994). These stages are in themselves underpinned by an irreversible genetic mutation which occurs in DNA (Barrett, 1993), which leads to the DNA replication error during the synthetic phase of the cell cycle or changes in cellular metabolism, such as the release of free radicals and reactive oxygen species (Chen et al., 2007, Pray, 2008). Some of the environmental factors outlined previously clearly have the potential to initiate mutations in DNA, for example, UV radiation, X-Rays and smoking. Exposure to these factors creates covalent bonds within DNA segments, called DNA adducts, and this process can lead to mutation if not eliminated though the DNA repair system prior to replication. Direct DNA damage is classified into four different groups depending on the error that appears within the DNA; substitution, loss or addition of bases, modification in chemical structure of DNA itself and breaks in the DNA backbone (Pitot, 1993, Barrett, 1993, Frank, 2007).

In the promotion stage, cellular function maybe compromised resulting in an increase in the average rate of cell growth and division that increases the production of daughter cells and errors at the cell cycle checkpoint. At this stage the cancer promoters are not binding directly with DNA as in the initiation phase, but rather are linked to proliferation through activation of cell signalling pathways (Pitot et al., 1981). For example, the enhanced production of growth factors which bind to specific receptors on the target cell membrane can promote gene expression via

activation of cell signalling pathways, or indeed mutation of the intracellular pathways themselves which in turn promotes changes in gene expression without the need for activation a receptor (Pitot et al., 1981, Troll and Wiesner, 1985).

The final stage includes the ability of cells to form neoplastic cells or masses, which includes more spontaneous DNA damage, mutation or epigenetic change. Cells in this stage have a high rate of proliferation with a greater potential to metastasise, through the processes of migration and matrix de-regulation. These three stages can take a long time thus giving the opportunities for interventions to delay or prevent cancer progression (Pitot, 1993, Barrett, 1993, Frank, 2007).

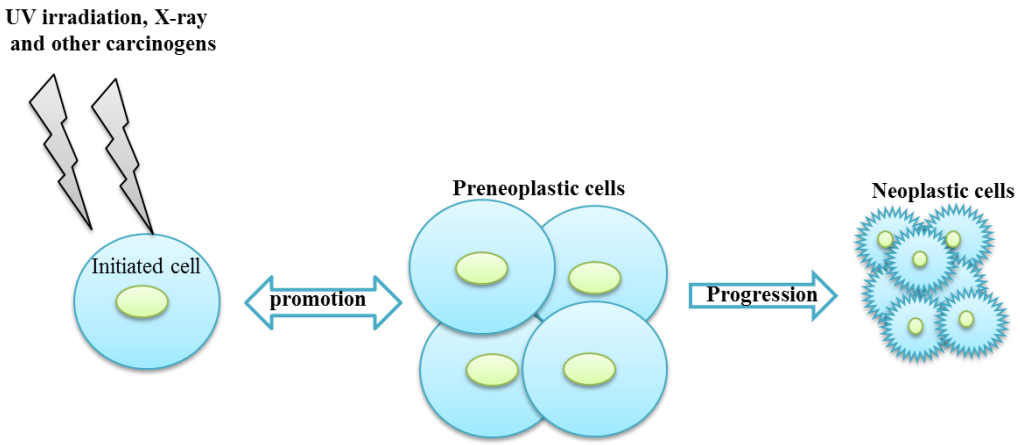

Figure 1.1 Three major steps in the carcinogenesis process; initiation, promotion and progression

(Barrett, 1993, Pray, 2008, Surh, 2003).

1.3 Cellular modifications related to cancer development

Under normal conditions cellular functions are tightly regulated, and the rates of cellular division and death are well balanced. At a cellular level there are numerous changes which affect this overall balance and are related to all three stages described above. According to Hanahan and Weinberg (Hanahan and Weinberg, 2011), there are a number biological modifications in cancer that causes transformation of normal cells to tumour cells. These modifications have been expanded to give a comprehensive view of the cancer process and are indicated in Figure 1.2.

Figure1. 2 Hallmarks of cancer

Adapted from (Hanahan, and Weinberg, 2011).

1.3.1 Sustained proliferative signalling

In normal cells the growth process is regulated through signalling by hormones and growth factors however, cancer cells have the ability to produce these signals through a number of different ways. For example, by producing growth factors themselves, called autocrine signalling, or altering intracellular proteins that independently mediate cell growth in the absence of an external signal (Hanahan and Weinberg, 2011). This is encapsulated in the second stage of the carcinogenesis process and is associated with oncogenic mutations leading to enhanced growth factor production, increased growth factor receptor expression and hyperactive intracellular signalling pathways such as Extracellular Signal-regulated Kinase (ERK) and Phosphoinositide 3-kinase (PI3 Kinase) (Hanahan and Weinberg, 2011, Evan and Vousden, 2001).

1.3.2 The evasion of growth suppressors

There are a group of genes called tumour-suppressor genes that regulate cell division by regulating the entry of cells into specific stages of the cell cycle, by ensuring genetic competency (McClatchey and Yap, 2012). Tumour cells undergo mutation in these genes which allow continual unregulated growth. Furthermore, normal cells are able to sense when the available space is filled and contact inhibition occurs stopping proliferation. In cancer these sensors are missing and cells grow and divide continuously over each other (McClatchey and Yap, 2012).

1.3.3 Resistance to cell death processes

Normal cells are programmed to die in cases where the cell becomes infected or damaged. This characteristic is absent in tumour cells, these cells have direct

alterations in the mechanisms that induce apoptosis or undergo changes in signalling pathways or proteins that have a role in the apoptotic process, for example p52, a check point kinase (Elmore, 2007). These and other components also link to replication immortality (Hanahan and Weinberg, 2011). There is a specific segment at the end of chromosomes, called telomeres. These telomeres become shorter each time the cell divides, after a specific numbers of divisions this segment becomes so short that the cell is unable to divide again. However, cancer cells have specific enzymes that make the telomeres length longer, thus allowing continuous cell growth (Hanahan and Weinberg, 2011, Kawai et al., 2007, Hansel et al., 2006).

1.3.4. Induction of Angiogenesis

There are a number of components that relate to the environment supporting tumour growth and metastatic spread. Angiogenesis, the formation of new blood vessels, is an essential process in embryo development and wound healing where capillary formation provides a source of oxygen and nutrients to the cells nearby. Since tumour cells are in continuous growth they need to develop additional sources of oxygen and nutrients to allow survival (Bergers and Benjamin, 2003). This is when the angiogenic process is activated to support neoplastic growth and expansion. There are a number of mediators released by tumour cells which mediate angiogenesis through binding to specific receptors on the surface of endothelial cells. One of these proteins is vascular endothelial growth factor (VEGF), encoded by the VEGF gene. Overexpression of VEGF by oncogenic transformation or by hypoxia gives cancer cells the ability to promote the growth of new blood vessels. In addition, there are other proteins which alter during the development of cancer to induce

angiogenesis, such as, thrombospondin type 1 (TSP-1) (Ferrara, 2009, Mac Gabhann and Popel, 2008).

1.3.6. Activation of invasion and metastasis

Cancer cells have the ability to make changes in their shape, attach to each other or bind to the extracellular matrix (ECM) (Michel et al., 2010), which helps them to invade local tissue spaces or metastasize to other tissues or organs (Berx and van Roy, 2009). One of the most effective proteins that restricts mesenchymal transition, E-cadherin, is missing in cancer cells. E-cadherin is an adhesion molecule which has a key role in the formation of the junction between adjacent epithelial cells and is thought to help the cells form tissue sheets and retain the cells within the tissue. Many studies have indicated that overexpression of E-cadherin in cells functions to antagonise invasion and metastasis of tumours (Berx and van Roy, 2009, Cavallaro and Christofori, 2004).

1.3.7 Genome instability and mutation

Recently, the molecular genetic analysis of the main types of cancer cell genomes have shown such a wide range of mutations suggesting that genomic instability is enhanced during tumour development. In their study, Korkola and Gary used a genetic analysis method called comparative genomic hybridization (CGH) and they found both increases and decreases in gene copy in cell genomes of a numbers of tumours. In the same study they concluded that there is failure in the control of genomic integrity in cancer cells; this disordering of genomic maintenance reflects defects in DNA repair systems and is thought to be a key component of tumour progress (Korkola and Gray, 2010).

1.3.8 Tumour promoting inflammation

More recent evidence has shown that inflammation has a role in cancer progression; it has been demonstrated that inflammation is active in the earliest stage of cancer development (Qian and Pollard, 2010). Furthermore, Grivennikov and co-workers found that during the inflammation there are several chemical mediators released near the tumor cells, which supports the proliferation of the cells themselves and promotes the invasion of specific immune cells or progenitor cells which aid tumour growth, this can also include endothelial cell progenitors (Grivennikov et al., 2010).

1.3.9 Deregulating cellular energetics

In normal cells there are two ways to produce the energy that is required for cell activity. In the presence of oxygen cells produce energy through conversion of glucose to pyruvate within the cytosol and. Then inside the mitochondria carbon oxide is produced. However, in the absence of oxygen, cells use glycolysis to release the pyruvate. In cancer, cells have ability to use glycolysis process even in in the presence of oxygen which is called aerobic glycolysis (Warburg, 1956a, Warburg, 1956b), thus the tumour cells produce energy that supports their development via this mechanism.

Cancer cell classification depends on the energy generation pathway, the first group uses lactose to produce energy, whilst in the second group, cells use lactose produced by neighboring cells to produce energy (Kennedy and Dewhirst, 2010, Feron, 2009, Semenza, 2008).

1.3.10 Avoiding the immune system

In the normal body, cells and tissues are continuously monitored by the immune system, abnormal cells or early initiated tumor cells are routinely destroyed. Increasingly, tumour cells have the ability to avoid the detection process through one arm of the immune system where expression of cell surface proteins, which the immune system can normally recognise, are altered. There are a number of cancers which can develop following a breakdown in the immune system (immunocompromised individuals) indicating the key regulatory role of this system (Teng et al., 2008, Kim et al., 2007).

1.4. The pancreas and pancreatic cancer

The pancreas is a large gland found in all vertebrates which includes two types of gland cells; endocrine cells which form islets of Langerhans and represent only 2% of the cell mass and the second type is the exocrine cell that forms acini. The pancreas is responsible for production of various hormones such as glucagon, amylin and insulin, and also produces the digestive enzymes: trypsin, lipase and amylase. Cancer can arise from both types of cells, but is commonly exocrine in origin.

Pancreatic cancer (PC) is linked with a poor prognosis and is ranked as the fourth cancer worldwide in terms of death rates (Siegel et al., 2015). Generally, resection is the only effective treatment but only 20% of PC patients are usually suitable for surgery, dependent on the stage of cancer, with an 80% possibility of the tumour returning (Sohn et al., 2000). One of reasons that make PC a fatal cancer is that the diagnosis is usually only made during the later stages because no symptoms or signs are apparent earlier. In addition, there is no clear guidance for checking people who

have a high risk of PC such as those with a family history or pancreas diseases such as diabetes (Sohn et al., 2000, Von Hoff et al., 2013). Only 5% of patients diagnosed with pancreatic cancer survive to 5 years (Jemal et al., 2008, Li et al., 2004), and there are approximately 50,000 new causes of PC annually in the United States (Saluja et al., 2016). During 2010 there were 43,140 people diagnosed with pancreatic cancer and 36,800 deaths, confirming the high mortality rate (Jemal et al., 2010). In a study over three years in Eastern Spain, which included 185 samples from patients diagnosed with pancreatic cancer, the results demonstrated that 6% of these cases involved the tail of the pancreas, 10% in the body and 62% in the head of pancreas whilst the rest of cases were not specified (Porta et al., 2005).

1.4.1. Risk factors

As with many other cancers, the factors that cause pancreatic cancer remain unknown. However, there are many studies which have identified a number of factors related to the development of pancreatic cancer. One research study, which included a number of different countries from Europe, the United States and others worldwide, showed pancreatic cancer to be more common in men than woman, with the risk being 30% greater in males (Ferlay et al., 2010, Andren-Sandberg et al., 1999). The reason for this difference maybe because of gender differences in lifestyle, for example smoking and alcohol intake is more prevalent in males (Lynch et al., 2009). Furthermore, the risk increases with advancing age in both men and women (Ferlay et al., 2010). In addition, ethnicity is considered a risk factor for PC. Silverman and co-workers found that in the US the highest percentage of pancreatic

cancer is in African Americans followed by white Americans, whilst Asian Americans have the lowest percentage (Silverman et al., 2003).

Furthermore, there are many modifiable factors which have been linked to pancreatic cancer, the most important one is smoking. Many studies suggest smoking to be causative agent in 20-30% of PC cases (Parkin et al., 2011, Parkin, 2011a). The risk increases between 2.5 to 3.6 times in comparison to non-smokers, and increases with the number of cigarettes and exposure time to smoke (Hassan et al., 2007). According to Parkin study found about 29% of pancreatic cancer cases in the UK were thought to be related to tobacco use (Parkin, 2011b).

Another modifiable factor related to PC cancer is vitamin D, and there are three different aspects to consider. Many studies suggest that Vitamin D intake through the diet or synthesis during exposure to the sun, may have a role as a protective factor in PC (Grant, 2007, Mohr et al., 2010, Bao et al., 2010a, Giovannucci, 2009). Indeed, one study has also shown that low levels of vitamin D can increase the risk of PC (Stolzenberg-Solomon et al., 2009). In contrast, another study from the same group suggests that there is no relationship between low levels of vitamin D and PC, but observed that high levels of vitamin D doubled the risk of PC (Stolzenberg-Solomon et al., 2010). Clearly more studies are required to resolve this issue.

There is no clear evidence regarding the role of alcohol intake as a risk factor in PC. One study has showed that consuming about 30g or more a day (Genkinger et al., 2009) or consuming three or more drinks of alcohol increased the risk of pancreatic cancer (Tramacere et al., 2010). However, another study noted that there was no correlation between alcohol and pancreatic cancer even for a person consuming 60g

or more each day (Michaud et al., 2010). These finding suggest the role of alcohol intake in development of pancreatic cancer still requires further investigation.

There are also very limited studies regarding the relationship between other modifiable factors, such as aspirin use and intake of coffee and the development of pancreatic cancer (Streicher et al., 2014, Dong et al., 2011). However, there is much more evidence linking pancreas diseases to the development of cancer. Several studies demonstrate an increase in disease progression if the patient has a history of chronic pancreatitis, diabetes, previous incidence of gastric ulcers, obesity and intestinal cancers (Batty et al., 2009, Landi, 2009, Lowenfels and Maisonneuve, 2006, Genkinger et al., 2009, Bao et al., 2010b, Aune et al., 2012, Shen et al., 2006).

Whilst the majority of pancreatic cancers involve somatic mutations due to external factors, about 5% to 10% of pancreatic cancers are hereditary. The risk increases three time in people who have a family history of pancreatic cancer (first degree relative) and 57 times in cases where there are four or more relatives who have pancreatic cancer (Hidalgo, 2010, Shi et al., 2009). Many studies suggest there are numbers of germ line mutations related to increased risk of pancreatic cancer in families who have history of the disease, for example, BRCA2, STK11, PRSS1 and P16 (and others see section 1.1.3). Nevertheless, these genes are responsible for developing pancreatic cancer in only a small portion of patients with a familial susceptibility, the genetics underpinning pancreatic cancer development remain largely unknown (Shi et al., 2009, Giardiello et al., 2000, Goldstein et al., 1995, Lowenfels et al., 1993, Whitcomb et al., 1996).

Interestingly, one non-modifiable genetic factor linked to pancreatic cancer is blood group. A person who has blood type O has the lowest rate of PC compared with the other three main blood groups A, B and AB (Wolpin et al., 2009, Pelzer et al., 2013). Overall the large number of studies outlined above indicates a complex array of factors which result in the development of pancreatic cancer.

1.4.2. Symptoms

The most common symptoms of pancreatic cancer are; abdominal pain, anorexia non-logical weight loss, back pain, vomiting, diarrhoea, jaundice, and asthenia. Studies demonstrate a correlation between the location and size of tumour within the pancreas and the symptoms. The jaundice, weight loss and steatorrhea were found to be closely related with head tumours, but did not occur with tail tumours (Modolell et al., 1999, Kalser et al., 1985, Bakkevold et al., 1992). Porta and co-workers showed that 60-70 % of tumours are at the head of the pancreas, whilst, 20-25% are localised within the body and tail. In a study involving 185 pancreatic cancer patients, they found that 71% of these patients suffered from epigastric pain and 79% of patients had abdominal pain (Porta et al., 2005). Furthermore, it has been found that PC caused death in approximately 90% of those diagnosed with the disease, 70% of patients died because of spread to other organs, the remaining 30% died with limited metastases at the time of death, however most patients had bulky primary tumours (Iacobuzio-Donahue et al., 2009).

1.4.3. Types of pancreatic cancer cell tumour

Since the pancreas contains two types of secretory cells, there are two types of pancreatic cancer which can develop. Endocrine tumour cells are formed from

endocrine cells (cells producing hormones) and exocrine tumour cells are derived from exocrine cells (cells producing enzymes). Endocrine tumours are also referred to as neuroendocrine tumours,which have the potential to be malignant, and are considered to be the less common type of pancreatic cancer. The second group, which are called exocrine tumours, are further subdivided into two types; adenocarcinomas and mucinous.Tumours of Adenocarcinomas are considered the most prevalent type of exocrine pancreatic tumour and have a poor prognosis with a high level of malignancy. Mucinous tumours have a slightly better prognosis when compared with adenocarcinomas (Goodman et al., 2012).

1.4.4. Stages of pancreatic cancer

According to the Tumour Node Metastasis system from the American Joint Committee on Cancer (AJCC), pancreatic cancer is staged and the main distinguishing features used for staging the pancreatic cancer is resectability of the tumour and whether or not it has metastasised. There are four main stages of pancreatic cancer with subdivisions; in stage I and II the tumours are classified as resectable, stage III tumour are classified as unresectable and locally advanced tumours, while the tumour in stage IV exhibit distant metastases (Edge and Compton, 2010) (see table 1.2).

Table 1.2 Stages of pancreatic cancer.

*M indicates distant of metastasis, ** N for lymph nodes (Bilimoria et al., 2007, Hidalgo, 2010).

Stage	characteristics	Distant metastases *	Nodal status **	Median survival (Months)
I A	Tumour is limited to pancreas and the size of tumour <2cm	M0	N0	24.1
IB	Tumour is limited to pancreas and the size of tumour >2cm	M0	N0	20.6
IIA	Tumour spread beyond the pancreas	M0	N0	15.4
IIB	Different size of tumour	M0	N1	12.7
III	The tumour involves the celiac axis or superior mesenteric artery	M0	N0 or N1	10.6
IV	Distance metastasis with any zise of tumour	M1	N0 or N1	4.5

Over recent years the genetic analysis of pancreatic cancer has been developed by many research groups and has the potential to guide future personalised and more effective treatment. Studies analysing 456 pancreatic cancer patients samples, using integrated genomic analysis, showed 32 gene mutations implicated in ten cellular pathways and related processes including; KRAS, transforming growth factor beta (TGF-β), NOTCH, ROBO/SLIT, WNT signalling, chromatin modification,

SWTch/surose non-Fermentable (SWI/SNF) event linked to G_1/S phase of the cell cycle, DNA repair and RNA processing (Bailey et al., 2016).

Further expression analysis has determined four key subtypes of tumours. Firstly squamous tumours, the most common genes mutations in these tumours, include genes involved in hypoxia response, inflammation and metabolic reprogramming (Hoadley et al., 2014), The second subtype called a pancreatic progenitor tumour, involves upregulate of genes that are involved in early pancreatic cancer development, for example, FOXA2/3, MNXI and PDX1 (Hale et al., 2005). A third subtypes called Aberrantly Differentiated Endocrine Exocrine (ADEX), includes genes involved in KRAS activation, exocrine differentiation and endocrine differentiation (von Figura et al., 2014). An immunogenic tumour is the fourth subtype, featuring upregulation of genes and their downstream products required for suppression of the immune system (Rooney et al., 2015).

1.4.5. Treatment of pancreatic cancer

As implicated above surgery is considered the main treatment for pancreatic cancer, patients with rececTable tumours have an increased probability of recovery (Jones et al., 2008). The surgery used is dependent on the location of the tumour, for a tumour

in the head of the pancreas the typical procedure is a pancreaticodoudenectomy, whilst the common treatment of a recetable tumour in the tail of pancreas is a distal pancreatectomy (Yokoyama et al., 2009). Only stage I and II tumours will be considered amenable for surgery, however even at these early stages survival after five years is only 30%.

Therefore, because of the limitations in using surgery to treat pancreatic cancer other approaches such as chemotherapy are needed (Jones et al., 2008, Li et al., 2004). Chemotherapy is used primarily as an adjuvant after surgery. The European Study Group of Pancreatic Cancer 1 (ESPAC-1) found that there was a relationship between the survival rates of patients who had surgery followed by chemotherapy or radiotherapy treatment. The five year survival rate was 21% in the group who had taken chemotherapy after resection compared with 8% in the group without chemotherapy, the average survival time was 20.1 months in the chemotherapy group compared to 15.5 months without chemotherapy. Interestingly, the study also found that for the group of patients who used a combination of chemo and radiotherapy, in addition, to surgery, their general health became worse (Neoptolemos et al., 2004).

For treatment of the advanced local (III) or metastatic (IV) stages of pancreatic cancer, chemotherapy or radiation is the only option available and is only useful in reducing the symptoms of the disease. Burris and co-workers studied the effect of chemotherapy and survival rate of PC patients, the data suggested that gemcitabine is the optimum option. Gemcitabine reduced the symptoms such as pain in about 23.8% of patients compared with 5% with 5-Fluorouracil. Additionally, the study found that the survival average was increased to 5.7 months compared to 5-Fluorouracil which

was 4.4 months (Burris et al., 1997). However, this study did not compare either of drugs with untreated control patients.

More recent studies have exploited the potential of targeting different cellular pathways for example, using Erlotinib which is an inhibitor of the epidermal growth factor receptor. In a study conducted by the National Cancer Institute of Canada Clinical Trials Group using new targeted therapies such as Erlotinib and other tyrosine kinase inhibitors, they found there was a significant increase in survival rates in stage III patients compared with those using gemcitabine alone (Kelley, et al 2008) (see Table 1.3).

Table 1.3 Chemotherapy agents that use as treatment of pancreatic cancer

TDrug	pharmacological class	Mechanism of action	Common adverse effect
Erlotinib	Tyrosine kinase effect	Inhibition phosphorylation of numbers tyrosine kinase	Anorexia, vomiting, rash, diarrhoea and fatigue
Gemcitabine	Antimetabolite pyrimidine analogue	There are two action; diphosphate and triphosphate, and inhibit the ribonucleotide reductase that inhibition of DNA synthesis process.	Anemia, nsusea, myelosuppression and vomiting
5-fluorouracil	Antimetabolite pyrimidine analogue	Inhibit the thymidylate synthase, therefore inhibition DNA synthesis	Anemia, nsusea, myelosuppression and vomiting

1.5. Signalling pathways in pancreatic cancer

The studies outlined above demonstrate the limitation of current therapeutic approaches for the treatment of pancreatic cancer. A better understanding of the role of different signalling pathways in the development of pancreatic cancer may allow better targeting of the disease and lead to the development of a new treatment. The

next section outlines a number of signalling pathways linked to pancreatic cancer which include; Nuclear Factor Activated T-cell (NFAT), Nuclear Factor like-2 (NRF2), Wnt, ERK and the Nuclear Factor Kappa B (NFκB) pathway (Martin, 2003). The reader is directed to a number of excellent reviews of this general topic (Arlt et al, 2012, Karanikas, et al., 2016).

1.5.1 Extracellular signal-regulated kinases ERK

Over 30 years ago, four similar signalling pathways were identified as Mitogen-activated protein kinases (MAPKs) each containing a different cascade of kinase signalling. These are known as the: ERK, the c-Jun amino-terminal kinases (JNK), p38 and ERK5 pathways. Usually, the JNK, p38 and ERK5 pathways are activated by growth factors or stress, whilst the ERK pathway is activated by growth factors through cell surface receptors (Benson et al., 2006). There are two predominant isoforms of ERK, ERK1 (44 kDa) and ERK2 (42kDa) which show a 70% similarity in the amino acid sequence of their chemical structure. Phosphorylation of both threonine and tyrosine residues of ERK leads to its activation (Downward, 2003). One activated, ERK dissociates from MEK and is translocated into the nucleus and through activation of transcription factors regulates a variety of genes. For example, increased expression of cyclin D1 and Myc, which have a role in cell progression, inhibits the pro-apoptotic process by induced overexpression of Bcl2-associated death promoter (BAD) and Bcl2-like protein 11 (Bim). In addition, this increases the expression of Bcl2 and Blc-x expression which promotes the anti-apoptotic process (Neuzillet et al., 2014). Furthermore, activation of ERK kinases promotes regulation of many biological processes such as, cell survival, proliferation, migration and

angiogenesis through promoting phosphorylation of a number of transcription factor and cytoskeletal proteins (Yoon and Seger, 2006).

Unsurprisingly, activation of ERK kinases are implicated in cancer development through driving a number of signalling pathways and through the regulation of gene expression (Yoon and Seger, 2006). Therefore, targeting ERK has become the aim of many studies. The ERK pathway can be mediated through MEK inhibitors such as PD184352, PD0325901 and AZ6244 which have been developed as potential therapeutic strategy for cancer treatment (Sun et al., 1993, Sebolt-Leopold et al., 1999). Some of these inhibitors are under current examination in pre-clinical and clinical studies (McCubrey et al., 2010, Zhao and Adjei, 2014), whilst some have shown promising result with survival benefits in a clinical setting (Flaherty et al., 2012).

Interestingly, KRAS mutations have been found in 95% of pancreatic cancers (Bryant et al., 2014) and many studies have reported that the KRAS mutation is essential for pancreatic cancer development (Cox et al., 2014, Stephen et al., 2014). However, a recent study concluded that MEK inhibitors are inactive or have limited effect on cancers that have KRAS mutations and this attributed to the loss of feedback inhibition and reactived ERK pathways through REK compensatory mchanisms (Samatar and Poulikakos, 2014). Therefore, other proteins downstream of KRAS have been investigated in a number of studies as potential therapeutic strategy for cancer treatment (Rachagani et al., 2011, Maier et al., 2013, Ling et al., 2012, Chiao and Ling, 2011, Pak and Miyamoto, 2013) see Figure 1.3.

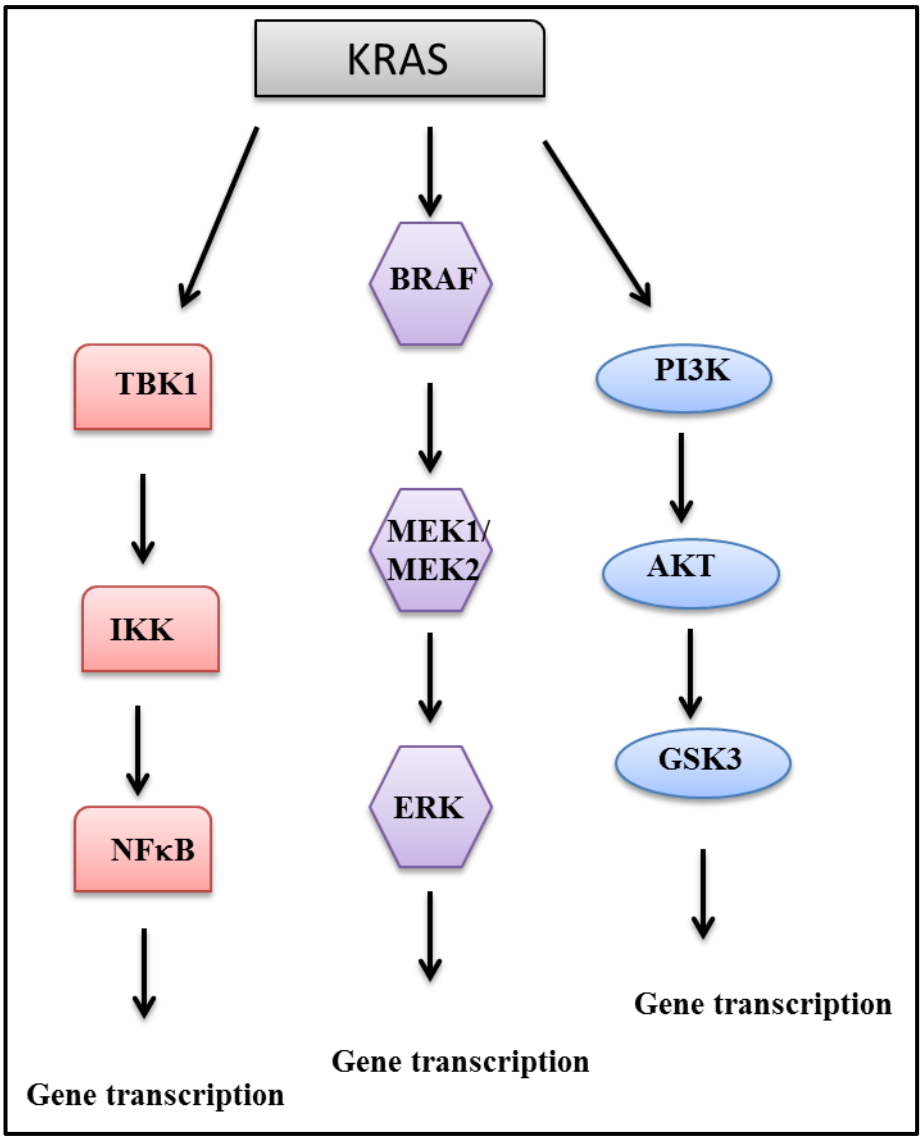

Figure 1.3 The Major interaction of KRAS pathway

Proteins downstream of KRAS signaling which could be promising therapeutic targets for pancreatic cancer treatment in patients with KRAS mutation. Adapted from (de Castro Carpeno and Belda-Iniesta, 2013).

1.5.2 The Nuclear Factor Activated T-cell (NFAT) pathway

The NFAT family consists of a group of calcineurin-responsive transcription factors, which play key role in the regulation of cellular apoptosis and growth, particularly in the context of T lymphocyte activation (Konig et al., 2010, Baumgart et al., 2013). There are four isoforms; NFATc1 (NFAT2/NFATc), NFATc2 (NFAT1/NFATp),

NFAT3 (NFAT4/NFATx) and NFATc4 (NFAT3) and activation is under control of the Ca^{2+} / calcineurin signalling pathway (Mancini and Toker, 2009). At rest NFAT is located within the cytoplasm and is highly phosphorylated on a number of serine residues. Stimulation leads to the dephoshorylation by calcineurin, entry of NFAT2 into the nucleus and subsequent regulation of gene transcription (Nayak et al., 2009)

Many reports have shown that the NFATc1/NFAT2 and NFATc2/MFAT1 pathways play a role in chemo-resistance and carcinogenesis in different stages of pancreatic cancer (Baumgart et al., 2013, Baumgart et al., 2012, Shibasaki et al., 2002). Additionally, others have documented that NFAT plays a role in the regulation of growth and development of pancreatic tumours, however, there is very limited data about the appropriateness of NFAT as a molecular target for therapies (Mancini and Toker, 2009, Medyouf and Ghysdael, 2008).

NFκB and NFAT both belong to the superfamily of transcription factors and have the same DNA binding site (Serfling et al., 2004). Additionally, the regulation of COX-2 expression depends on the balance between both the NFκB and NFAT pathways (Hai et al., 2011, Cai et al., 2011). A role for COX-2 has been demonstrated in the chemo- resistance of pancreatic tumour cells (Jendrossek, 2013, Li et al., 2008) however, how these pathways interact in pancreatic cancer is still not understood.

1.5.3 The Nuclear Factor like-2 Nrf2 pathway

Nrf2 is a transcription factor, also known as nuclear factor (erythoid- derived)-like 2 or NFE2L2, is part of the cap'n' collar basic leucine zipper protein family and plays a key role in cellular defence to oxidation (Moi et al., 1994, Gold et al., 2012, Nguyen et al., 2009, Martin-Montalvo et al., 2011). In normal conditions, Nfr2 is

located within the cytoplasm of cells, anchored to kelch-like ECH-associated protein 1 (Keap1) (Martin-Montalvo et al., 2011, Nguyen et al., 2009). Nrf2 can be activated by many cellular conditions or oxidative stress, and the interaction of both Nrf2 and cysteine residues of Keap1 results in the translocation of Nrf2 into the nucleus (Tian et al., 2012).

DeNicola and co-workers proposed a critical role for Nrf2 in the oncogenesis of pancreatic tumours (DeNicola et al., 2011). Furthermore, another study demonstrated up-regulation of cytoplasmic Nrf2 either in pancreatic cancer cell lines or from patient's samples (Lister et al., 2011). Even in the absence of mutations in mediators which activate this pathway an additional study noted that increasing Nrf2 through cellular stimulation or over expression of Nrf2 gives pancreatic cancer cells resistance against apoptotic stimuli (Hong et al., 2010).

Most of the research which has studied the interaction between the NFκB and NRF2 pathways concentrates on the activation of NRF2 through oxidative stress which enhances NFκB signalling and in turn causes the inhibition of NRF2 through an unknown mechanism (Li et al., 2008). Arlt and co-workers found that NRF2 has the ability to facilitate the release of NFκB from IκBα (Arlt et al., 2009). However, there is a study which demonstrated that the p65 NFκB subunit of NFκB could interact with Keap 1 to modulate the activity of NRF2 (Yu et al., 2011).

1.5.4. Wnt signalling pathway

The Wnt signalling pathway plays an essential role in growth and differentiation of cells during development and is strongly implicated in cancer. This pathway can be

activated through binding of ligands to the Wnt receptor on the cell membrane and activation of β-catenin. In cells at rest β-Catenin is inactivated through binding to GSK3-β kinase. Phosphorylation of this complex triggers the release of β-Catenin, accumulation within the cytosol and translocation into the nucleus where subsequent expression of target genes occurs. (Zeng et al., 2006, Pasca di Magliano et al., 2007).

Many studies have linked activity of this pathway to pancreatic cancer development where increased levels of β-Catenin was noted in pancreatic cancer patient samples but not in normal pancreas (Pasca di Magliano et al., 2007, Zeng et al., 2006). Lowy and co-workers found there was a high level of β-Catenin expression only in the early stage of pancreatic cancer (Lowy et al., 2003). In addition, it was demonstrated that blocking the expression of β-Catenin resulted in decreased proliferation of pancreatic cancer cells (Pasca di Magliano et al., 2007). These findings provide strong evidence that the Wnt pathway has an involvement in pancreatic cancer development.

1.5.5. The Nuclear factor kappa B pathway (NFκB)

In 1986, NFκB was identified as a transcription factor in B cells by Sen and Baltimore (Sen and Baltimore, 1986). Today the NFκB pathway is recognised as a key signalling mechanism found in many types of cell and plays an important role in a diverse range of cellular processes, including: inflammation and immune function, growth and apoptosis, cell division and fetal development (Brand et al., 1997, Brand et al., 1996, Oeckinghaus and Ghosh, 2009).

There are two main NFκB pathways within the cell, the classical or canonical pathway and the non-canonical (described in more detail later in this section). These two pathways comprise of three main components; the NFκB transcription factor, a regulatory inhibitory protein called Inhibitory kappa B (IκB) and thirdly a key regulatory kinase called inhibitory kappa kinase B (IKK). A common mechanism of activation exists where, at rest, the NFκB transcription factor resides within the cytosol bound or partially bound to an IκB. Following cellular activation this complex dissociates due to the activity of the IκB kinase and the transcription factor translocates to the nucleus, where it binds to promotor regions of different genes. Variations exist within each pathway although the molecular structures of each of the components share similar features which are outlined below (Gamble et al., 2012a).

1.6 Genes regulated by NFκB

There are many cellular functions in which NFκB plays a key role, such as apoptosis, growth, survival, development and differentiation. As outlined above this is dependent upon transcriptional regulation of a number of NFκB-dependent genes, following binding of heterodimers or homodimers to specific DNA binding motifs. The core sequence for specific DNA binding has been identified for p65/p50 as 5-GGGRNYYYCC-3' (where R=A or G; Y=C or T; W=T or A; V=A, C or G) (Chen and Ghosh, 1999, Chen et al., 1998). Through this basic binding motif, many genes are regulated, for example, vascular cell adhesion protein 1 (VCAM-1), COX-2 and intercellular adhesion molecule-1 (ICAM-1)(Denk et al., 2001). Additionally, p65/RelA is also responsible for regulation of anti-apoptotic genes such as cellular inhibitor of apoptosis protein (c-IAP) and X-linked inhibitor of apoptosis protein

(XIAP) (Chu et al., 1997, Stehlik et al., 1998). Furthermore, RelA is responsible for the regulation of other genes that influence cell proliferation and survival, called pro-survival factors, for example, BCL-1 and 2 (Catz and Johnson, 2001).

Genes which mediate immune cell trafficking, angiogenesis and lymphoid development are regulated by Rel B. The sequence that Rel B binds to within DNA has also been identified as 5'-GGGRVWTTYY-3'; (Britanova et al., 2008). In 2002 it was found that several chemokines, including: B lymphocyte chemoattractant (BLC), secondary lymphoid tissue chemokine (SLC), stromal cell- derived factor-1α (SDF1α) and EBI-1 ligand chemokine factors that are responsible for the regulation of lymphoid organogenesis, were regulated by Rel B subunits (Dejardin et al., 2002). Another study conducted by Wharry and colleagues, investigated pancreatic ductal adenocarcinoma (PDAC), and identified that some genes, for example, cytokine B cell activation factor (BAFF) and chemokine C-X-C motif ligands CXCL12, CXCL13 and C-C motif ligands CCL19 and CCL12 are up-regulated through activation of RelB in this tumour type. The development of lymphoid malignancies and solid tumours are also strongly linked to the expression of several of these genes (Wharry et al., 2009).

1.7 Structure and function of NFκB

In normal cells NFκB proteins are found as heterodimer or homodimer complexes formed from five proteins located in the cytoplasm (Figure 1.4) which include p52/p100, c-Rel, RelB, p65/RelA and p50/p105. p65/RelA is also known as p65 NFκB while p50 is also named NFκB1, the molecular weight of these two proteins are 65 kDa and 50 kDa respectively. NFκB isoforms including p50 and p65 NFκB

shuttle from the cytoplasm to the nucleus where they bind to the consensus DNA sequence. These sites are called κB sites and through this binding, transcriptional activity is regulated (Zabel et al., 1991, Kawakami et al., 1988, Ghosh et al., 1990, Sen and Baltimore, 1986).

Structurally, all NFκB subunits have an N-terminal Rel homology domain termed (RHD) which is highly conserved, this site is not only responsible for DNA binding but also nuclear translocation, protein dimerisation and interaction with specific IκB proteins (Hayden and Ghosh, 2004). Furthermore, NFκB is further classified depending on the presence of C- terminal transactivation regions. Class 1 NFκB isoforms do not have a C- terminal transactivation region, these subunits, p50/p105 and p100/p52 instead have a trans-repression domain (Brown et al., 1994, Kang et al., 1992, Lernbecher et al., 1993, Plaksin et al., 1993, Schmitz and Baeuerle, 1991). In contrast, class 2 NFκB subunits have C- terminal transactivation regions and these include RelA, c-Rel and RelB subunits (Dobrzanski et al., 1993).

1.7.1. IκB structure

There are a number of IκBα proteins which regulate NFκB isoforms within the cytosol. The family includes IκBα, IκBβ (Thompson et al., 1995), IκBγ (Inoue et al., 1992) IκBε (Li and Nabel, 1997), and p100/p105 (Mercurio et al., 1993, Perkins et al., 1992). The first time IκB was identified as an inhibitor for NFκB, by Baeuerle and his co-workers, they demonstrated that binding retained NFκB subunits within the cytoplasm of cells (Baeuerle and Baltimore, 1988).

The binding site for IκB is located in the RHD within the Rel/ NFκB dimer,requires an ankyrin repeat (Cervantes et al., 2009, Henkel et al., 1992) and according to many studies each IκB protein is specific for each Rel/NFκB protein. For example, RelB only binds to p100/p105 (Dobrzanski et al., 1995), whilst IκBα and IκBβ bind to and strongly inhibit the RelA/p52 dimer (Baeuerle and Baltimore, 1989, Beg et al., 1992, Thompson et al., 1995).

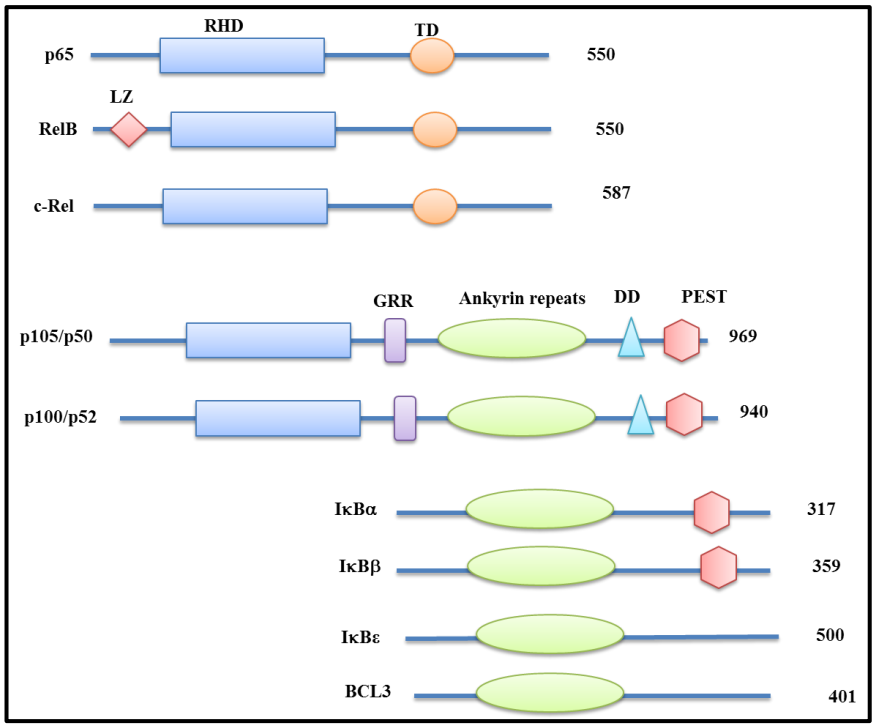

Figure 1.4 NFκB/IκB subunits.

The structures of various subunits of NFκB and IκB members, a number of amino acid is display next to each NFκB and IκB members. LZ, leucine zipper; TD, transactivation domain, Rel homology domain, PEST region, polypeptide sequence enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) region, GRR, glycine-rich region, DD, death domain adapted from (Beinke and Ley, 2004).

1.7.2. Regulation of IκBα

In 1990 Zabel and Baeuerle discovered that there were two main isoforms of IκB in resting cells that associated with NFκB; these two isoforms are named as IκBβ and IκBα. Several studies indicate that IκBα but not IκBβ has an important role in the canonical NFκB pathway. When cells are at rest and the NFκB pathway is not activated, IκBα works as an inhibitor by binding to NFκB (p65/p50) to prevent

translocation into the nucleus (Jaffray et al., 1995). The affinity of IκBα for each NFκB isoform has enabled classification into three groups; highest affinity when the IκBα binds to the heterodimer (p65/p50), medium affinity for IκBα binding to the p65 NFκB homodimer and the lowest affinity for IκBα binding to the p50 homodimer (Malek et al., 1998). Ganchi and co-workers showed that the site where IκBα binds to p65 NFκB also contains the Nuclear localization sequence (NLS) because when there is a mutation at this site the binding of p65/IκBα is disrupted. There are two sites for IκBα binding within the p65 NFκB homodimer; aa 450-500 and aa 151-312. Mutation within these regions leads to dissociation of the p65/IκBα complex but does not disrupt DNA binding (Ganchi et al., 1992)

Following cellular activation, IκBα is phosphorylated at serine residues 32 and 36 within the N-terminus. This has been shown to occur following stimulation by agents, such as TNFα, ionomycin and phorbol 12-myrisated 13-acetate (PMA) (Brockman et al., 1995, Brown et al., 1993). The IκBα phosphorylation functions to initiate ubiquitination and targets the protein for proteasomal degradation which leads to liberation of the NFκB subunits. The ubiquitination of IκBα is considered to be an important and fundamental step before degradation of IκBα (Li et al., 1995). Lysine residues 22 and 21 have been identified to be the primary site for signal-induced ubiquitination of IκBα, when mutations occur in these sites the phosphorylation of IκBα by the IKK complex persists (see below) however, ubiquitination and degradation is inhibited and as a consequence NFκB remains in

the cytoplasm because it cannot be separated from IκBα (Scherer et al., 1995, Chen et al., 1996).

1.7.3. NFκB2 (p100/p52)

NFκB2 (p100/p52) was originally called p98 because the molecular weight of this protein is 98 kDa. The activation of this subunit is unique when compared to the other NFκB subunits but p100 can function as an IκB family member (Mercurio et al., 1992, Mercurio et al., 1993). Both p100 and p105 are similar to IκBα in that these subunits have ankyrin repeats within the C- terminal region, and the C-terminal domains of both p105 and p100 function to retain NFκB in the cytoplasm (Rice et al., 1992, Mercurio et al., 1993). The formation of NFκB is mediate via proteasomal-mediated degradation of the C-terminus of both p105 and p100; in this process both p100 and p105 are spilt in half forming the two NFκB subunits, p50 and p52 respectively (Fan and Maniatis, 1991, Siebenlist et al., 1994).

1.7.4. Inhibitory Kappa B kinases

As indicated previously an essential step in the activation of NFκB is phosphorylation of IκBα. Chen and co-workers discovered a complex with a molecular weight of approximately 700-900 kDa that was responsible for IκB phosphorylation (Chen et al., 1996), and called it IκB kinase (IKK). The role of the complex was confirmed by studies carried out in HT-29 cellswhere TNFα was use to stimulate the activation of the IKK complex and this led to IκBα phosphorylation and degradation. Overexpression of IKK also increased NFκB activation (DiDonato et al., 1997).

It is now understood that the IKK complex is comprised of both IKKβ and IKKα which are catalytically active kinases and IKKγ which is inactive. Woronicz *et al* found similarity in protein structure within IKKβ and IKKα; both proteins have a C-terminal helix-loop-helix (HLH) domain, leucine–zipper motif and NH_2-terminal kinase domain (see Figure 1.4) (Woronicz et al., 1997). Significantly, it was found that there was a diversification in the NFκB pathways; IKKβ regulates the canonical pathway whilst IKKα regulates the non-canonical NFκB pathway (May et al., 2010).

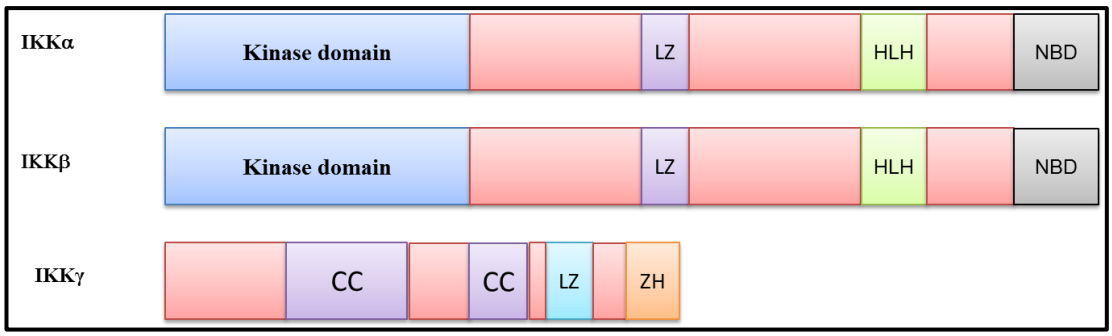

Figure 1. 5 Structures of IKKα, IKKβ and IKKγ.

Abbreviations; LZ :leucine zipper motif, HLH: helix-loop-helix domain, NBD: MEMO-binding domain and CC: coiled coli region adapted from (Pham and Tenoever, 2010).

1.8 Cellular regulation of the canonical pathway

The structural modules described for each NFκB cascade and their control by two distinct molecules implicates distinctiveness at the cellular regulatory level. In terms of physiological responses, the canonical NFκB pathway is activated principally by cytokines, for example, TNFα and IL-1β. However, for this pathway in particular the range of stimuli is quite diverse and includes multiple activators of Toll like

Receptors (TLRs) such as: UV light, reactive oxygen species and neurotransmitters (Hayden and Ghosh, 2008).

Irrespective of the stimuli a key integration point of this pathway is the activation of IKKβ, which in turn mediates phosphorylation of IκBα promoting ubiquitination and degradation. However there are a number of key intermediates that link the IKK complex to the cognate receptor, for example TNFα stimulates receptor (TNFR1) which via TNFα receptor-associated factor 2 (TRAF2) leads to activation of the pathway (Plotnikov et al., 2011). Another independent study supported the above interaction by showing there was increased p65 NFκB binding to DNA during the over-expression of TRAF2 (Devin et al., 2001). Transforming growth factor β – activated kinase (TAK-1), which is a member of the mitogen-activated protein/FRK kinase 3 family (MAP3K), plays a role in the phosphorylation and activation of the IKK complex. The association between TAK-1 and IKKα, IKKβ and TRAF2 was induced by TNFα (Takaesu et al., 2003) (see Figure 1.5).

In contrast, activation of the classical NFκB pathway by IL-1 occurs after TRAF6 engagement and not TRAF2. After stimulation by IL-1 (see Figure 1.6), the response to this agent is mediated by the recruitment of TAB2 which functions as an adaptor protein. TAB2 translocates from the plasma membrane to the cytosol, linking to TAK-1 and TRAF6 (Takaesu et al., 2000). Kishida and his co-workers found that TAK-1 facilitated the ubiquitination of TRAF6 during stimulation of cells with IL-1 (Kishida et al., 2005). TAB3 and TAB2 binding to TRAF6 was mediated through the zinc finger (ZnF) domain (Kanayama et al., 2004), where mutations in this domain

made TAB2 and TAB3 unable to bind to TRAF6 and led to the failure of TAK1 activation of the IKK complex (Kanayama et al., 2004).

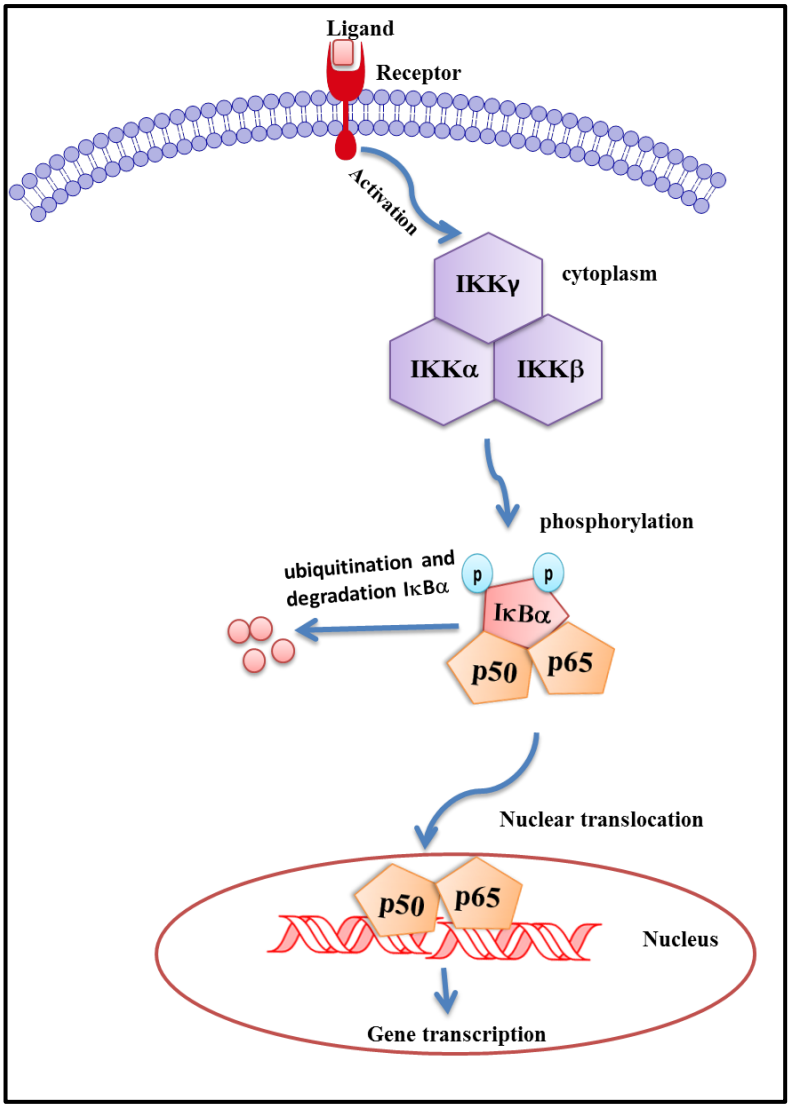

Figure 1.6 The classical NFκB pathway.

A simplified model diagram of the canonical NFκB pathway where, following activation via a membrane receptor, IKKβ induces degradation of IκBα and allow to p65 NFκB phosphorylates then p65 translocation to the nucleus and promoting expression of target genes. Adapted from (da Silva et al., 2013).

1.9 The regulation of the non-canonical NFκB pathway

A number of studies have demonstrated that IKKα plays a key role in activation of the non-canonical NFκB pathway, (see Figure1.7) (Claudio et al., 2002, Dejardin et al., 2002, Liang et al., 2006, Senftleben et al., 2001). Activation of the non-canonical NFκB pathway requires phosphorylation of the p100 subunit which is regulated by IKKα. Phosphorylation and processing of p100 is crucial for formation of the RelB/p52 complex and translocation of p52 into the nucleus to initiate gene transcription (Luftig et al., 2004, Muller and Siebenlist, 2003). There have been studies in mice which conclude that this pathway has an important role in B-lymphocyte function and lymphoid organogenesis (Senftleben et al., 2001, Bonizzi and Karin, 2004), however, the role of this pathway in inflammation remains unclear (Bonizzi and Karin, 2004, Lawrence and Bebien, 2007).

The *TNFR* superfamily including CD40 (Coope et al., 2002), B cell-activation factor receptor (BAFFR) (Claudio et al., 2002, Kayagaki et al., 2002), receptor activator for nuclear factor κB ligand (RANKL) (Novack et al., 2003), lymphotoxin β receptor (LTβR) (Dejardin et al., 2002) and TNFR2 (Rauert et al., 2010), have all been shown to couple in the non-canonical NFκB pathway. Furthermore, this pathway also can be activated by the cognate ligands for each receptor, for example, stimulation by RANK in osteoclast precursors leads to processing of p100 to p52 (Novack et al., 2003).

Following receptor activation, there are a series of events that occur prior to phosphorylation of p100. Both TRAF3 and TRAF2 are degraded which leads to

elevated expression and activation of NFκB-inducing Kinase (NIK). This in turn activates IKKα which regulates the p100 processing (Xiao et al., 2001a). Ling and co-workers found that phosphorylation of IKKα at residue 176 was under the control of NIK, they confirmed this through mutating residue 176 of IKKα which prevented IKKα phosphorylation.

In addition, the failure of IKKα phosphorylation blocked TNFα and IL-1 activation of the non-canonical NFκB pathway (Ling et al., 1998). Another study showed that NIK has the ability to regulate processing of p100 through a region named NIK responsive domain (NRD), a mutation in NIK led to decreased processing of p52 in alymphoplasia mice (Xiao et al., 2001b). It seems that IKKα and NIK both have an essential role in the activation of the non-canonical NFκB pathway (Razani et al., 2010) and both of them could be potential targets for the inhibition this pathway.

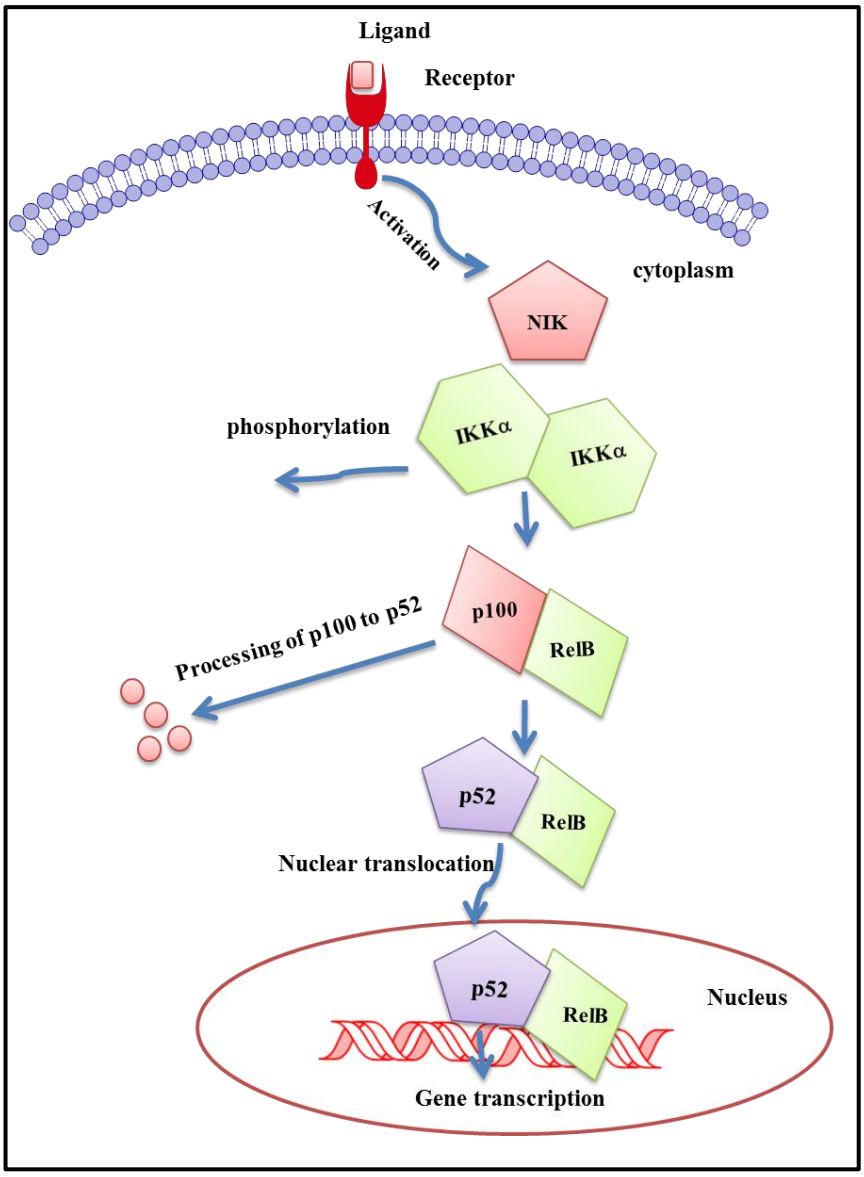

Figure 1.7 The non-canonical NFκB pathway.

A model diagram of non-canonical NFκB pathway. This activation leads to the phosphorylation of IKKα which induces procession p100 to p52 and formation RelB/ p52 dimer. The dimer then translocate to the nucleus which promote the transcription of target genes. Adapted from (Beinke and Ley, 2004)

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1.10 Nuclear Factor kappa B pathways in cancer

An increasingly large body of evidence suggests that NFκB could contribute to a number of cellular processes related to cancer development such as proliferation and cell survival (Valentine et al., 2010, Gilmore, 1999). In 2010 Tysnes highlighted two cellular processes that linked NFκB activity and tumour development. These were, increased expression of survival genes which made cells resistant to apoptosis and synthesis of cell cycle genes such as cyclin D1 and cyclin D2 (Tysnes, 2010).

Cellular activity of NFκB in cancer has been demonstrated to correlate with clinical outcomes. For example, NFκB has been shown to be active in 95% of cancers such as pancreatic, colorectal and lung cancer (Lu et al., 2004, Lu and Stark, 2004, Senegas et al., 2015). Furthermore, high-levels of NFκB were observed in primary breast cancer tissue and mammary carcinoma cell lines derived from both human and rodents (Cogswell et al., 2000). High levels of NFκB have also been measured in melanoma, the most aggressive type of skin cancer (Kashani-Sabet et al., 2004). Karin and co-workers found a strong link between NFκB activity and the development of melanoma metastatis, as well as leukemia and lymphoma (Karin, 2006). In addition, a high level nuclear p65 NFκB, indicative of activation, was found to be associated with head and neck cancer. This role is thought to be related to a loss in epithelial-mesenchymal transition (Arun et al., 2009).

More recently, studies have sought to examine the role of the IKKs themselves in cancer development (Lee and Hung, 2008). Constitutive activation of the IKK complex has been observed in a number of cancer cell lines such as breast (Romieu-Mourez, 2001), prostate (Gasparian et al., 2002) and colorectal (Charalambous et al.,

2003). However, there is limited evidence regarding somatic or inheritable mutations in IKK genes that may lead to hyperactivity of IKKs; to date only mutations in genes for intermediates of the non-canonical NFκB pathway have been observed, for example in myeloma (Keats et al., 2007). Nevertheless, a reduction in the activity of IKKβ has been linked with impaired development of myeloma (Yang, 2010). Moreover, pretreatment with PS1145 an IKKβ inhibitor which blocks the classical NFκB activity resulted in the death of myeloma cancer cells (Castro et al., 2003, Hideshima et al., 2002). Lee and co-workers also demonstrated that hyperactivity of IKKβ leads to cancer progression and increase of angiogenesis in the breast (Lee et al., 2007), another a study have invrstigated the role of IKK/NFkB signalling pathway in metastasis of breast cancer (Fusella et al., 2017)

Many studies have observed a potential role for IKKα in a number of cancers such as breast, prostate, lung, colorectal and pancreatic cancer through the impact on cell cycle progression and apoptosis (Fernandez-Majada et al., 2007b, Park et al., 2005, Hirata et al., 2006, Luo et al., 2007). Studies show that IKKα can regulate cyclin D1 independently of NFκB activation and can promote oestrogen receptor α (ERα) phosphorylation and activation that leads to increased expression of cyclin D1 in breast cancer (Park et al., 2005). Furthermore, it has been demonstrated in Hela cells that silencing of IKKα results in the accumulation of cells in the G_2/M phase of the cell cycle, produced by the effects upon cyclin D1, PLK1 and phosphorylation of Aurora (Prajapati et al., 2006). Koch and Radtke have indicated a role for non-cannonical NFkB signalling in a prostate cancer mouse metastatic model, through inhibition of the expression of tumour suppressor genes such as Maspin (Koch and

Radtke, 2007). Several other studies have implicated IKKα in the regulation and phosphorylation of a number of proteins which enhance cell proliferation in different cancer models including again cyclin D1, but also Amplified in breast cancer 1 (AIB1), β-catenin and others (Pui, 2009, Zardawi et al., 2010, Qi et al., 2003, Cantarini et al., 2006). These and a host of additional studies strongly implicate a role for NFκB signalling and increases in the non-cannonical cascade in the regulation of multiple processes that lead to cancer development (Lee and Hung, 2008, Fernandez-Majada et al., 2007a, Luo et al., 2007).

Margalef and co-works investigated the role of both IKKα isoform in patient tissue samples, animal and cell lines from colorectal cancers. They reported that both IKKα isoforms p84 and p54 were located within the cytoplasm of tumour cells as well as normal cells. However, p45 IKKα isoform was overexpressed within both cytoplasmic and nuclear fractions of tumour cells compared with normal cells and they linked the translocation of the p52 isoform to increased cellular the malignancy (Margalef et al., 2012). These results were confirmed in other study by the same research group (Margalef et al., 2015). In considering issues related with siRNA knockdown the potential of the existence of two different isoform of IKKα may be relevant factor.

1.10.1 Nuclear Factor kappa B pathways in pancreatic cancer

There have been a growing number of studies investigating the role of NFκB in pancreatic cancer from both a clinical perspective and from work conducted in cell lines. Groups from the Anderson Cancer Centre were the first to report an increase in

NFκB signalling in the activation phase of pancreatic cancer through testing of 11 human pancreatic cancer cell line samples and 20 pancreatic adenocarcinomas. The results of this study showed that in 9 out of 11 cell lines and 14 out of 20 tumour samples NFκB was activated (Wang et al., 1999). The same group demonstrated suppression of metastasis and tumour malignancy following infection with an IκBα mutant (Fujioka et al., 2003a, Fujioka et al., 2003b). Vimalachandran and co-workers also examined the nuclear distribution of p65 NFκB in pancreatic cancer cells; in 96% of benign duct cells p65/RelA was detected in the cytoplasm and in 26% it was found in the nucleus, whilst in pancreatic cancer cells nuclear p65/RelA was detected in 57% of cells (Vimalachandran et al., 2005). Expression of p65/RelA was studied in 82 samples of pancreatic adenocarcinomas by Weichert and colleagues, they observed high expression of p65/RelA in the nucleus of 37 samples and in the cytoplasm of a further 42 (Weichert et al., 2007). This suggests association between the nuclear translocation of p65/RelA and cancer development.

Pancreatic cancer is similar to other tumour types in that the activation of NFκB is not linked with genetic mutation. However, it is influenced by proinflamantory cytokines operating within paracrine loops between cancer cells and tumour-infiltrating immune cells (Grivennikov et al., 2010). The inflammatory cytokines which most notably affect the tumour environment are IL-1β and IL-1α (Dinarello, 1996, Apte et al., 2006). Niu and co-workers noted that activation of activator protein 1 (AP1) induced autocrine secretion of IL-1α, leads to enhanced activity of NFκB in metastatic pancreatic cancer cells but not in non-metastatic cells (Niu et al., 2004).

Although, evidence supports a role for the classical NFκB pathway in pancreatic cancer, there are very few studies examining the potential contribution of the non-canonical NFκB pathway in pancreatic cancer development. For example, Doppler and co-workers showed that the high level of NFκB activity is underpinned activation by the non-canonical cascade in pancreatic cancer. A high level of the p52/RelB complex was due to the overexpression of NIK in both Panc-1 and MiaPaCa-2 cells (pancreatic cancer cell lines). They found that degradation of TRAF2 led to increased levels of NIK and eventually activation of the non-canonical NFκB pathway, which drives the anchorage-independent growth and proliferation of pancreatic cells (Doppler et al., 2013).

A recent study have showed that treated pancreatic cancer cell lines with the NEMO-banding domain peptide (NBDP) which is a NFκB inhibitor promoted increased PARP and caspase 3 cleavage and suppressed growth of these cells, in addition treated xenograft mouse model with NBDP combined with gemcitabine was resulted in significantly reduced NFκB activity and induced apoptosis pathway (Zhuang et al., 2017). Taken together, NFκB is a promising target for pancreatic cancer treatment.

1.11 development a IKKα inhibitors

As described above, there is body of evidence that supports a role for NFκB pathways in the development and progression of pancreatic cancer as well as other cancers. A number of NFκB inhibitors have been developed and their potential effect as anti-cancer treatment in different cancers including pancreatic cancer investigated.

TPCA-1 (Cataldi et al., 2015), NDRG1 (Hosoi et al., 2009), IMD-0354 (Ochiai et al., 2008) and Apigenin (Wu et al., 2014) are NFκB inhibitors, which have been examined in pancreatic cancer cells lines, however these inhibitors have either a wide range of actions within cells or lose their selectively through inhibition of other kinases. As outlined above, IKKα has a crucial role in the expression of a number of genes that regulate cellular processes linked with cancer development such as, invasion, metastasis, proliferation and resistance to the chemotherapy (Fernandez-Majada et al., 2007a, Hirata et al., 2006, Park et al., 2005, Doppler et al., 2013). Today there are a number IKKα/IKKβ or selective IKKβ inhibitors, however, these inhibitors have a number of issues which preclude clinical effectiveness. For example, the reduction in the expression of a number of anti-apoptotic genes in normal cells as well as tumour cells (Chariot, 2009, Li et al., 1999, Gamble et al., 2012b, Shukla et al., 2015). In addition, targeting IKKα resulted in inhibition of the non-canonical pathway which is IKKα dependent, activation of this pathway leads to a slow and maintained NFκB signal in comparison with IKKβ dependent pathway (canonical NFκB pathway), which is a rapidly generated response. This difference may allow for the generation of selective IKKα inhibitors (Sun, 2012).

1.12 Aims of study

Pancreatic cancer has a very poor prognosis, with surgery currently being the only solution for prolonged survival (maximum 5 years) beacause most patients are either resistant to chemotherapy or become so over time. Many studies have demonstrated that the NFκB pathway plays a role in the resistance to chemotherapy and found that a better response is achieved when standard treatments are combined with NFκB inhibitors. In addition, recent studies have sought to investigate these pathways as a new strategy for developing new anti-cancer drugs. IKKα has a key role in the activation of the non-canonical NFκB pathway, furthermore it is implicated in the regulation of growth, invasion and metastasis, and therefore, targeting IKKα may be a promising route to the development of new anti-pancreatic cancer drugs.

The aims of this study are to:

1- characterise the non-canonical and canonical NFκB pathways in pancreatic cancer cell lines.
2- Investigate the pharmacological effect of first-in-class selective IKKα inhibitors through examining their selectivity within both the non-canonical and canonical NFκB pathways and their effect on IKKα-dependent cellular processes.
3- Assess the regulation of IKKα-dependent genes in Panc-1 cells and the effect of pharmacological IKKα inhibitors on the expression of these genes.

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 General reagents

All reagents and materials used were supplied by Sigma-Aldrich Chemical Company Ltd. (Pool, Dorest UK) or other high-rank companies unless otherwise stated.

Thermo Fisher Scientific UK Ltd (Leicestershire, UK)

Bovine Serum Albumin (BSA)

L – Glutamine Gibco™ Penicillin-Streptomycin

Gibco™ Fetal Bovine Serum

Dulbecco's Modified Eagle Medium (DMEM)

Cell culture plates and dishes

60mm Nunclon™ Delta Surface plates

Bio-Rad Laboratories (Hertfordshire, UK)

Bio-Rad DC^{TM} Protein Assay Dye Reagent Concentrate

Pre-stained SDS-PAGE molecular weight markers.

Insight Biotechnology Limited (Wembley, UK)

Recombinant Human Tumour Necrosis Factor-alpha (TNF-α)

GE Healthcare (Buckinghamshire, UK)

Amersham Hybond ECL Nitrocellulose Membrane

Carl Roth GmbH + CO. KG (Karlsruhe, Germany)

Rotiphorese® Gel 30 (37.5:1) acrylamide

Whatmann (Kent, UK)

Nitrocellulose Membrane, 3MM blotting paper

Corning B.V (Buckingghamshire, UK)

All tissue culture flasks, dishes, graduated pipettes and multi-well plates.

Sarstedt AG & Co LTD (Leicester, UK)

Serological pipette 5ml and Serological pipette 10ml

Thermo Fisher Scientific Inc (Surrey, UK)

Multidish 6 wells and Multidish 12 wells

3.1.2 Antibodies

Santa Cruz Biotechnology Inc (California, USA)

Rabbit polyclonal anti-NFκB. p65 (C-20)

Rabbit polyclonal anti-NFκB-inducing kinase NIK (H-248)

Rabbit polyclonal anti TRAF2 (H-10)

Abcam (Cambridge, UK)

Rabbit polyclonal anti-NFκB-inducing kinase NIK (ab7204)

Rabbit monoclonal anti-IKKβ (ab32135)

Cell signalling Technology Inc (Hertfordshire, UK)

Rabbit polyclonal anti-TRAF3 (4729s)

Rabbit polyclonal anti-TRAF2 (4724s)

Rabbit monoclonal anti- p-NF-kappa B2 p100 (4810L)

Rabbit polyclonal anti-p-NF-kappa B2 p65 (3031L)

Rabbit monoclonal anti-IκBα (92424L)

Rabbit monoclonal anti-p-IκBα (SAS)

Rabbit monoclonal anti-p-p105 (5933)

Rabbit monoclonal anti-GAPDH (14C10)

Millipore (U.K.) Limited (Watford, UK)

Anti-NFκB p52-(32534)

Mouse monoclonal anti-IKKα (14A231).

Calbiochem (Nottingham, UK)

Mouse monoclonal anti-IKKα (14A231)

Jackson ImmunoResearch Laboratories Inc (PA, USA)

Horseradish Peroxidase (HRP)-conjugate goat anti-Rabbit IgG (111-035-144)

Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG

SU compounds (University of Strathclyde)

SU1261, SU1087, SU1432, SU1434, SU1436, SU1438, SU1411, SU1392, SU1499, SU1433, SU1399, SU1497, SU1489 and SU1394

NIK inhibitors

CW15337, CW15420 and CW15407

2.2 Cell Culture

All cells were cultured in 75 cm^2 flasks and all cell culture work was performed using aseptic techniques in a Class II Safety Flow Hood.

2.2.2 Panc-1 and Mia-Paca-2 cell lines

All pancreatic cancer cell lines were supplied by Prof. Andrew Biankin (Wolfson Wohl Cancer Research Centre, University of Glasgow, Glasgow). MiaPaCa-2 cells are an epithelioid carcinoma cell line, derived from the pancreatic cells of a 65-year-old man, with tumours present the body and the tail of the pancreas. Panc-1 cells are also epithelioid carcinoma in organ but these cells were derived from a 56-year-old male with tumour only in the head of the pancreas. These two pancreatic cancer cell lines were used in this study supplied. The normal medium for these cell lines was DMEM medium supplemented with 10% (v/v) bovine calf serum, 1%(v/v) penicillin/streptomycin and 1%(v/v) L-glutamine. Cells were incubated at 37°C in presence of a mixture of 95% air, 5% CO_2.

2.2.2 Sub-culturing Panc-1 and Mia-Paca-2 by Trypsinisation

When the cells reached approximately 90%, conflurncy then they were sub-cultured. Firstly the media was removed and the cells washed with a sterile solution of 0.5% (w/v) trypsin, 0.2% (w/v) EDTA in phosphate buffered saline (PBS). After trypsin was removed, the flask was placed in the incubator at 37 $^{\circ}$C, at 5% CO_2 for 2-4 min. Once the cells displayed a round morphology, which indicated they could be detached from the surface of flask, media was used gently to detach the cells completely from the surface of the flask. Removed cells were used for seeding the cells into fresh flasks (75 cm^2) or multi-well culture plates (12 well or 6 well) as required. The cells were placed in the incubator at 37^{o}C, and the media changed every two days, until the growth reached 90% and then cells were starved for 24 h before any stimulation using free FSC media.

2.3 Western Blotting

2.3.1 Preparation of whole cell extracts

Cells grown on 6 or 12 well plates were exposed to suitable agonists for specific periods of time, plates placed on ice to terminate the reaction, and then the media aspirated. Cells were washed twice with 250 µl cold PBS, and 150-250 µl of Laemmli's sample buffer (63mM Tris-HCl, (pH 6.8), 2mM Na-4P2O7, 5mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50mM DTT, 0.007% (w/v) bromophenol blue). The cells were scraped and the chromosomal DNA sheared by repeatedly passing through a 21 gauge needle. Cell extracts were transferred into Eppendorf tubes, then boiled for five minutes and stored at -20 °C until use.

2.3.2 Amidoblack protein assay

To assay unknown protein samples a standard curve was prepared using a 1gm/ml stock of BSA (in H_2O), in a series of 1:1 dilutions in sample buffer. An aliquot (5µl) of each BSA dilution and samples was spotted onto a strip of cellulose acetate and then samples allowed to dry. The strips were bathed in Amido black stain (0.25%Amido black, 45% Methanol, 45% dH_2O, 10% glacial acid) for 10 min with shaking at room temperature, washed using midoblack de-stain buffer (45% (v/v) Methanol, 45% (v/v) dH_2O, 10% (v/v) glacial acid) then left to dry at room temperature. An aliquot of each sample was placed in 1.5ml Eppendorfs and incubated in 500µl dissolving solution (80ml formic acid, 10ml glacial acid and 1ml 100% TCA) at 60°C for 30 min. 250µl from each sample was added to 96 well plate and measured at 620nm.

2.3.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

An analytic method was used to measure the change in proteins levels in extracts from both cell lines. Western blot was used the determine the change in the intermediates of the canonical and non-canonical of NFκB pathway following exposure of pancreatic cell lines to specific agonists with or without treatment with IKKα inhibitors (SU compounds) or siRNA transfection.

The gel plates were washed with 70% (v/v) ethanol before assembly. Distilled water was added to the assembled plates for 30 min to check the glass plates were not leaking or flush. The resolving gels were prepared by mixing a suitable value (10%

or 7.5% (v/v)) of acrylamide (acrylamide 30%: N, N'- methylenebis-acrylamide 0.8% (37.5: 1), 0.375M Tris base (pH8.8), 0.1% (w/v), SDS, ammonium persulfate (APS) 10% (w/v) and TEMED added to initiate the polymerisation process. The solution was poured between the two glass plates assembled in vertical slab configuration according to the manufacture's instruction (Bio-Rad) and 180µl of 1% (v/v) SDS was overlaid onto the gel solution. When the gels were polymerised, the layer of SDS was removed, during this time stacking gels were prepared; 10% (v/v) acrylamide 30% (37.5:1), 0.125M Tris base (pH 6.8), 0.1% (w/v) SDS and 10% (w/v) ammonium persulfate (APS) and 0.05% (v/v) TEMED) and directly added above the resolving gel, and wall combs inserted immediately into the stacking gel solution. After the polymerization of the gel, the combs were removed and the gel assembled in a Bio-Red Mini-PROTEAN IITM electrophoresis tank. Aliquots of samples (10- 30 µl) were then loaded into the wells using a microsyringe. A prestained SDS-PAGE molecular weight marker of known molecular weights was run concurrently in order to identify the polypeptides of interest. Samples were electrophoresed at a constant voltage of 130V, until the bromophoenol dye had reached the bottom of the gel.

2.3.4 Electrophoretic transfer of proteins to nitrocellulose membrane

Gels were transferred to the nitrocellulose membrane by the method of Towbin and co-workers (1979). The gels were firmly pressed against a sheet of nitrocellulose and assembled in a transfer cassette sandwiched between two pieces of paper (Whatman 3MM) and two pieces of sponge. The cassette was submersed in transfer buffer (19mM glycine, 25M Tris and 20% (v/v) methanol) in a Bio-Rad mini Trans-Blot^{TM}

tank and a constant current of 300mA was applied for 105 min. During this time the tank was cooled by inclusion of an ice reservoir. The presence of SDS in the resolving gel confers a negative charge on the proteins so the cassette was oriented with the nitrocellulose towards the anode.

2.3.5 Immunological detection of proteins

Following the transfer of proteins to nitrocellulose membranes , the membranes were removed and the proteins were blocked by incubation in a solution of 2% or 5% (w/v) BSA in NATT buffer (20 mM Tris , 150 mM NaCl (pH 7.4) , 0.2% (v/v) Tween-20) for 2h on a rotary shaker at room temperature. Blocking buffer was then removed and the membranes incubated with specific antibody for the target protein in 0.2 % (w/v) BSA in NATT overnight. The following day the membranes were washed in NATT every 10 minutes 3-4 times with gentle shaking. Following this the membranes were incubated for two hours with secondary horseradish peroxidase-conjugated IgG directed against the first immunoglobulin diluted to approximately 1:10000 in NATT buffer containing 0.2% (w/v) BSA. The membrane was then washed in NATT every 10 minutes for 3-4 times and the bands of immune-reactive protein were detected by incubation in enhanced chemiluminescence (ECL) reagents (reagents 1+2) for 3 min with gentle shaking. The membranes were then placed in photographic cassette and covered with cling film, then exposed to X-ray film for the required time under darkroom conditions and developed using a X-OMAT processor (KODAK M35-M X-OMAT).

2.3.6 Reprobing and stripping of nitrocellulose membrane

To reprob the nitrocellulose membrane for additional proteins the membranes were stripped. The nitrocellulose was incubated in 15ml of stripping buffer (0.05M Tris-HCl, 2% (v/v) SDS, and 0.1M of β-mercaptoethanol). This stage included incubation of the membrane at 60^{o}C for 1 h with shaking. The stripping buffer was discarded and the membrane washed 3 times every 15 min in NATT to remove the residual stripping buffer. After the last wash the membrane was incubated with primary antibody overnight with 0.2% (w/v) BSA in NATT. The blots at this stage were then ready for the immunological detection protocol as explained previously.

2.4.1 Preparation of nuclear extracts

To preparing nuclear extracts, cells were grown in 6 well plates, 90% cells confluent were rendered the cells quiescent followed cells were exposed to appropriated agonists over a specific time course. The reaction was terminated by washing on ice with 1ml of cold PBS, the cells scraped into 0.5 ml PBS and transferred into Eppendorf tubes. The tubes were centrifuged at 4°C for 13,000 rpm for 3 min. 400µl of buffer 1 (10mM Hepes pH 7.9, containing 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF, 10µg/ml aprotinin, 10µg/ml leupeptin and 10µg/ml pepstatin) was added to each pellet, and following resuspension incubated for 15 min. Following the addition of 25µl of 10% (w/v) NP-40 the samples were vortexed for 10 sec. at full speed. The nuclear fraction was separated after spinning the samples at 13,000 rpm for 1 min and discarding the supernatant. The pellet was resuspended in 50 µl of buffer 2 (20mM Hepes, (pH 7.9), 25% (v/v) glycerol, 0.4M

NaCl, 1mM EGTA, 1mM DTT, 0.5mM PMSF, 10µg/ml aprotinin, 10µg/ml leupeptin and 10µg/ml pepstatin) with shaking for 15 min at 4 °C. The samples were sonicated twice for 30s, and the nuclear extracts collected after centrifugation at 13,000 rmp for 15 min. Supernatants (the soluble nuclear extracts), were stored at -80 °C until further use.

2.4.2. Determination of proteins concentration in the nuclear extracts

The concentration of protein in nuclear extracts was detection by using the Bio-Rad DC™ protein assay dye reagent by the Bradford assay method. A range of standards were prepared using different concentrations (2-20 µg) of BSA. The dilutions of the standards and the nuclear extracts samples were made up in sterile dH_2O (790-795 µl) and mixed with 200 µl of dye agent. The samples then were transferred into a cuvette and the colour development quantified at 595nm on an Ultrospec®2000 UV/visible spectrophotometer. The protein concentration of each sample was calculated from the standard curve.

2.5 siRNA transfection of IKKα and IKKβ in pancreatic cancer.

Small interfering RNA (siRNA) is a molecular biology method that is used to study the functions and role of different proteins in cellular processes in cells. It is a double-stranded of RNA consisting of (20-25 pairs base). siRNAs are complementary nucleotide sequences of target genes that lead to the switching off of expression of these genes (Elbashir et al., 2001). For examination of the knockdown of IKKα and IKKβ in Panc-1 cells, cells were transfected with siRNA against IKKα and IKKβ in comparison to non-target siRNA (NT), the siRNA was purchased from

Thermo Fisher Scientific® (Thermo Fisher Scientific, Surrey, UK), the target sequences are shown in table 2.1.

A 20nM stock of each of siRNAs was prepared through suspension of the siRNA in RNase- free 1X siRNA buffer (Dharmacon, Buckinghamshire, UK). 500µl of 1X siRNA buffer were added to 10nmol of siRNA then mixed by pipetting and the suspension was placed on an orbital mixer at room temperature for 30 min, and then aliquoted and stored at -20°C. Panc-1 cells were seeded in 12 or 6 wells plates and cultured until reached approximately 50-60% confluence prior to transfection. Two Eppendorf tubes were prepared for each well that was transfected with siRNA. Tube A contained the siRNA mixture, value of siRNA was required to prepared the specific concertation of siRNA then completed the value in tube A to 100µl by added Optimem (Life Technologies, Paisley, UK). Tube B was to be content 5µl of Lipofectamine RNAiMAX® (Invitrogen®, Paisley, UK) and it was diluted into 95µl of Optimem. Tube A was subsequently added to Tube B and mixed gently. Tubes were left for 20-25 minutes to allow complex formation. Full media was removed from the cells and replaced with 800µM of Optimum and transfection mixture was added to the appropriate wells dropwise. Plates were incubated for 6-8h at 37°C at 5% CO_2, after which the transfection mixture was aspirated off and replaced with full DMEM media. Cells were then incubated for 72h at 37°C at 5% CO_2. Thereafter whole cells extracts were prepared as outlined in Section 2.3.1. Western Blotting was used to determine levels of expression IKKα and IKKβ or related NFκB pathways markers.

Table 2.1 siRNA transfection agents, target gene, siRNA origin and target sequence

Target Gene	On-tatget plus siRNA	Target sequence
IKKα (CHUK)	Human CHUK Cat. NO: J-003473-09	GCGUGAAACUGGAAUAAU
IKKβ(IKBKB)	Human IKBKB (3551), Cat-No: J-003503-13	GAGCUGUACAGGAGAUAA
Non-target (NT)	Non-target, Cat No: D-001810-01-20	UGGUUUACAUGUCGACUA A

2.6 Cell Cycle Analysis

Cells were grown to around 70% confluence for routine experiments. Plates were media aspirated and trypsinized cells, and then the contents transferred to 15 ml tubes and centrifuged at 1500rpm for 10min. Following aspiration of the supernatant, on ice cold, 1ml of 70% (v/v) ethanol was added and stored at -20 ^{o}C until analysis. On the day of analysis, samples were centrifuged at 3000 rpm for 10 min, and then supernatant discarded and 250µl of cold PBS was added. Materials were transferred into FACS tube and 5µl of RNase A solution (100µg/ml) was added and incubated 37°C and 5% CO_2 for 30-60 min. 13.5µl of Propidium Iodide (PI) and the cell cycle analysis was carried out using a FACScan (Becton Dickinson Systems, Cowley, UK).

2.7 Clonogenic assay

Cells were grown to around 70% confluence, the media was removed and the cells were washed with 2ml trypsin. Cells were detached using 1ml of trypsin (0.05%) then the cells were collected in 15ml tube and 5-10ml of media was added. Cell number was determined using a haemocytometer and the appropriate number of cells (150 cells) seeded onto 60 mm petri dishes in 3mls of media in triplicate for each treatment. After 24 h, cells were treated with different concentrations of compounds

for 24h and the media removed and 5ml of growing media added. Following 7-14 days of incubation, the media was removed and the colonies washed with 3ml of cold BPS and the cells fixed with 100% methanol for 15min. The cells were stained with 3ml of Giemsa for 15 min then washed with tap water and dried. The number of colonies were accounted manually. Plating efficiency (PE) and survival fraction (SF) were calculated from the following equations:

$$\mathrm{PE} = \frac{\text{average no. of control colonies formed}}{\text{no. of seeded cells}}$$

$$\mathrm{SF} = \frac{\text{average no. of colonies formed after treatment}}{\text{no. of seeded cells (untreated cells) X PE}}$$

2.8 Polymerase chain reaction (PCR) amplification

2.8.1. Harvesting Cell of total RNA preparation

Cells were exposed to suitable agonist or treatment, then cell lysis were prepared through mixed an equal values of both lysis buffer and 70% (v/v) ethanol (RLY supplemented with β-mercaptoethanol (β-ME).The suspension was transferred to RNeasy Mini spin column (2ml collection tube) and centrifuged, Next the membrane was washed using 350μM of Membrane desalting buffer (MEM). DNase digestion buffer (90μM of DNase RDB, plus 10μM of DNase free water) was added directly to the centre of membrane and incubated for 15mine at room temperature. The membranes were washed three times by wash buffer1 (RW1) and wash buffer2 (RW2). Next, collection tubes was placed into a nuclease-free 1.5 ml tube and 60μl

of RNase free water was added directly onto centre of membrane, total RNA was stored at -80 until use.

2.8.2. RNA concentration

RNA concentration of prepared samples was measured using NanoDrop (ND-2000C, Thermo Scientific) (220-750 nm). 2µl of RNase-free water was used as blank. The sampling platform was then cleaned prior to adding 2µl from each RNA sample to measure the concentration. The NanoDrop is a program displaced s the sample absorption curve, the RNA concentration and the ratios (A_{260}nm/A_{280}nm; A_{260}nm/A_{230}nm). The ratio of A_{260}nm/A_{280}nm should be around 2.0 of absorbed was indicated purity RNA whilst A_{260}nm/A_{230}nm around 2.0 indicates for level of salt contamination.

2.8.3 cDNA Saynthesis using Reverse Transcription (RT)

To quantify the mRNA transcripts of genes under investigation, RNA was reverse transcribed to complementary DNA (cDNA) using Tetro cDNA synthesis kit (Bioline, USA), as described in the manufacturer's manual. The volume which contained 1µg mRNA from each sample was added in tube and the volume was adjusted to 12µl by adding RNA-free water followed by 1µl of oligo (dt) as first-strand synthesis primer, 1µl of 10mM dNTP mixture, 4 µl of 5x RT buffer, 1µl of RiboSafe RNase Inhibitor and 1 µl of Tetro Reverse Transcriptase (200u/µl). The reaction where was mixed gently by pipetting, and incubated at 45°C for 30 min and the reaction was terminated by incubating at 85°C for 5 min. Samples then stored at -20 °C until processed for quantitative real-time PCR for gene expression analysis.

2.8.4. Primers for SYBR select Master mix assay

Primers for PCR were designed to bind to selected target genes and to avoid primer dimers and non-specific products in the SYBR® assay, see Table 2.2. Using the gene Runner function as described via the National Centre for Biotechnology Information (NCBI) Genome Browser https://blast.ncbi.nlm.nih.gov/Blast.cgi m

Table 2.2 Nucleotide sequences of the primers used for the analysis of gene expression by qPCR

Primer name/ gene target	Sequence
IL-6	Forward: 5'-GCCTCTTTGCTGCTTTCACA-3' Reverse: 5'-ACCCCAATAAATAGGACTGA-3'
VCAM-1	Forward: 5'-CTCTGGGGGCAACATTGACA-3' Reverse: 5'-CTAGCGTGTACCCCCTTGAC-3'
EZH2	Forward: 5'-CCAAGTCACTGGTCACCGAA-3' Reverse: 5'-GTTGGCGGAAGCGTGTAAAA-3'
GAPDH	Forward: 5'-AAATGAGCCCCAGCCTTCTC-3' Reverse: 5'-GTCAAGGCTGAGAACGGGAA-3'
NCL	Forward: 5'-AAGTGTTCTCGCATCTCGCT-3' Reverse: 5'-AAGCGTTGGAACTCACTGGT-3'
MAP3K14	Forward: 5'-GACACACAGCATGGCCAAAG-3' Reverse: 5'-CAAAGCTGACAACGTGCTCC-3'
18S	Forward: 5'-CTGCCTTCCTTGGATGTGGT-3' Reverse: 5'-CCATTCGAACGTCTGCCCTA-3'
TNFAIP3	Forward: 5'-CACCCTTGGAAGCACCATGT-3' Reverse: 5'-TCTGGTTGGGATGCTGACAC-3'
SERPINB5	Forward: 5'-CGGTCTCTTCGTAGAGCTGA-3' Reverse: 5'-TCCACCTCTCTGTCACTTGC-3'
BBC3	Forward: 5'-CCACAAATCTGGCAGGGGAC-3' Reverse: 5'-AATTTGGCATGGGGTCTGCC-3'

2.8.5. Quantitative Real-Time Polymerase Chain Reaction amplification (qRT-PCR)

The qRT-PCR assay was carried out in triplicate by placing the samples in a sterile PCR Cycle plate (Applied Thermo Fisher). Each 30µl PCR reaction contained; 15µl SYBR select Master mix (Applied Biosystems), 0.3µl of 1 pmol/µl of both reverse and forward primes, 3µl of the cDNA template and 11.4µl of DEPC-treated water, triplicate were generated for each sample. The thermal cycling and detection was carried out on StepOne Plus real-time PCR system. The thermal cycle consisted of an initial uracil-DNA glycosylase activation of 5 min at 50°C. To control PCR product carryover contamination the DNA polymerase activation of 10 min at 95°C, followed by 40 cycles 15 sec at 95 °C, 1min at 60°C.

2.8.6. Quantification method (the relative quantification (ΔΔCt) method

The quantification method selected to confirm the QRT-PCR result was the relative quantification (ΔΔCt) method (Livak and Schmittgen, 2001). This method compares Ct values between the target gene and endogenous reference gene to examine the changes in genes expression between untreated and treated samples. To compare expression levels in samples using this method, the following equations were followed:

1- Calculating the ΔC between the target gene and reference gene for both treated and untreated samples

   $\Delta C = Ct_{\text{target}} - Ct_{\text{reference gene}}$

2- Calculating the ΔΔC between the target gene and reference gene for both treated and untreated samples

$\Delta\Delta C = \Delta C$ treated sample - ΔC untreated sample

3- Fold change in gene expression in treated sample

Fold change = $2^{-\Delta\Delta C}$

2.8 Data Analysis

All data shown were expressed as mean ± S.E.M and were representative of at least three separate experiments. Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, California). The statistical significance of differences between mean values from control and treated groups were determined by one-way analysis of variance (ANOVA) with Dunnett's post-test ($p<0.05$ was considered significant). For calculating the IC_{50} for SU compounds, GraphPad Prism version 5.0 (GraphPad Software California) was used. X values were the logarithms of compounds concentrations and Y values were the reading of concentrations. Non-linear regression analysis was used to determine the concentration response curve and calculation of the IC_{50}.

Chapter Three

Characterisation of non-Canonical NFκB pathway in Panc-1 cells

3.1 Introduction

As previously mentioned in Section 1.5.3, NFκB has an important role in several cellular functions such as growth and apoptosis (Brand et al., 1997, Brand et al., 1996, Oeckinghaus and Ghosh, 2009). In addition, NFκB also plays a key role in many processes that leads to tumour progression, for example, proliferation, anti-apoptotic responses, cellular motility, angiogenesis, metastasis and resistance to anticancer drugs (Hoesel and Schmid, 2013). Whilst the vast majority of such studies are linked to the canonical pathway, activation of the alternative or non-canonical pathways is becoming a more prominent feature. Stimulation of the non-canonical NFκB pathway by different stimuli, for example, lymphotoxin β ($LT\alpha_1\beta_2$) leads to phosphorylation of IKKα and subsequent phosphorylation of p100, processing to p52 then formation of p52-RelB heterodimers. These dimers translocate into the nucleus and bind to specific sites within DNA to activate the transcription process for a number of genes relevant to cancer (Oeckinghaus and Ghosh, 2009). In particular, IKKα is thought to have an important role in the regulation of a number of genes that are related to angiogenesis, Birbrair and co-workers found that IKKα mediated regulation of gene products such as Matrix Metallo-Protienases-9 (MMP-9), Interleukin-8 (IL-8) and VEGF (Birbrair et al., 2015). In addition, another study has demonstrated that exposure of fibrosarcoma tumours to lymphotoxin leads to enhanced release of CXC chemokines and macrophage inflammatory protein 2 (MIP-2) in a xenograft model (Hehlgans et al., 2002). Furthermore, it was demonstrated that IKKα activation increased, by over 3 fold, the expression of number of genes such as, Chemokine (C-C motif) ligand 19 (CCL19), CCL21 and

CXCL12 in a pancreatic cancer cell line (BxPC-3 cells) (Wharry et al., 2009). Many studies have implicated NFκB in the pathogenesis of different types of cancers including pancreatic cancer (Fernandez-Majada et al., 2007a, Shiah et al., 2006, Govindarajan et al., 2016). Evidence indicates that NFκB pathways are more active in cell lines and specimens derived from pancreatic cancer patients, compared to normal cell types (Pan et al., 2008, Chandler et al., 2004, Wang et al., 1999). Furthermore, these findings are correlated with increased resistance to chemotherapy in pancreatic cancer patients (Long et al., 2011). However, to date there has been relatively little characterisation of the status of the non-canonical NFκB pathway in a pancreatic cancer setting, or any potential contribution to disease progression. Therefore, the aim of this chapter was to interrogate components of the non-canonical NFκB pathway in pancreatic cancer cell lines and study the role of IKKs in regulation of NFκB pathways in pancreatic cell lines.

3.2 Characterisation the non-canonical of NFκB pathway

The aim of the initial stage of this study was to examine the kinetics of activation of the non-canonical and canonical NFκB pathways. This was examined using three agonists; FCS, $LT\alpha_1\beta_2$ and additionally TNFα which can activate both canonical and non-canonical NFκB pathways (Oeckinghaus and Ghosh, 2009). In our laboratory, these three agonists have the ability to activate NFκB pathways in other cancer cell lines, for example, U2OS, an osteosarcoma cell line and PC3M and LNCaP cells, both prostate cancer cell lines.

3.3 Characterisation of the non-canonical of NFκB pathway using $LT\alpha_1\beta_2$

3.3.1 Phosphorylation of p100 in pancreatic cancer cell lines

As mentioned in Chapter One, the key marker for activation of the non-canonical NFκB pathway is phosphorylation of p100 NFκB2. Panc-1 cells were exposed to $LT\alpha_1\beta_2$ over a 24 h time course, as shown in Figure 3.1. After a delay of 2h phosphorylation of p100 increased, reaching a peak at 4h (fold increase; 14.44 ± 0.12, Figure 3.1B), and remaining elevated for up to 24h at approximately 10 fold of basal values. Whilst not shown here the same outcome was observed in another pancreatic cell line, MiaPaCa-2 (Figure 3.1C). This indicates that $LT\alpha_1\beta_2$ can activate the non-canonical NFκB pathway in pancreatic cell lines.

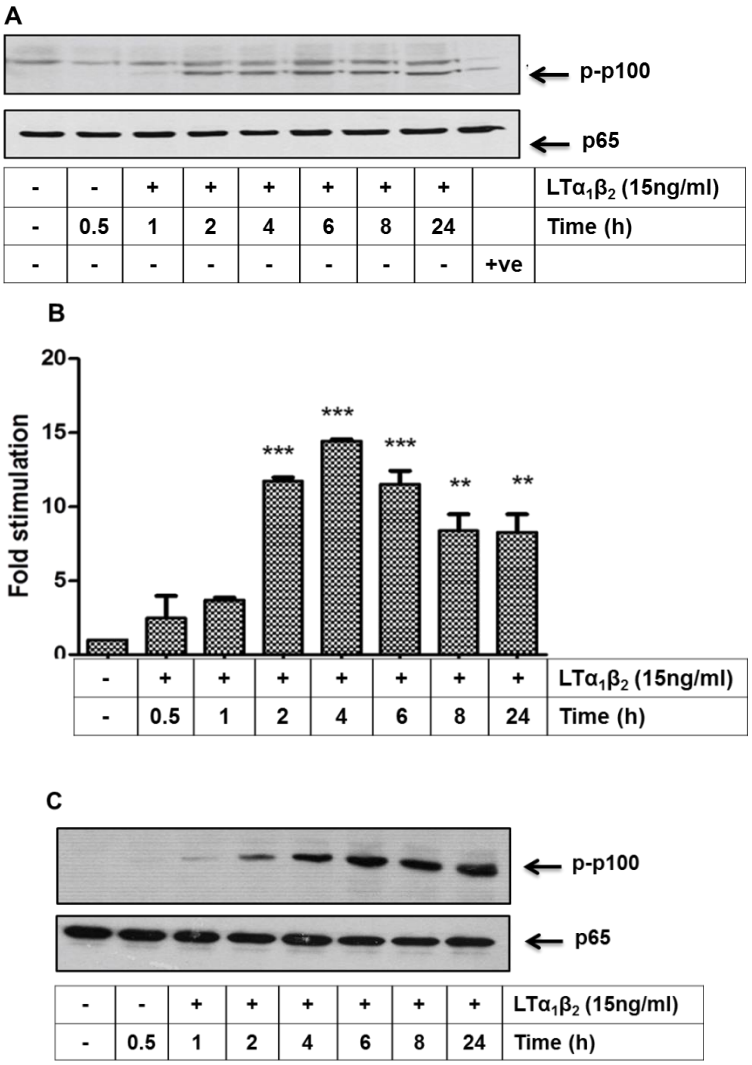


Figure 3. 1 LTα$_1$β$_2$-induced phosphorylation of p100 in pancreatic cancer cell lines

Panc-1 cells were exposed to LTα$_1$β$_2$ (15ng/ml) for the times indicated. Whole cell extracts were prepared and separated by SDS-PAGE and assessed for p-p100 (100kDa) and p65 NFκB (65kDa) which was used as a loading control. In panel B blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control. Each blot is representative three experiments. Each value in panel B represents the mean ± SEM of three experiments. ***P< 0.001, **P< 0.01 compared to control. In panel C MiaPaca-2 cells were exposed to LTα$_1$β$_2$ (15ng/ml) for the times indicated. Whole cell extracts were prepared and separated by SDS-PAGE and assessed for p-p100 (100kDa) and p65 NFκB (65kDa) which was used as a loading control, the blot represents two experiments.

3.3.2 Characterisation of p100-p52 processing in whole cell extracts

After the optimal time for phosphorylation of p100 in pancreatic cancer cell lines was determined, the next step was to find the optimal time for processing of NFκB2 (p100) to form p52 using the same agonist. Panc-1 cells were incubated with $LT\alpha_1\beta_2$, over a time course between 30 min to 24h. The results of analysis by Western blot demonstrated a significant increase in the levels of p52 in whole cell extracts, following 4h exposure to $LT\alpha_1\beta_2$, which continued to rise up to 24h, the longest time point studied (43.46 ± 1.48 fold), (Figure 3.2B). Furthermore, another effect which was observed following exposure to $LT\alpha_1\beta_2$ was a significant increase in the level of expression of p100, the results show that after 4h of stimulation the level of p100 was significantly increased and again rose further, continually increasing up to the 24h time point (5.94 ± 0.42 fold, Figure 3.2C). When p52 formation was compared to the increase in p100 expression during exposure to $LT\alpha_1\beta_2$ (Fig 3.2D), this ratio increased over the alltime points suggesting that possibility that p52 and p100 formation are linked. This suggests that $LT\alpha_1\beta_2$ mediates both processing of p100 and its expression in Panc-1 cells.

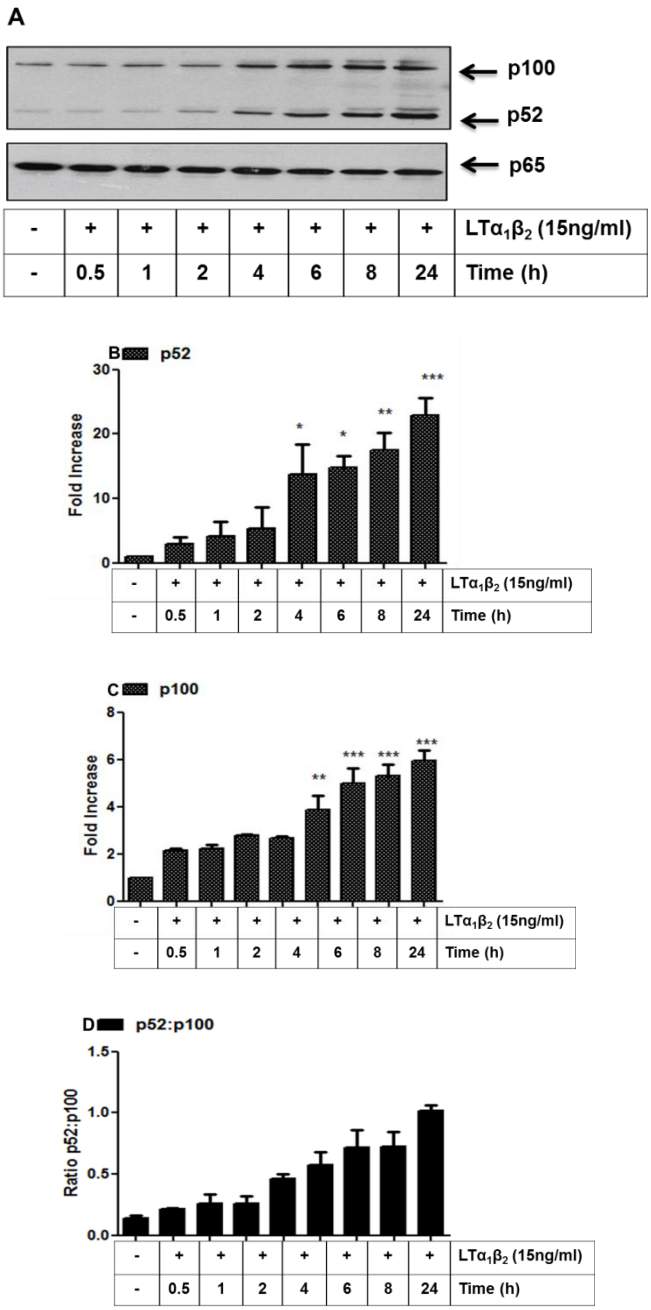


Figure 3.2 LTα$_1$β$_2$-induced formation of p52 in Panc-1 cells.

Panc-1 cells were exposed for LTα$_1$β$_2$ (15 ng/ml) for the times indicated (h), whole cell lysates were prepared and separated by SDS-PAGE, and then blotted for p52 (52 kDa), p65 NFκB (65 kDa) was used as a loading control, Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p52 (panel B), p100 (panel C) and p52:p100 (panel D). Each blot is representative three experiments. Each value represents the mean ± SEM of three independent experiments. ***P< 0.001, **P< 0.01 and *P< 0.05 compared to control

In order to investigate the effect of longer-term exposure to $LT\alpha_1\beta_2$, Panc-1 cells were exposed to $LT\alpha_1\beta_2$ for time points between 4h to 88h. The 4h time point was chosen to be the earliest point of degradation of p100 to p52 was first observed (Figure 3.2B). The results in figure 3.3 indicate that a significant increase in the levels of p52 formation was observed after 4h of stimulation and increase over time, reaching it is zenith at 40h after $LT\alpha_1\beta_2$ exposure (23.96 ± 2.8 fold). After this time point the level of stimulation began to decrease however, p52 levels remained high after 72 and 88h of exposure to $LT\alpha_1\beta_2$, (Figure 3.3B). In addition, the levels of p100 expression also increased over time reaching a peak after 40h of stimulation (2.16 ± 0.34 fold, figure 3.6C). As expected, the ratio between p52 formation and p100 expression increased over the time period (Figure 3.3D).

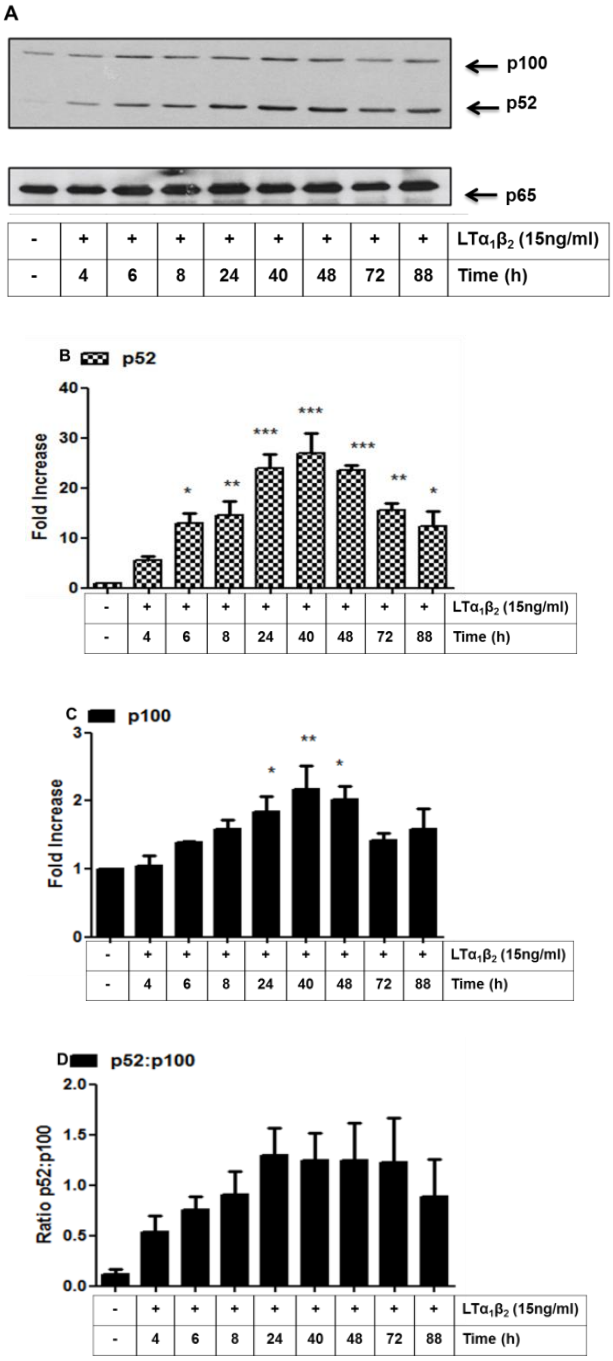


Figure 3. 3 Prolonged $LT\alpha_1\beta_2$-induced formation of p52 in Panc-1 cells.

Cells were exposed to the $LT\alpha_1\beta_2$ (15ng/ml) for the times indicated. Whole cell extracts were prepared and separated by SDS-PAGE and assessed for p-p100 (100kDa) and p65 NFκB (65kDa). Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p52 (panel B), p100 (panel C) and ratio p52:p100 panel D, Each value in panels B-D represents the mean ± SEM of three independent experiments. ***p< 0.001, **P< 0.01 and *P< 0.05 compared to control.

3.3.3 $LT\alpha_1\beta_2$ induced nuclear translocation of p52 in Panc-1 and MiaPaCa-2 cells

The results obtained in Figure 3.2 and 3.3 demonstrate that exposure to $LT\alpha_1\beta_2$ induced the formation of p52 in whole cell extracts from Pac-1 cells. In order to investigate if the $LT\alpha_1\beta_2$ induced p52 translocated to the nucleus of Panc-1, and also MiaPaC-2 cells as a comparison, nuclear extracts from cells treated with $LT\alpha_1\beta_2$ were prepared as outlined in Section 2.4.1. The results in Figure 3.4 showed that 4h after $LT\alpha_1\beta_2$ stimulation, a significant increase in the level of nuclear p52 was observed. In addition nuclear p52 was increased further at later time points, the highest nuclear accumulation of p52 was observed after 24h of stimulation, the longest time point studied (27.5 ± 2.22 fold). These results suggest that $LT\alpha_1B_2$ can mediate the processing of p-p100 to p52 and translocation to the nucleus.

MiaPaCa-2 cells were compared with Panc-1 cells and the results shown in Figure 3.5 were very similar to Panc-1 cells. Again there was a delay in the response to $LT\alpha_1\beta_2$ before nuclear p52 levels started to rise to a maximum by 24h (22.9 ± 3.7 fold).

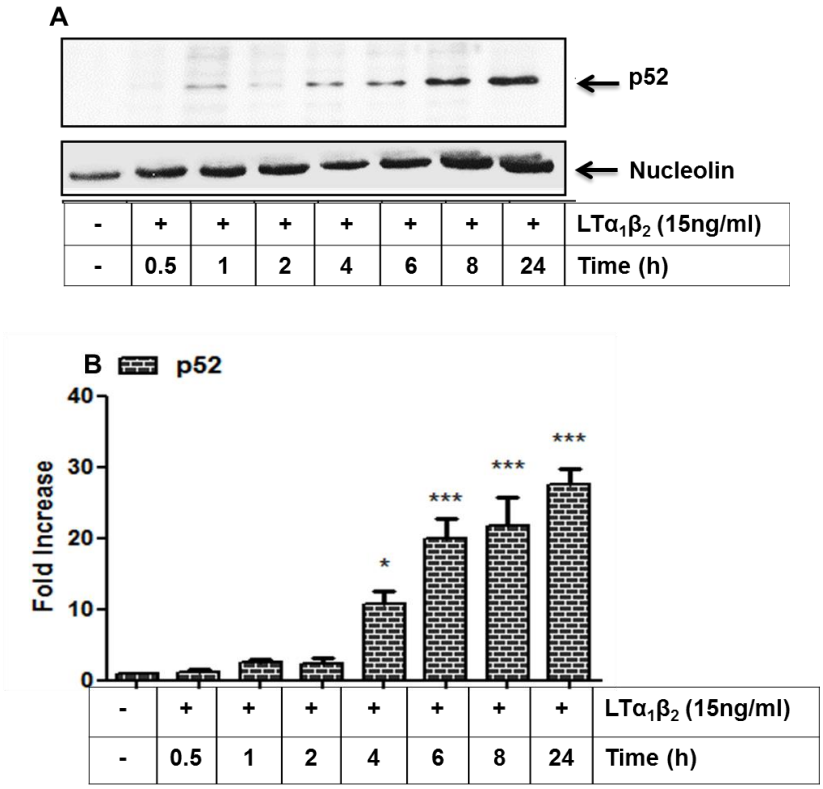


Figure 3. 4 $LT\alpha_1\beta_2$-induced nuclear translocation of p52 in Panc-1cells

Panc-1 cells were exposed to $LT\alpha_1\beta_2$ (15ng/ml) for increasing time periods (h), nuclear lysates prepared, separated by SDS-PAGE, then blotted for p52 (52 kDa). Nucleolin was used as a loading control (100 kDa). In panel B blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p52. Each value represents the mean ± SEM of three independent experiments. ***p< 0.001, and **p< 0.05 compared with control.

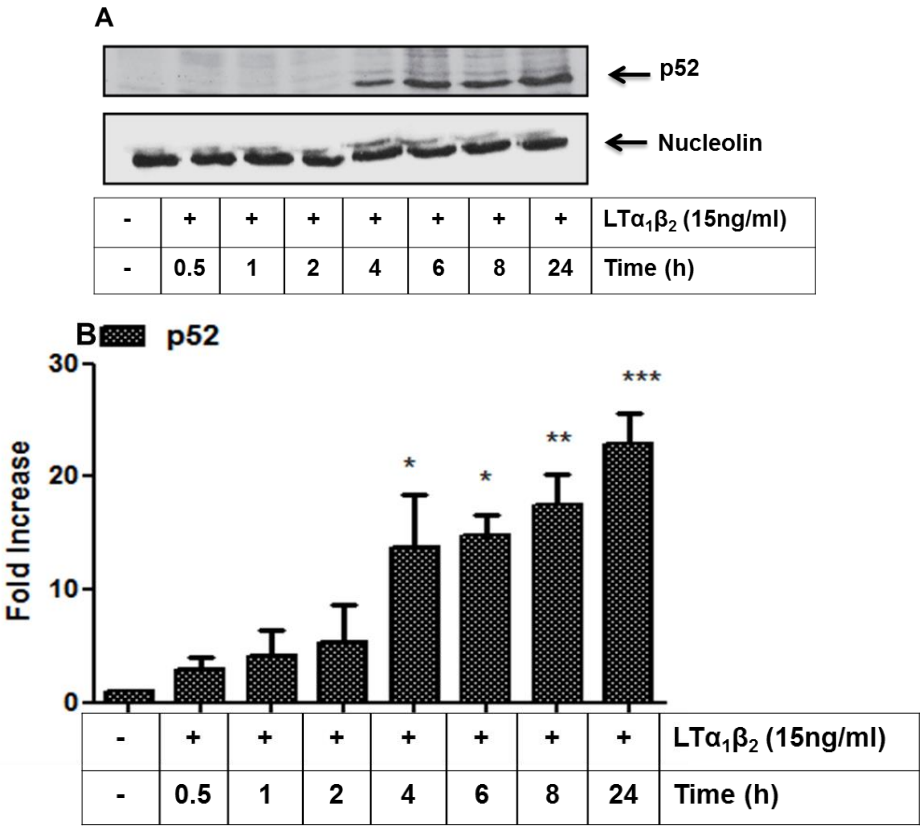


Figure 3. 5 $LT\alpha_1\beta_2$-induced nuclear translocation of p52 in MiaPaCa-2 cells

MiaPaCa-2 cells were exposed to $LT\alpha_1\beta_2$ (15ng/ml) for increasing time periods (h), nuclear lysates prepared, separated by SDS-PAGE, then blotted for p52 (52 kDa). Nucleolin was used as a loading control (100 kDa). In panel Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p52. Each value represents the mean ± SEM of three independent experiments. ***p< 0.001, and **p< 0.05 compared with control.

Next, the effect of longer $LT\alpha_1\beta_2$ stimulation times was assessed; Panc-1 cells were stimulated with $LT\alpha_1\beta_2$ over 88h starting with the 4h time point. The results, shown in Figure 3.6., demonstrate that increased nuclear p52 levels were observed between 24h and 48h, following stimulation by $LT\alpha_1\beta$ and the peak of nuclear accumulation of p52 was at 24h (fold stim 6.54 ± 0.2), after which p52 levels declined and returned to the basal level after 88h. The fold stimulation observed in these experiments was largely similar to those obtained in previous experiments which examined responses over 24h.

In addition, MiaPaCa-2 cells were also studied; cells were exposed to $LT\alpha_1\beta_2$ over a similar time course to Panc-1 cells (Figure 3.7). The results showed that as with Panc-1 cells, there was an increase in the accumulation of nuclear p52 starting at 4h and reaching a maximum between 24 h (fold stim 18.28 ± 3.67) and 48 h (fold stim 19.56 ± 4.29) compared with control, then gradually returning back to the basal level after 88h. The results of previous experiments for both cell lines stimulated with $LT\alpha_1\beta_2$ showed a similar optimal time for phosphorylation of p100, formation p52 and translocation p52 to the nucleus. These data suggest that activation of the non-canonical of NFκB pathway is a feature of pancreatic cancer cell lines.

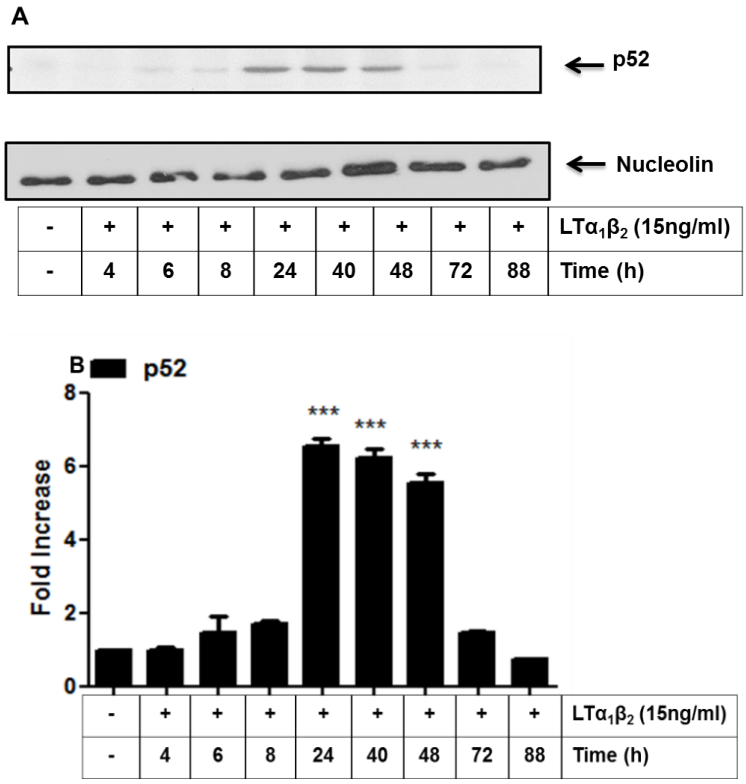


Figure 3.6 Prolonged $LT\alpha_1\beta_2$-induced nuclear translocation of p52 in Panc-1 cells

Panc-1cells were exposed to $LT\alpha_1\beta_2$ (15ng/ml) for increasing time periods (h), nuclear lysates prepared, separated by SDS-PAGE, then blotted for p52 (52 kDa). Nucleolin was used as a loading control (100 kDa). In panel B blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p52. Each value represents the mean ± SEM of three independent experiments. ***p< 0.001compared with control.

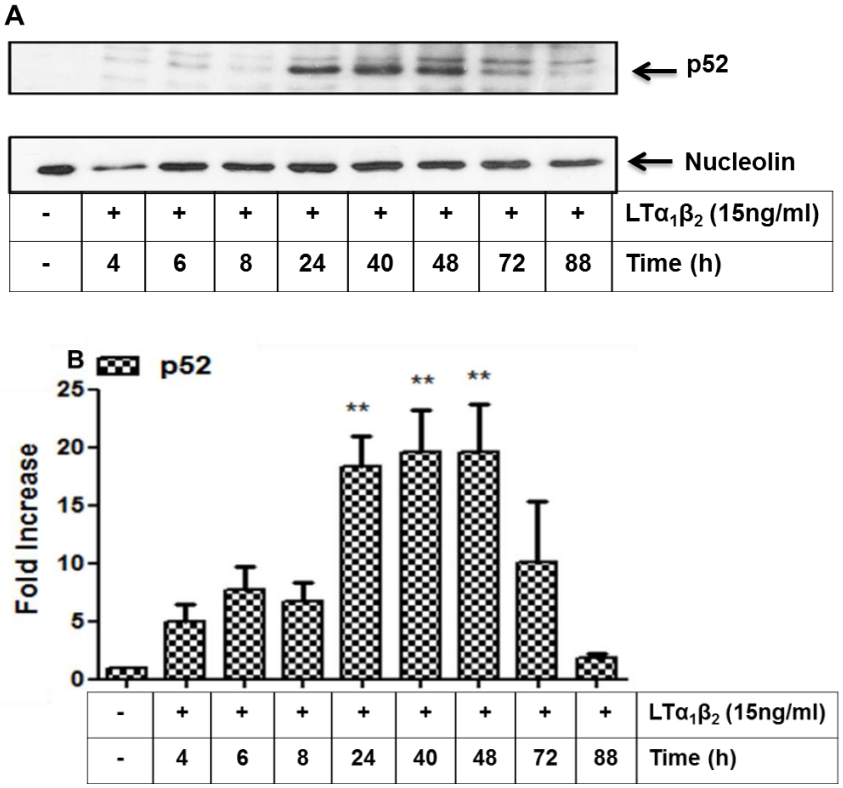


Figure 3. 7 Prolonged LT$\alpha_1\beta_2$-induced nuclear translocation of p52 in MiaPaCa-2 cells.

MiaPaCa-2 cells were exposed to LT$\alpha_1\beta_2$ (15ng/ml) for increasing time periods (h), nuclear lysates prepared, separated by SDS-PAGE, then blotted for p52 (52 kDa). Nucleolin was used as a loading control (100 kDa). In panel B blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p52. Each value represents the mean ± SEM of three independent experiments. ***p< 0.001, and **p< 0.05 compared with control.

3.3.4 Characterisation of RelB formation in Panc-1 inwhole cells extracts in panc-1 cells

In order to investigate the role $LT\alpha_1\beta_2$ in formation of RelB in Panc-1 cells. Panc-1 cells were exposed to $LT\alpha_1\beta_2$ between 30 min and 24h and RelB formation were assessed by Western blot. Increased formation of RelB was observed at 4 h of stimulation and increased in a time-dependent manner, reaching its peak at 24 h (fold stim 23.9 ± 3.9) compared with unstimulated control (Figure 3.8).

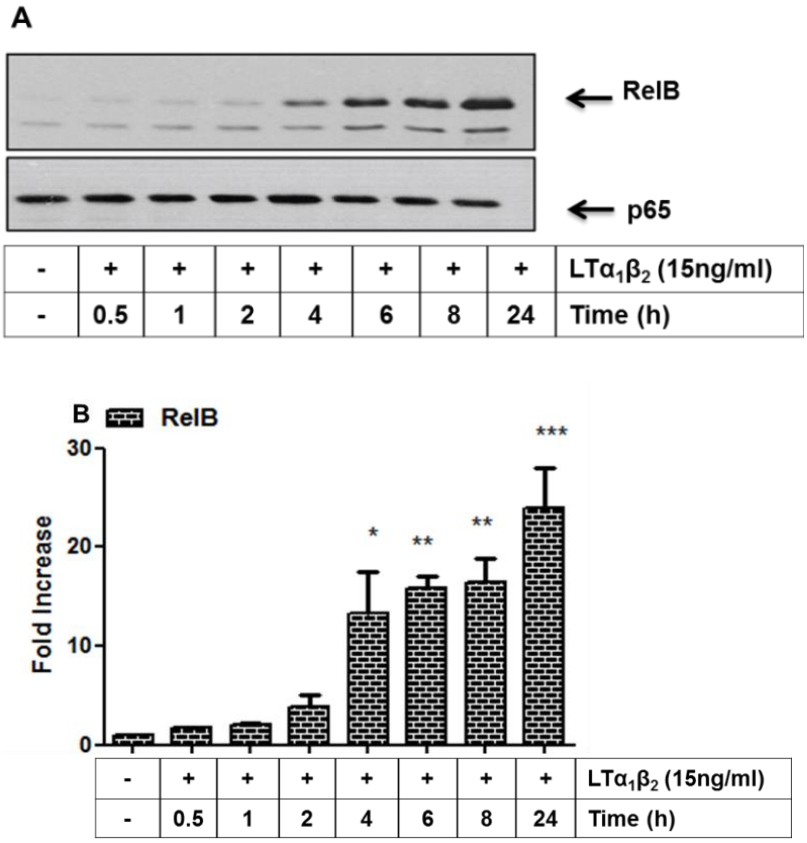


Figure 3.8 $LT\alpha_1\beta_2$-induced formation of RelB in Panc-1 cells

Panc-1 cells were exposed for $LT\alpha_1\beta_2$ (15 ng/ml) for time indicated (h), whole cell lysates were prepared and separates by SDS-PAGE, and then blotted for RelB (68 kDa) and p65 NFκB (65 kDa) was used as a loading control. In panel B blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for RelB. Each value represents the mean ± SEM of three independent experiments. ***p< 0.001, **p< 0.01 and *p< 0.05 compared with control.

3.3.5 TRAF2 and TRAF3 degradation in pancreatic cancer cell lines

TNF receptor-associated factors (TRAFs) are a family of proteins with multi-functions. These proteins have the ability to bind to the intracellular surface of membrane receptors to form proteins complexes which have a role in activation of a number of signalling pathways such as NFκB pathway (Chung et al., 2002). Studies have reported variable levels of TRAF2 and TRAF3 in pancreatic cancer cell lines, which are degraded following cellular activation (Doppler et al., 2013). Therefore, Panc-1 and Mia-Paca-2 cells were exposed to $LT\alpha_1\beta_2$ (15ng/ml) between 0.5-24 h and then whole cell extracts was prepared.

In Panc-1 cells it was found that there was a partial degradation of TRAF2 which occurred between 2-4 h of exposure to $LT\alpha_1\beta_2$ but this was not complete by the end of the time period, in fact levels recovered between 8-24 h of exposure (Figure 3.9). In contrast, TRAF3 levels increased at 24h of stimulation. However, as shown in Figure 3.10, when MiaPaCa-2 cells were compared with Panc-1 cells, it was found that degradation of TRAF2 only occurred at 24h of stimulation but levels were generally very low. When the blot was re-probed for TRAF3, an increase in the levels of expression was observed at two time points, following exposure to $LT\alpha_1\beta_2$ for 1 and 2 h of stimulation. Levels then retuned to basal level over the remainder of time course. This suggests that $LT\alpha_1\beta_2$ does have the potential to regulate the non-canonical NFκB pathway via the degradation of TRAF2 and 3 but the results were inconsistent and in contrast to the closely matched effects upon p100 phosphorylation and other parameters.

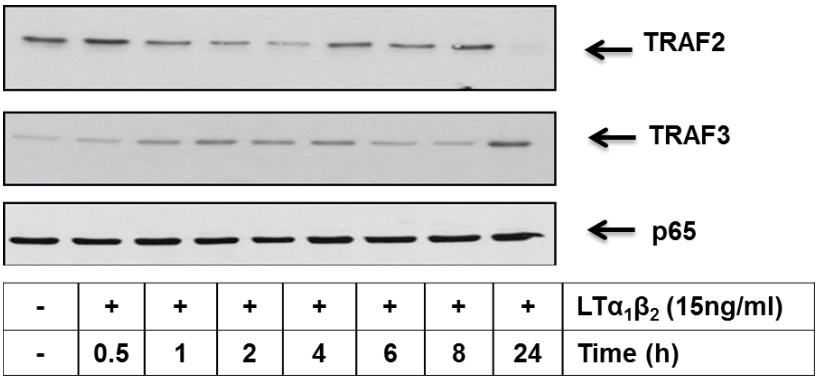


Figure 3. 9 TRAF2 and TRAF3 degradation in Panc-1 cells

Cells were exposed to $LT\alpha_1\beta_2$ (15ng/ml) for the times indicated. Whole cell extracts were prepared, and analysed by Western blot for expression of TRAF2 (53 kDa), TRAF3 (62 kDa) and p65 (65 kDa) which was used as a loading control. Each blot is representative of two independent experiments.

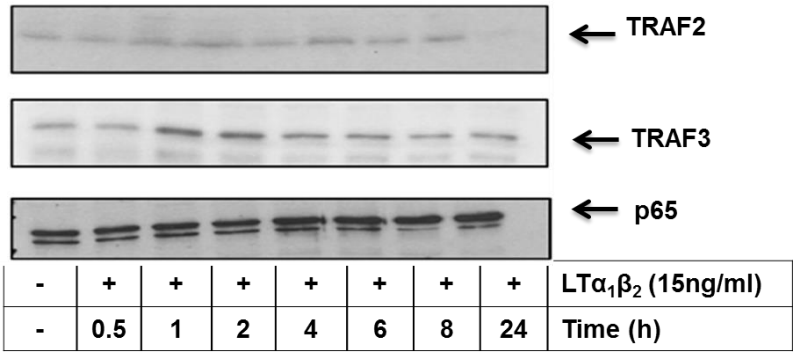
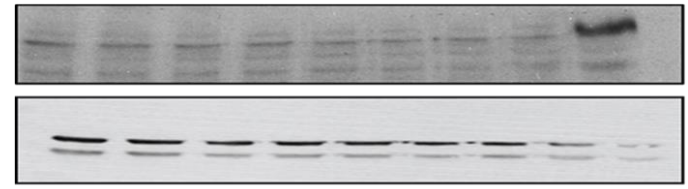


Figure 3. 10 TRAF2 and TRAF3 degradation in MiaPaC-1 cells

Cells were exposed to $LT\alpha_1\beta_2$ (15ng/ml) for the times indicated. Whole cell extracts were prepared, and analysed by Western blot for expression of TRAF2 (53 kDa), TRAF3 (62 kDa) and p65 NFκB (65kDa) which was used as a loading control. Each blot is representative of two experiments.

3.3.6 NIK expression in Panc-1 cells

Many previous studies have demonstrated that following cellular stimulation NIK is induced in Panc-1 cells, as mentioned in Section 1.6.2 chapter one, increased NIK levels lead to subsequent activation of the alternative NFκB pathway (Doppler et al., 2013). Therefore, following stimulation with $LT\alpha_1\beta_2$ (15ng/ml) whole cell extracts were prepared and blotted for NIK. For a positive control, a separate stock of Panc-1 cells were infected with NIK adenovirus (Adv. NIK), resulting in the over-expression of NIK protein. Figure 3.11 shows that endogenous levels of NIK were low in Panc-1 cells, compared with the positive control and not induced by $LT\alpha_1\beta_2$ over the time period studied.



-	-	+	+	+	+	+	+	-	$LT\alpha_1\beta_2$ (15ng/ml)
-	0.5	1	2	4	6	8	24	-	Time (h)
-	-	-	-	-		-	-	+ve	

Figure 3.11 NIK expressions in Panc-1 cells

Panc-1 cells were exposed to $LT\alpha_1\beta_2$ (15ng/ml) for the times indicated, whole cell extracts were prepared, and analyzed by Western blot for expression of NIK (120 kDa). A sample from Adv. NIK infected Panc-1 cells was used as a positive control, whilst p65 NFκB was included as a loading control. The blot is representative of two independent experiments.

3.3.7 Characterisation of IKKα in Panc-1 cells

As levels of p100 increased in response to $LT\alpha_1\beta_2$ stimulation, the levels of cellular IKKα and IKKβ in Panc-1 cells were investigated. Cells were exposed to $LT\alpha_1\beta_2$ for the same time points used in previous experiments. IKKα and IKKβ levels were assessed by Western blot. The results showed as hypothesis no change in the levels of both IKKα and IKKβ over all time points (Figure 3.12A).

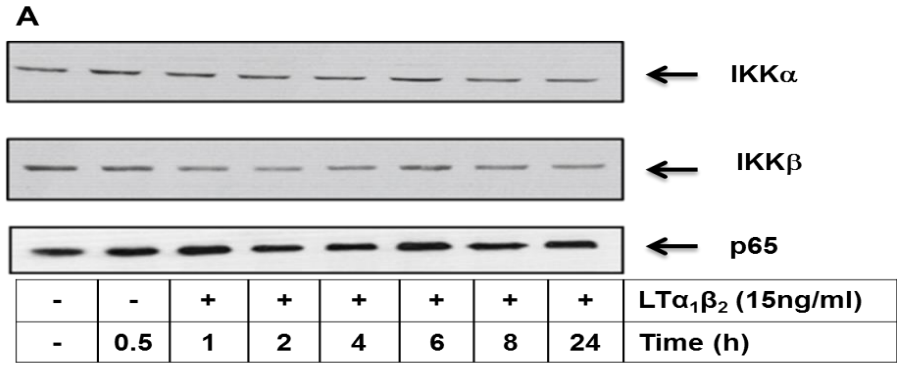


Figure 3.12 Effect of $LT\alpha_1\beta_2$ on expression of IKKα and IKKβ in Panc-1 cells

Panc-1 cells were exposed to $LT\alpha_1\beta_2$ (15ng/ml) for the times indicated, whole cell extracts were prepared and analyzed by Western blot for expression of IKKα (84 KDa), IKKβ(86 kDa) and then stripped and assessed for p65 NFκB (65kDa) which was used as a loading control. The blot is representative two independent experiments.

3.4 Characterisation of the non-canonical NFκB pathway using FCS

A number of experiments in our lab demonstrated that FCS has the ability to activate the non-canonical of NFκB pathway in many cells line such as U2OS and HUVECs cells. Therefore, the effect of FCS in both Panc-1 and MiaPaC-2 cells were investigated, through measurement of p100 phosphorylation, formation of p52 and translocation of p52 to the nucleus. Panc-1 cells were exposed to FCS 10% (v/v) over time course between 30 min and 24 h and assessed by Western blot. FCS stimulation showed a slight increase in levels of p52 and phosphorylation of p100 between 4 and 24h (Figure 3.13 A). Similarly, FCS stimulated only a marginal increase of p52 in nuclear extracts following 6 h of stimulation compared to untreated cells (Figure 3.13B). Next the effect of FCS in translocated p52 into the nucleus was investigated in MiaPaC-1 cells as a comparison with Panc-1 cells. There was a high level of p52 noted at two-time points, 4h and 8h compared with untreated sample (figure 3.13C) but in general stimulation was weak and inconsistent. This suggests that FCS is unable to mediate the activation of the non-canonical NFκB pathway in pancreatic cancer cell lines. Further preliminary results indicated that FCS was unable to activate the canonical pathway (see section 3.6.2) and was not used in further experiments.

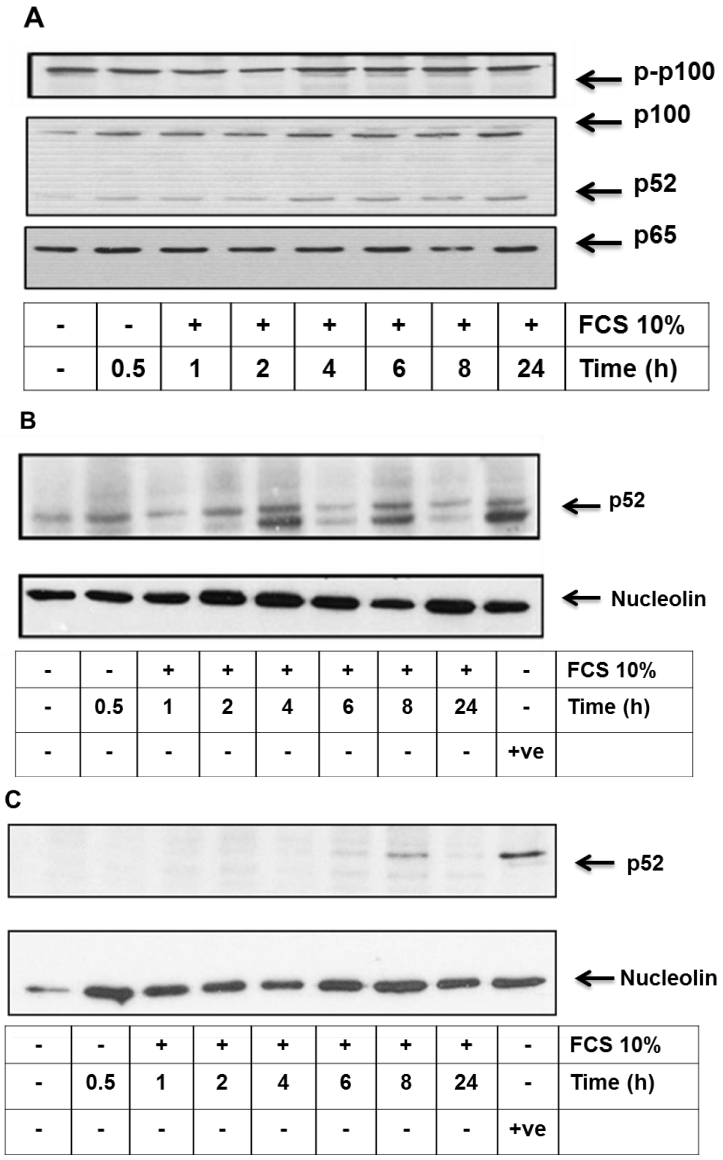


Figure 3.13 The effect of FCS on non-canonical NFκB signalling in Panc-1 and MiaPaCa-2 cells.

Panc-1 cells (panel A), cells were exposed to 10% (v/v) FCS for the times indicated (h). Whole cell extracts were prepared and then blotted for p-p100, p52 and p65 NFκB which used as a loading control. In panel B, Panc-1 cells were exposed to 10% (v/v) FCS for the times indicated (h). Nuclear extracts were prepared, and then blotted for p52 (52 kDa) and nucleolin (100 kDa) which used as a loading control. In panel C MiaPaCa-2 cells were stimulated with 10% (v/v) FSC for the times indicated (h), the nuclear extracts were prepared and botted for p52 (p52 kDa) and nucleolin (100kDa) which use as a loading control. One sample was used from the same cell line stimulated for 24 h with $LT\alpha_1B_2$ (15ng/ml) as positive control for p52 in panel B and C. Each blot is representative two experiments in panel A and B and one experiment in penal C.

3.5 Characterisation thr role of TNFα in NFκB pathways activation in pancreatic cancer cells lines

3.5.1. TNFα activation of the classical NFκB pathway in pancreatic cancer cell lines

The next part of the study examined the activation of the classical NFκB pathway. This was achieved by measuring the degradation of IκBα and phosphorylation of p65 NFκB NFκB in both Panc-1 and Mia-Paca-2 cells. In the Plevin laboratory, TNFα has been shown to activate the classical pathway in other cell lines, for example LNCaP, PC3M, HUVECs and U2OS cells. Initially, Panc-1 cell lines were stimulated with TNFα over an 8 h time course and IκBα degradation and phosphorylation of p65 NFκB measured IκBα degradation was rapid starting between 5 and 15 min and reaching a maximum after 30 min (2.29% ± 1.92 of basal expression) compared with untreated sample, before returning gradually to basal levels after 8 h (Figure 3.15B). Furthermore, TNFα stimulated also a rapid increase in p65 phosphorylation in Panc-1 cells which was maximal between 5 min (31.2 ± 2.47 fold) and 15 min (16.38 ± 1.16 fold) compared with basal values (figure 3.15C). A similar response was observed in MiaPaC-2 cells (Figure 3.16 A-C).

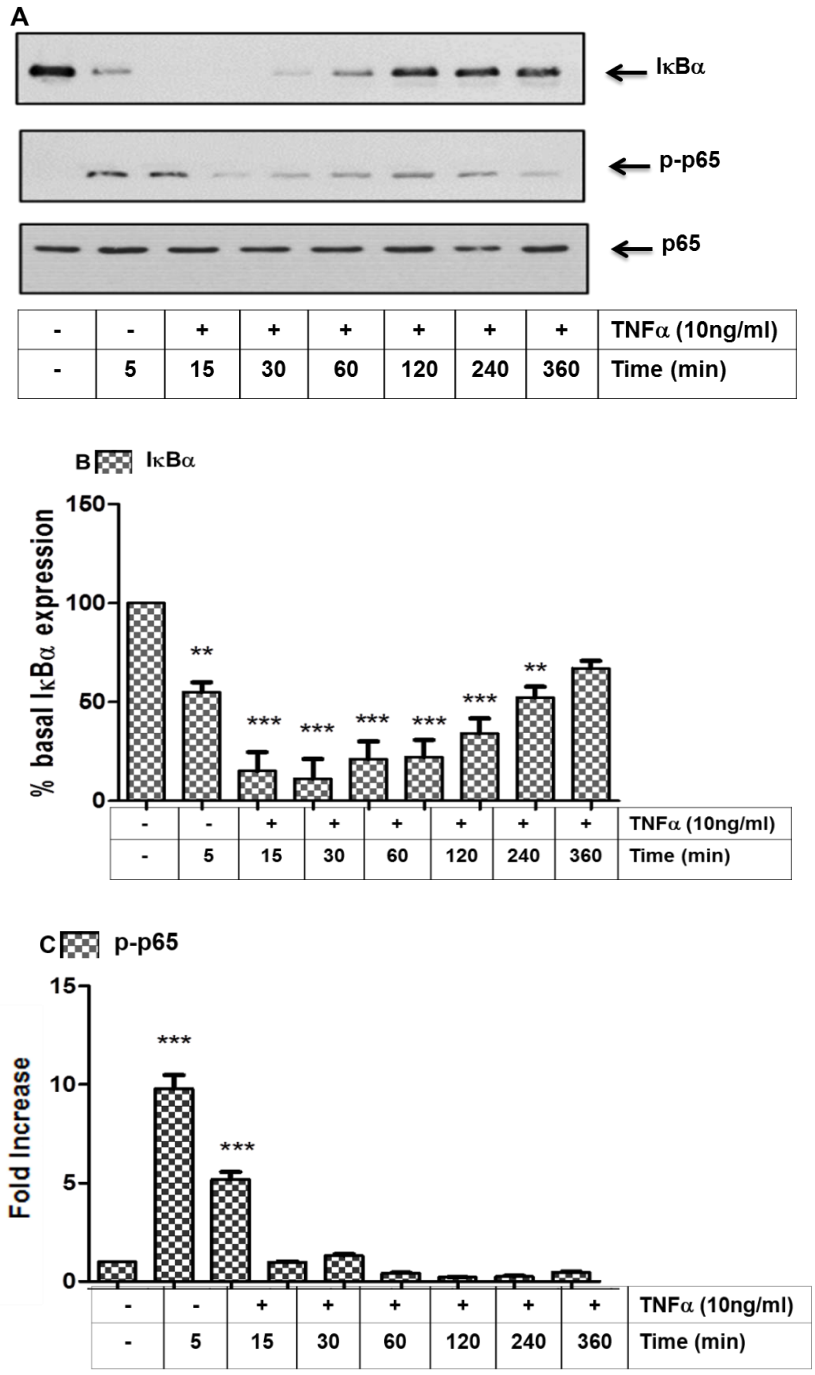


Figure 3.14 TNFα-induced degradation of IκBα and phosphorylation of p65 NFκB in Panc-1 cells

Cells were exposed to TNFα (10ng/ml) as indicated. Whole cell extracts were prepared, assessed for p-p65 (65kDa), and then re-probed for IκBα (37kDa) and also p65 NFκB (65kDa). Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for IκBα (panel B) and p65 NFκB (panel C). Each value represents the mean ± SEM of three independent experiments. *** $p<0.001$ and ** $p<0.01$ compared with control.

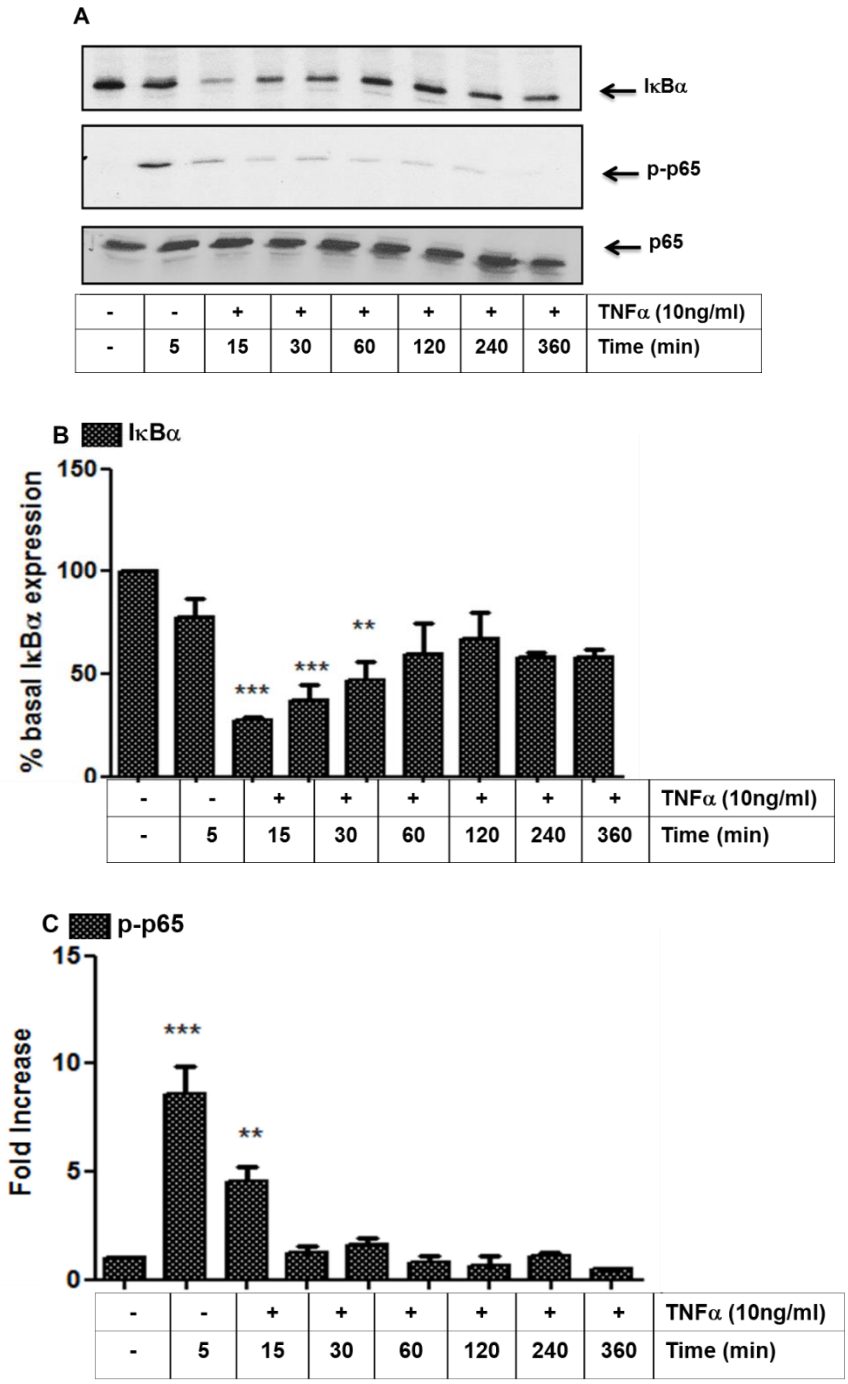


Figure 3.15 TNFα-induced degradation of IκBα and phosphorylation of p65 NFκB in MiaPaCa-2 cells.

Cells were exposed to TNFα (10ng/ml) as indicated. Whole cell extracts were prepared, then assessed for p-p65 NFκB (65kDa), then reprobed for IκBα (37kDa) and also p65 NFκB (65kDa) which was used as a loading control. Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for IκBα (panel B) and p65 NFκB (panel C). Each value represents the mean ± SEM of three independent experiments. *** $p<0.001$ and ** $p<0.01$ compared with control.

3.5.2 Role of TNFα in activation of the non-canonical of NFκB pathway

In the next set of experiments, the effect of TNFα on the activation of non-canonical NFκB pathway was examined. In contrast to $LT\alpha_1\beta_2$, TNFα was unable to induce an increase in p52 levels in whole Panc-1 cell extracts following 0.5-24 h exposure. A minor increase (not significant) in levels of p52 was observed following 24h stimulation (figure 3.14A). Furthermore, TNFα was unable to induce an increase in the levels of nuclear p52 compared to wells treated with $LT\alpha_1\beta_2$ (Figure 3.14B). A similar result was obtained in MiaPaCa-2 cells (Figure 3.14C). Taken together these experiments show that TNFα was unable to activate the alternative NFκB pathway in pancreatic cancer cell lines in contrast with U2OS and HUVECs cell lines.

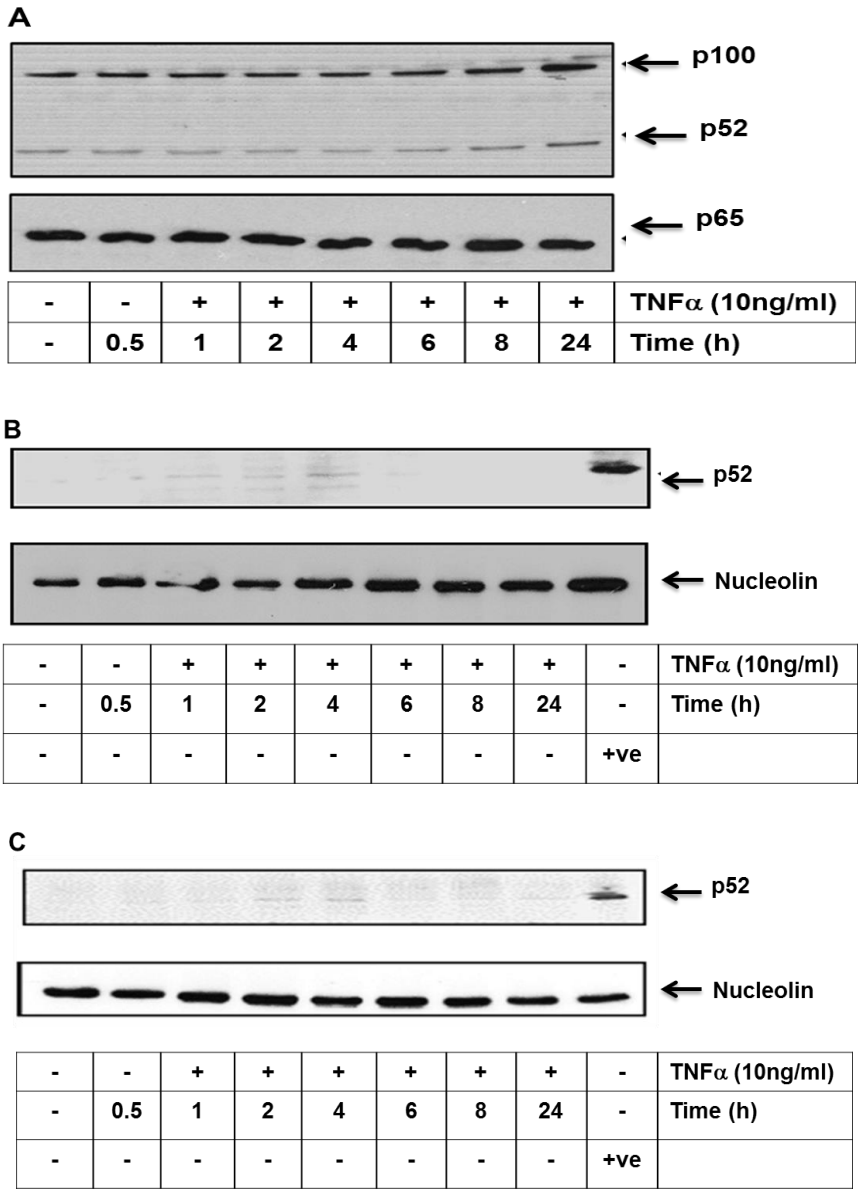


Figure 3.16 The effect of TNFα on the non-canonical of NFκB pathway in Panc-1 cells.

Panels A-C cells were exposed to TNFα (10ng/ml) for the times indicated (h). Whole cell extracts were prepared and then blotted for p52 and p65 NFκB which was used as a loading control. In Panel B Panc-1 cells were exposed to TNFα (10ng/ml). Nuclear extracts were prepared, then blotting for p52 (52 kDa) and nucleolin (100 kDa) was used as a loading control. Panel C, MiaPaCa-2 cells were exposed to TNFα (10ng/ml). Nuclear extracts were prepared, then blotted for p52 (52 kDa) and nucleolin (100 kDa) was used as a loading control. A positive control for p52 was prepared from the same cell line stimulated with $LT\alpha_1\beta_2$ (15ng/ml) for 24 h in panel B and C. Each blot is representative of two experiments in panel A and B, and one experiment in panel C.

3.7. The effect of LT$\alpha_1\beta_2$ on activation of the canonical NFκB pathway

The results in Section 3.3 above demonstrated that LT$\alpha_1\beta_2$ has the ability to activate the non-canonical NFκB pathway. Therefore, the effect of LT$\alpha_1\beta_2$ in activation of the canonical of NFκB pathway was investigated in Figure 3.17. Panc-1 cells were exposed to LT$\alpha_1\beta_2$ between 5 min to 6 h and the activation of classical of NFκB pathway was assessed by degradation of IκBα and phosphorylation of p65 NFκB using Western blotting. Whilst LT$\alpha_1\beta_2$ activated components of the canonical NFκB pathways there was a distinct profile of activation, the kinetics where substantially relative to TNFα. Degradation of cellular IκBα was first observed 15 min after LT$\alpha_1\beta_2$ stimulation reaching a maximum 1h after initial exposure. IκBα levels then recovered to the basal level over the rest of the stimulation period (2-6 h) (Figure 3.19B). A significant phosphorylation of p65 NFκB was observed 15 min after LT$\alpha_1\beta_2$ stimulation reaching a maximum at 2h (fold stimulation; 46.2 ± 4.64, figure 3.19C) Phosphorylation of p65 NFκB was remained elevated over the remainder of the time course compared with control.

Next the effect of FCS exposure on the activation of the classical of NFκB pathway was examined. Panc-1 cells were exposed to FSC 10% (v/v) for times points between 5 min and 6h. One sample was exposed to TNFα (10ng/ml) for 15 min which was the optimal time for degradation IκBα and phosphorylation p65 NFκB and this served as positive control for classical of NFκB pathway activation. The results show that FSC was unable to induce the degradation IκBα and phosphorylation p65 NFκB in Panc-1 cell (data not shown). Furthermore, MiaPaCa-2 cells were exposed

to $LT\alpha_1\beta_2$ and FCS and classical markers of canonical NFκB pathways were assessed and showed similar results to the Panc-1 cells results $LT\alpha_1\beta_2$ showed the ability to activate the canonical of NFκB pathway through inducing the IκBα and phosphorylation of p65 NFκB, whilst FCS did not.

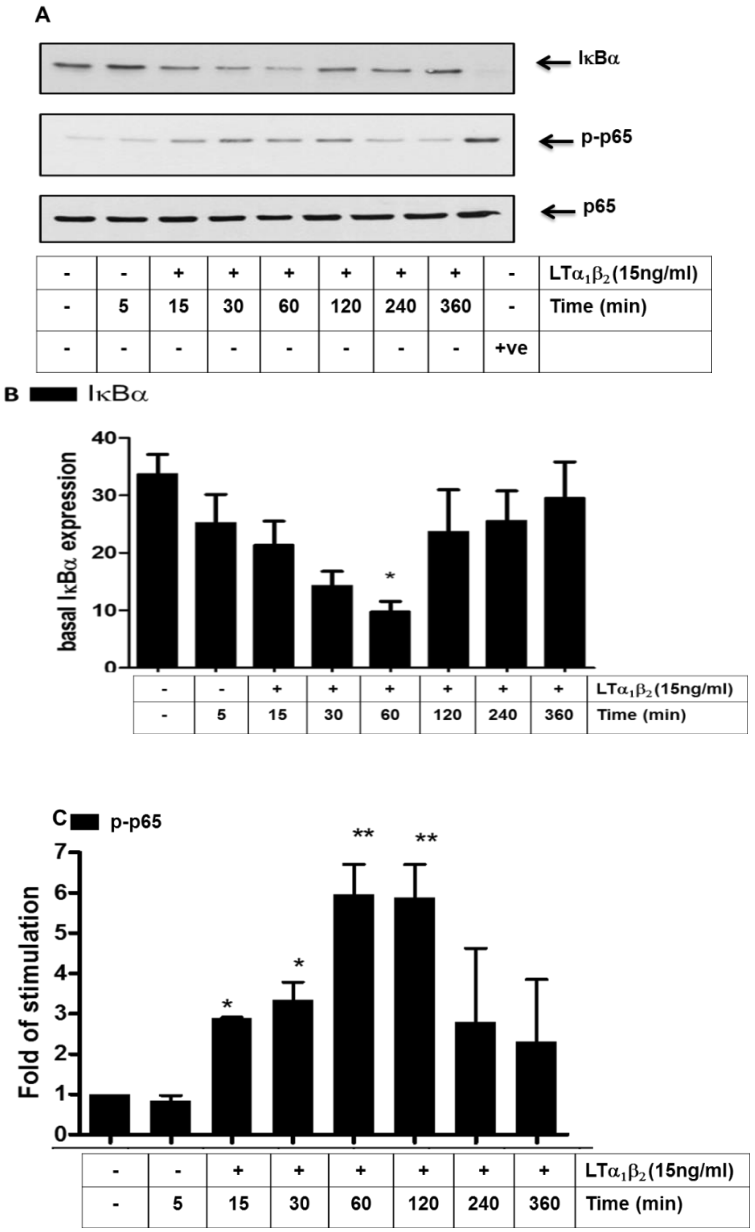


Figure 3.17 LTα$_1$β$_2$-induced degradation of IκBα and phosphorylation of p65 NFκB in Panc-1 cells

Cells were exposed to LTα$_1$β$_2$ (15 ng/ml) for the times indicated. Whole cell extracts were prepared, then assessed for IκBα (37kDa), then re-probed for p-p65 (65kDa), and also p65 NFκB (65kDa) which was used as a loading control, one sample from the same cell line stimulated with TNFα (10 ng/ml) for 15min was used as positive control for activation of classical of NFκB pathway. Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for IκBα (panel B) and p65 NFκB (panel C). Each value represents the mean ± SEM of three independent experiments. *** $p<0.001$, ** $p<0.01$ and * $p<0.05$ compared with control.

3.7 The effect of silencing cellular IKKα expression on the non-canonical NFκB pathway in Panc-1 cells

In order to confirm that phosphorylation of p100 and the formation of p52 were the result of IKKα activation, experiments using siRNA induced knockdown were attempted.

3.7.1 siRNA silencing of IKKα and IKKβ in Panc-1 cells

To optimise the knockdown of IKKα and IKKβ with siRNA, Panc-1 cells were transfected with increasing concentrations of IKKα and IKKβ siRNA (25-100nM), for 72h which in preliminary experiments was found to be the optimal time for silencing. Western blotting was used to evaluate the protein expression level of both IKKα and IKKβ. Using 50-100nM of non-targeting sequence (NT) resulted in a minimal effect on the of endogenous expression of IKKα, whilst surprisingly, the lowest concentration (25nM) of siRNA of IKKα, effectively reduced the endogenous of IKKα expression by greater than 95% (see Figure 3.18). The level of IKKα expression decreased further as the concentration of siRNA increased. Furthermore, treatment of cells with siRNA IKKα (25–100nM) showed no effect on expression of IKKβ even with the maximum concentration (100nM).

Next, the optimal concentration of siRNA-IKKβ for knockdown of IKKβ in Panc-1 cells was investigated. Figure 3.19, shows that there were a sharp decrease in the expression of IKKβ, the lowest concentration (25nM) caused a substantial and significant reduction in the level of IKKβ expression, basal expression was reduced to 3.52 ± 1.55% compared with control (100%). Moreover, inhibition of IKKβ

expression was increased to more than 99% at the maximum IKKβ siRNA concentration (100nM) without compromising cellular integrity by examination the morphology and appearance of cells using microscope, this further suggested that IKKα and IKKβ could be knocked down selectively in Panc-1 cells see Figure 3.19.

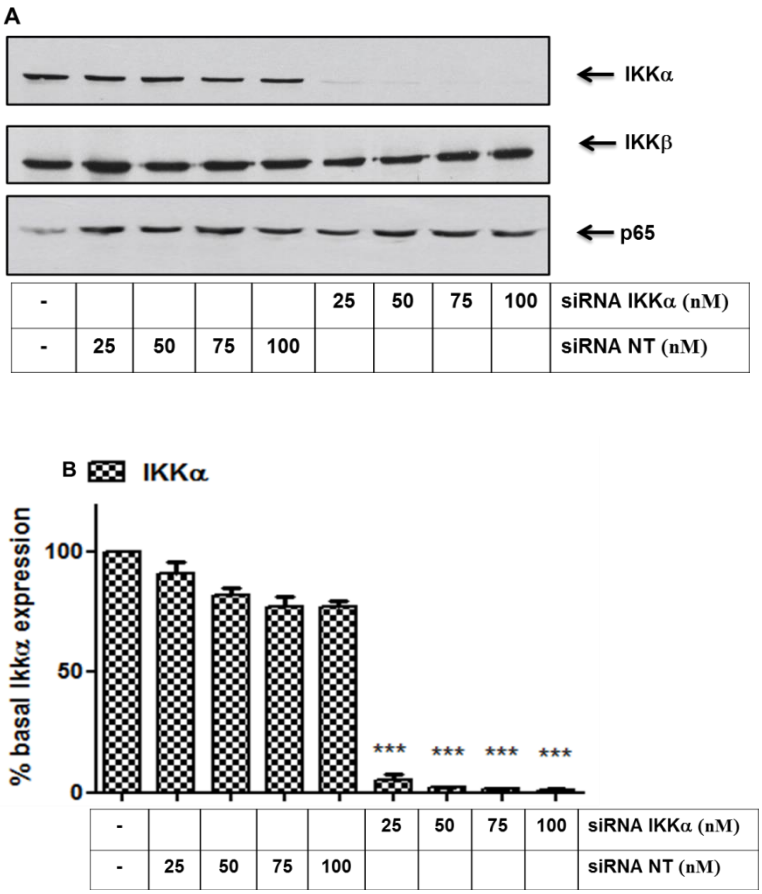


Figure 3.18 The effect of IKKα siRNA on IKKα expression in Panc-1 cells

Cells were transfected with nontargeting siRNA (NT) and IKKα up to 100nM for 72h. Whole cells extracts were prepared and separated by SDS-PAGE and then assessed for IKKα (84 kDa), IKKβ (86 kDa) and p65 NFκB (65kDa) which was used as a loading control. In panel B blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for IKKα. Each value represents the mean ± S.E.M of three independent experiments, *** $p<0.001$ compared with control.

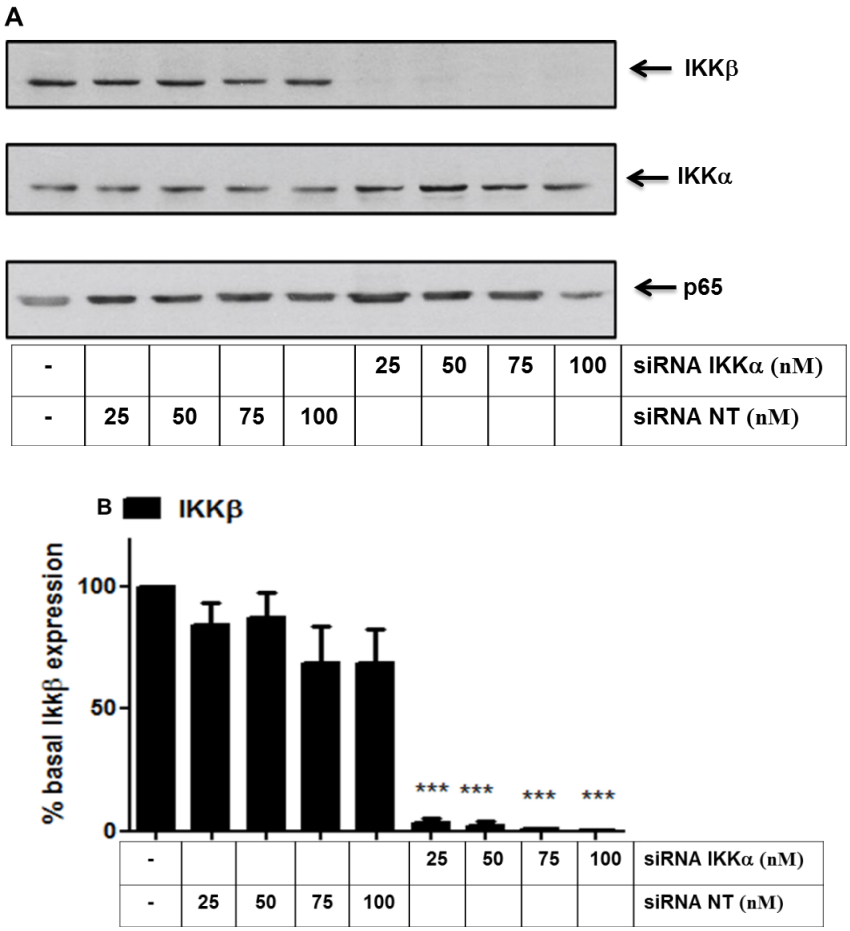


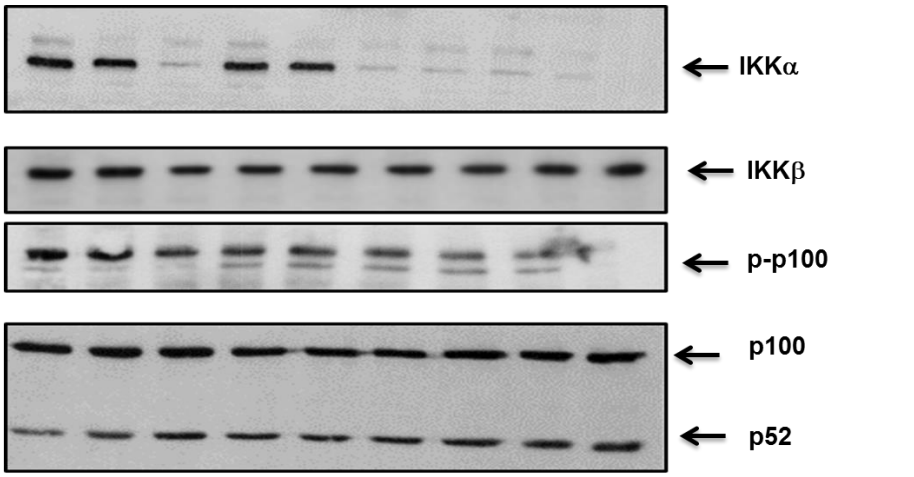
Figure 3.19 The effect of IKKβ siRNA on IKKβ expression in Panc-1 cells

Cells were transfected with non-targeting siRNA (NT) and IKKα up to 100nM for 72h. Whole cells extracts were prepared, separated by SDS-PAGE and then assessed for IKKβ (86 kDa), IKKα (84 kDa) and p65 NFκB (65kDa) which was used as a loading control. In panel B blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for IKKβ. Each value represents the mean ± S.E.M of three independent experiments, *** $p<0.001$ compared with control.

3.7.2 The effect of silencing of cellular IKKα expression on the non-canonical NFκB pathway in Panc-1 cells

The effect of IKKα knockdown on the alternative NFκB pathway was examined through assessment of three markers, phosphorylation of p100, expression of p100 and formation of p52. Panc-1 cells were treated with siRNA against IKKα (50–300nM) for 72h and then stimulated with $LT\alpha_1\beta_2$ for 4 h which is the optimal time for phosphorylation of p100 in (see Figure 3.1). Figure 3.20 shows a high level of p100 when the cells were exposed to $LT\alpha_1\beta_2$ alone, the levels of phosphorylation was decreased and returned to the basal levels only at the maximum concentration used, 300nM.

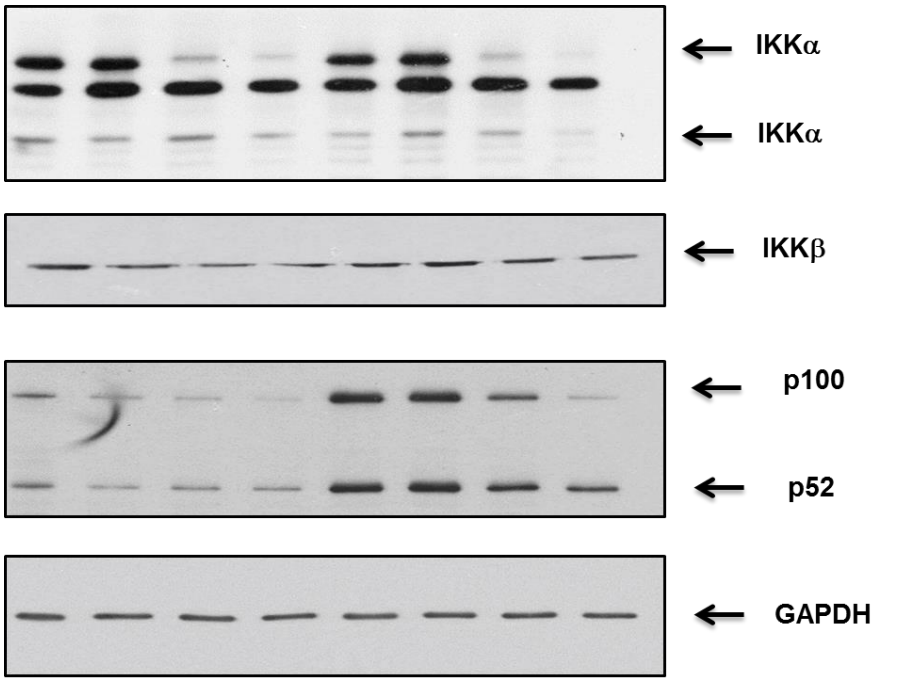
Next the effect of silencing of IKKα on formation of p52 in Panc-1 cells was investigated using two higher concentrations of siRNA (300 and 500nM). The cells were transfected with siRNA against of IKKα for 72h and then stimulated with $LT\alpha_1\beta_2$ for 24h, the optimal time for formation of p52 (see Figure 3.2). The results have showed there was an increase in p52 level in response to $LT\alpha_1\beta_2$ alone, the level of both p100 expression and of p52 started to decreased, but not completely following by pre-treatment with of 300 and 500nM of IKKα siRNA (Figure 3.21). In these experiments a new antibody for IKKα was used (Milllipore UK) which has the ability to detect both isoforms of IKKα, 84 kDa and 45 kDa, to study the effect of siRNA against IKKα on both IKKα isoforms this antibody was used to investigate the effect on both isoforms.



-	-	300	-	-	50	100	200	300	siRNA IKKα (nM)
-	300	-	-	300	-	-	-	-	siRNA NT (nM)
-	-	-	+	+	+	+	+	+	LTα$_1$β$_2$(2ng/ml)

Figure 3.20 The effect of IKKα siRNA on the phosphorylation of p100 in Panc-1 cells

Panc-1 cells were treated with siRNA for 72h and then stimulated with LTα1β2 (2ng/ml). Whole cells extracts were prepared, and analyzed by Western blot for expression of IKKα (84 kDa), IKKβ (86kDa), p-p100 (100 kDa) and p52 (52kDa) which used as a loading control. The blot is representative three independent experiments.



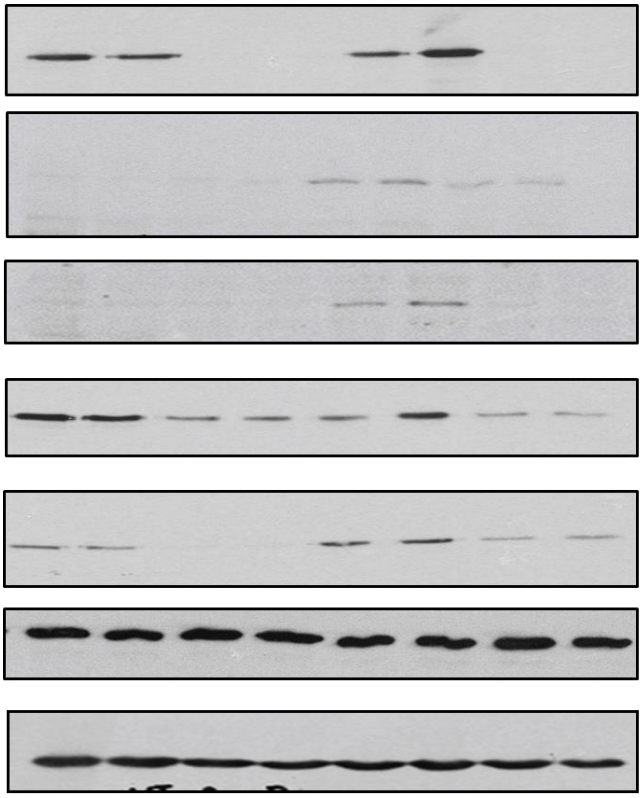
-	-	300	500	-	-	300	500	siRNA IKKα (nM)
-	500	-	-	-	500	-	-	siRNA NT (nM)
-	-	-	-	+	+	+	+	$LT\alpha_1\beta_2$ (2ng/ml)

Figure 3.21 The effect of IKKα siRNA on the p100 expression and formation p52 in Panc-1 cells

Panc-1 cells were treated with siRNA for 72h and then stimulated with LTα1β2 (2ng/ml). Whole cells extracts were prepared, and analyzed by Western blot for expression of IKKα (84 kDa), IKKβ (86kDa), p52 (52kDa) and GAPDH (37kDa) which used as a loading control. The blot is representative three independent experiments.

3.7.3 Effect of silencing cellular IKKβ expression on the canonical NFκB pathway in Panc-1 cells

Having established effective knockdown of IKKβ by siRNA, the next step was examine the effect of IKKβ silencing on the classical NFκB pathway. Panc-1 cells were transfected with 300nM and 500nM siRNA against IKKβ for 72h and then stimulated with TNFα for 15 min which was the optimal time for degradation of IκBα and phosphorylation of p65 NFκB (see Figure 3.15); rundown of IKKβ induced a reduction in the basal levels of degradation of IκBα and phosphorylation of p65 NFκB, however, there was no effect on the basal level of p-IκBα or p-p105. Following exposed to TNFα, partial degradation of IκBα and an increase in phosphorylation of p-p105, IκBα and p65 NFκB was observed, compared with untreated sample. In addition, surprisingly, treatment with non targeting of siRNA following by stimulation with TNFα inducing an increase in the level of IκBα to the same level as that observed in untreated control. Treatment with siRNA against IKKβ induced a reduction in TNFα -induced increases in the level of p-IκBα and phosphorylation of p105 and p65 NFκB, although there was no effect on the level of IκBα degradation (see Figure 3.22). As expected the results did confirm that IKKβ has a role in regulation of the canonical of NFκB pathway, but need further experimentation is required to optimise conditions.



-	-	300	500	-	-	300	500	siRNA IKKβ (nM)
-	500	-	-	-	500	-	-	siRNA NT (nM)
-	-	-	-	+	+	+	+	TNFα (2ng/ml)

Figure 3.22 The effect of IKKβ siRNA on the canonical of NFκB pathway in Panc-1 cells

Panc-1 cells were treated with siRNA of IKKβ for 72h and then stimulated with TNFα (2ng/ml). Whole cells extracts were prepared, and analyzed by Western blot for expression of IKKβ (86 kDa), p-p105 (105 kDa) p-IκBα (37kDa), IκBα (37kDa), p-p65 (p65kDa) and p65 NFκB (65kDa) which used as a loading control. The blot is representative two independent experiments.

3.7.7 Effect of the IKKβ and IKKα silencing on Panc-1 cell cycle progression

In order to investigate the role of both KKα and IKKβ in cell cycle distribution in Panc-1 cells, the cell cycle population was evaluated using flow cytometry and propidium (PI) staining as outlined in Section 2.7. After 48h of transfection of cells with concentrations of siRNA between 50 and 300nM, Figure 3.23 and 3.24. indicated that no change in distribution of cells in all cell cycle phases, all concentrations of siRNA against IKKα and IKKβ used. Next experiments were designed for cell cycle using different time of transfection with siRNA for both IKKα and IKKβ, cells were transfected for 72 h which was the optimal time for transfection in preliminary experiments in Section 3.7.1. Again the results there was no alteration in percentage of cells in each cell cycle phase (data not shown). Further investigate the regulatory role of IKKs of cell cycle are required using different time points or different methods.

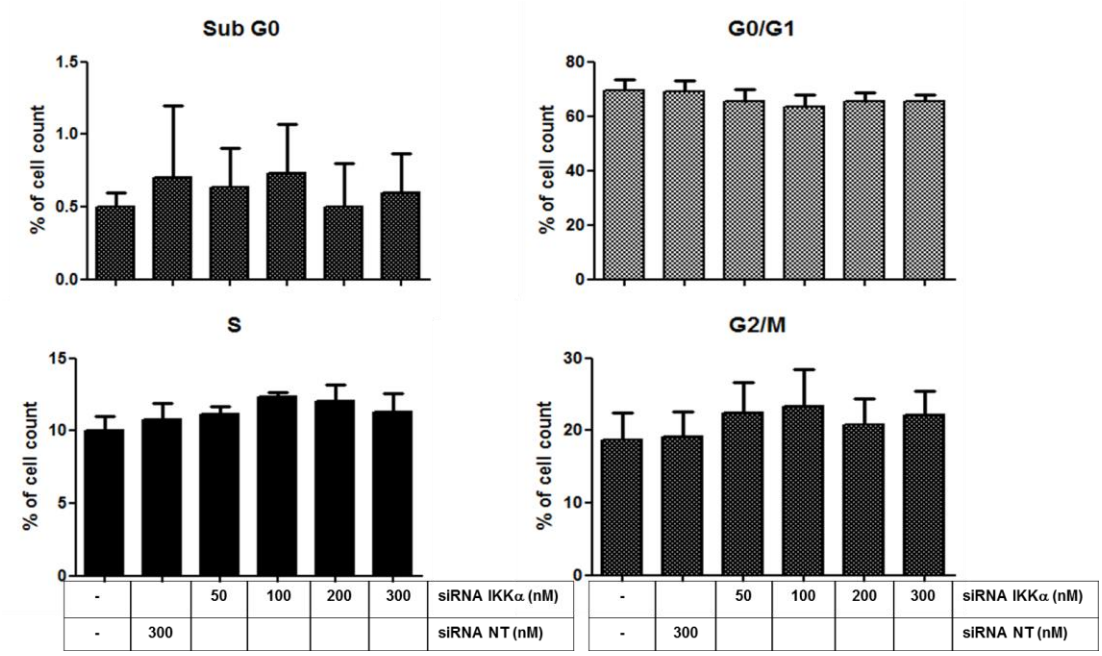


Figure 3.23 Effect of siRNA IKKα on cell cycle progression in Panc-1 cells

Cells were transfected either 300nM of non-targeting (NT), or increasing concentration of siRNA IKKα (50nM-300nM) for 48h, then assessed by flow cytometry using propidium iodide (PI) for determination of total DNA content. The charts demonstrate the distribution of cells in the different phases of the cell cycle following treatment. Data shown are expressed as % of cell count. Each value represents the mean ± E.S.M of three independent experiments.

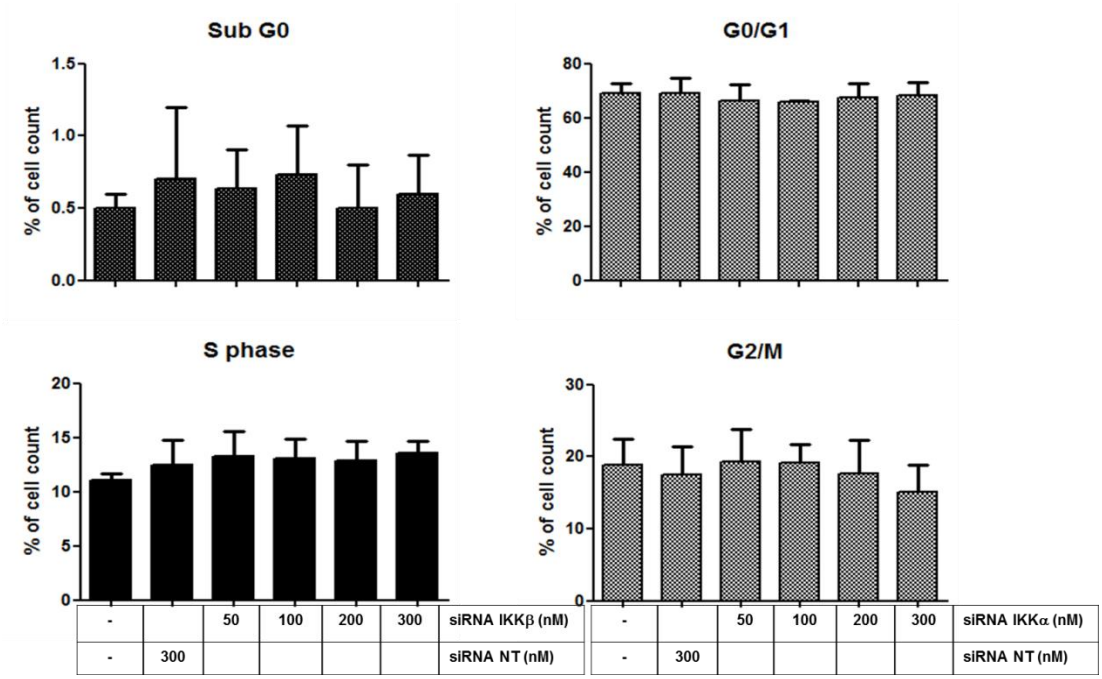


Figure 3.24 Effect of siRNA IKKβ on cell cycle progression in Panc-1 cells

Cells were transfected either 300nM of non-targeting (NT), increasing concentration of siRNA IKKβ (50nM-300nM) for 48h, then assessed by flow cytometry using propidium iodide (PI) for determination of total DNA content. The charts demonstrate the distribution of cells in the different phases of the cell cycle following treatment. Data shown are expressed as % of cell count. Each value represents the mean ± E.S.M of three independent experiments.

3.7.4 Effect of the IKKβ and IKKα silencing on Panc-1 cells clonogenicity.

Further examined whether the rundown of IKKα and IKKβ using siRNA have effect on Panc-1 cells survival, cells were transfected with siRNA against IKKα as well as IKKβ with (50-300nM), then cells were re-plated and incubated for 7 days, to investigate the effect in term of cells division and colony formation. In figure 2.25, rundown IKKα through siRNA against IKKα shown the number of colonies was slightly decreased not significant along the all concentrations that used in this experiment. Furthermore knockdown IKKβ in Panc-1 cells have showed there was no significant different between all the concentrations of siRNA against IKKβ that the cells treated with and untreated see figure 3.26, silencing both IKKα and IKKβ in panc-1 cells have no effect on cell division and colony formation, future experiment using different techniques are require for investigating the effect of knockdown of IKKα and IKKβ in cell proliferation of Panc-1 cells.

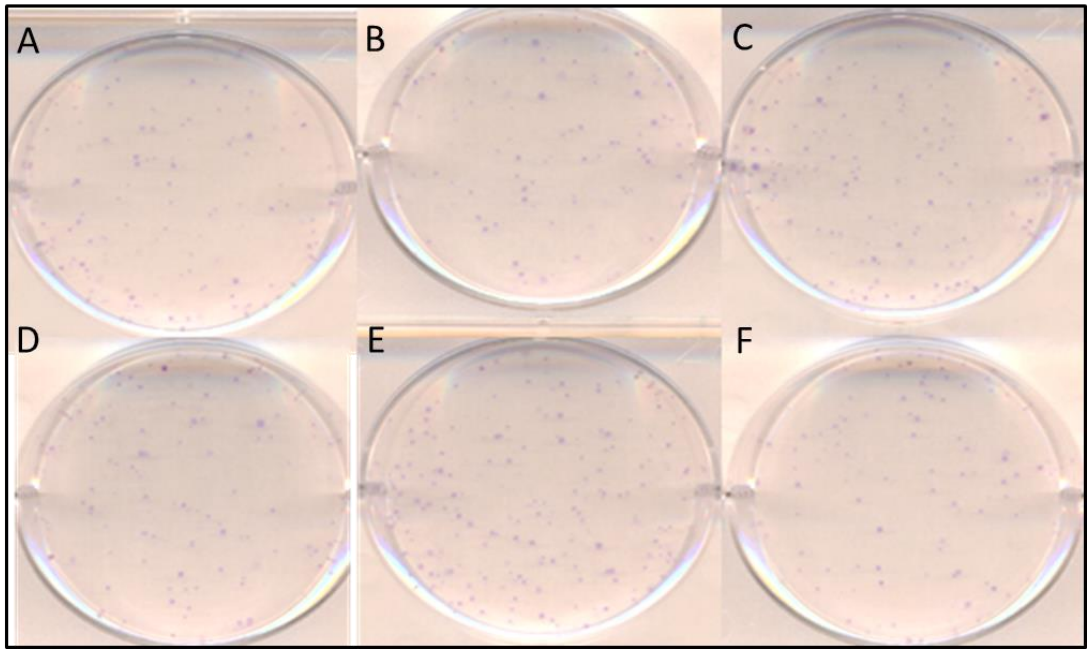


Figure 3.25 Effect of siRNA IKKα transfection on Panc-1 cells clonogenic survival.

Cells untreated (A) cells were transfected with non-targeting (NT 300nM) (B), IKKα 50nM (C), IKKα 100nM (D), IKKα 200nM (E) and IKKα 3000nM (F) for 48h, and then clonogenic survival assay were performed for each treatment group. The figure is representative three independent experiments

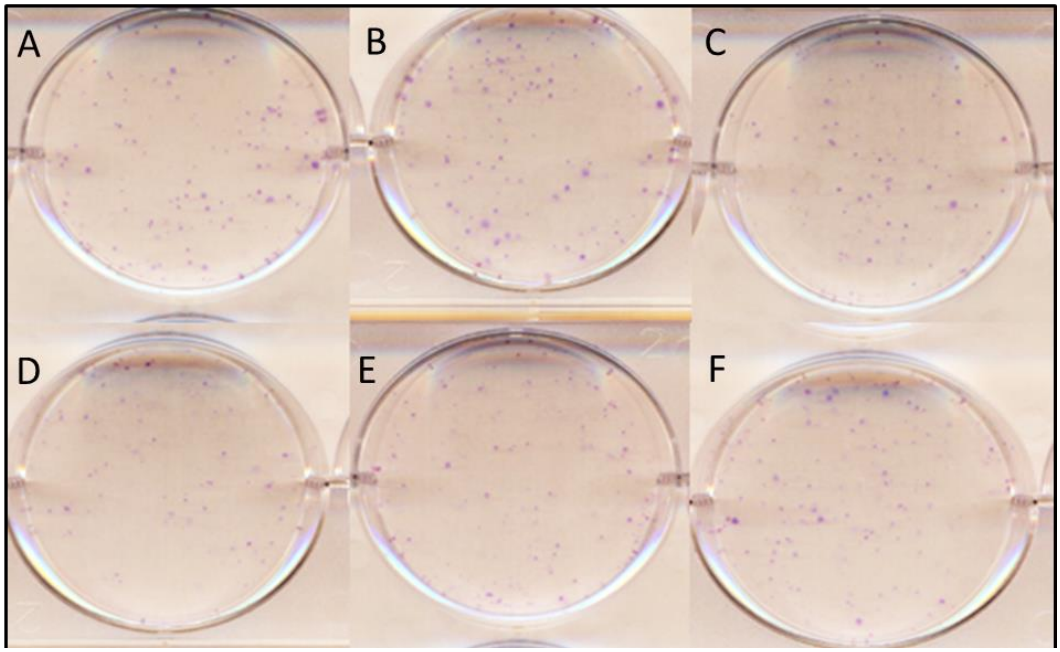


Figure 3.26 Effect of siRNA IKKβ transfection on Panc-1 cells clonogenic survival.

Untreated cell (A), cells were transfected with non-targeting (NT 300nM) (B), IKKβ 50nM (C), IKKβ 100nM (D), IKKβ 200nM (E) and IKKβ 3000nM (F) for 48h and then clonogenic survival assay were performed for each treatment group, the figure is representative three independent experiments

3.9 Discussion

As has been discussed previously, NFκB plays an important role in many cellular processes that lead to cancer progression (Baud and Karin, 2009, Hoesel and Schmid, 2013). Pancreatic cancer is different from many other cancers because of its very poor prognosis, the chance of survival after five years or more is less than 2 % (Jemal et al., 2008, Li et al., 2004). Recent evidence linked elevated NFκB signalling with pancreatic cancer but very few studies have investigated the involvement of the non-canonical NFκB pathway. In this chapter the activation of this pathway was examined in selected pancreatic cancer cell lines.

Two pancreatic cancer cell lines, Panc-1 and MiaPaCa-2, were chosen for this study, each cell line was derived from a different cancer, initiated in a different area of the human pancreas. Panc-1 cells were isolated from the head of the pancreas (Liber et al., 1975), whilst Mia-Paca-2 cells were obtained from the body and tail of the pancreas (Yunis et al., 1977). Panc-1 cells have previously exhibted metastatic growth *in vivo*, whilst according to Deer and co-workers, MiaPaCa-2 cells lack this ability (Deer et al., 2010). Both cell lines have many types of mutation, for example KRAS (encoding KRAS gene), TP53 (encoding the p53 protein) and CDKN2A/p16 (encoding tumour suppressor gene p16) (Moore et al., 2001).

Previous studies have shown that the $LT\alpha_1\beta_2$ could activate the non-canonical NFκB pathway (Madge et al., 2008, Ganeff et al., 2011). This study confirmed that $LT\alpha_1\beta_2$ could induce both phosphorylation of p100 and the formation of the p52 subunit and its translocation into the nucleus of Panc-1 and MiaPaC-2 cells. This suggests that

irrespective of the status of the cells in terms of mutations the pathways can be strongly activated in pancreatic cancer cells. Other studies have also shown the phosphorylation and degradation of p100 in other cell types including HEK 923, and HeLa cells (Ganeff et al., 2011). However, to date, no equivalent studies have been performed in pancreatic cancer cell lines and this study is the first to investigate p100 processing in this disease model.

There were however a number of interesting features of activation. At 4h phosphorylation and loss of p100 correlated with p52 formation, whilst over longer periods of time formation of p52 was associated with an increase in p100 formation. Whilst phosphorylation of p100 was still observed at these later times it is possible that p52 formation may be partly a result of constitutive p100 degradation. This is similar to that observed in other cells types such as HUVECs mediated by such as TNFα (Ka Ho Ho, PhD, thesis). Interestingly whilst TNFα stimulated a strong activation of the classical NFκB pathway in Panc-1 suggesting good receptor engagement there was no activation of the non-canonical NFκB pathway. This finding is in contrast to studies in RIP-/- MEFs cells which, demonstrated stimulation of non-canonical NFκB signalling following exposure to TNFα (Kim et al., 2011). Additionally, Lotzer and co-workers demonstrated that TNFα could stimulate the phosphorylation of p100 in aorta smooth muscle cells (Lotzer et al., 2010). This suggests differences in the coupling of the TNF receptor to intermediates of the non-canonical NFκB pathway in pancreatic cancer cell lines relative to others.

The findings above may also suggest that the non-canonical NFκB pathway in different cell types is constructed differently. This is further supported by studies with FCS which induced a minor stimulation of p100 phosphorylation and processing of p100 in both cells lines. This is in contrast to other studies in Plevin laboratory, which demonstrate strongly activation of p100 phosphorylation and degradation p100 to p52 in U2OS cells (personal communication).

As expected TNFα administration resulted in activation of the classical NFκB pathway in Panc-1 cells, by inducing the degradation of IκBα and phosphorylation of p65 NFκB. This finding is consistent with many other studies which have demonstrated TNFα activation of the classical NFκB pathway in many types of cell line, such as hypopharyngeal cancer cells (Yu et al., 2014), MLE cells (Schwingshackl et al., 2013) and ASME cells (Seidel et al., 2009). Interestingly, $LT\alpha_1\beta_2$ activates the classical NFκB pathway in Panc-1 and MiaPaCa-2 cells, many studies found that activation of the LTβ receptor couples to the canonical NFκB pathway (Drayton et al., 2006, Muller and Siebenlist, 2003, Haybaeck et al., 2009, Madge et al., 2008). A key feature was the relative delay in degradation of IκBα, which was maximal at 60 minutes in contrast to stimulation with TNFα which virtually peaked at 5 minutes. This again alludes to differences in coupling specificity and the overlap in stimulation of both arms of the pathway by a given agonist.

The differences in the characteristics of activation may be related to upstream modulation of the pathways by NIK and TRAF 2 and 3. Previous studies in Panc-1 cell lines have shown high levels of NIK and low levels of TRAF2 (Doppler et al., 2013), overexpression of NIK could induce activation of the alternative NFκB pathway through inducing phosphorylation of IKKα. This suggests that in pancreas the non-canonical NFκB pathway is normally hyper-active, in contrast with other cancers cell lines, for example colon cancer cells (Mladenova et al., 2013) and LNCaP cells (Parrondo et al., 2010).

Attempts to recapitulate this finding were not successful; NIK levels were low in Panc-1 and MiaPaCa-2 cells, relative to a virally infected control. One issue may be the quality of the antibodies used in the study, non-specific binding was substantial and it was difficult to demonstrate clear binding to endogenous NIK. Attempts to immunoprecipitate endogenous NIK were also not successful. Similarly, we found no changes in TRAF2 and 3; the data was not good enough to make a clear conclusion regarding the activation of the pathway. However, it should be noted that basal activation of the non-canonical pathway in Panc-1 was low with good stimulation by $LT\alpha_1\beta_2$ and if the basic model of IKKα activation is consistent for Panc-1 an increase in NIK expression and changes in TRAF may have been expected. Trauzold and co-workers (2005), have previously demonstrated that TRAF2 was overexpressed in Panc-1 cell lines but NIK was not present (Trauzold et al., 2005) and our findings would be more in keeping with this position.

As mentioned in Chapter One IKKα and IKKβ have been identified as the main activators for the classical and non-canonical NFκB pathways, in order to confirm that in particular IKKα mediated the activation of the non-canonical NFκB pathway we used siRNA (RNA interference). Overall the results were disappointing; whilst there was a significant inhibition of both IKKα and IKKβ of almost 90% (the results was assessed by Western blot) over a range of concentrations between (50-100nM) (see Figure 3.18 and Figure 3.19) there was no inhibition of either NFκB pathway. There was a possibility that the Western blotting technique underestimated the level of knockdown, therefore, it was decided to use higher concentrations of siRNA was inhibition of the pathway possible. This concentration of siRNA is very high and it as unclear why the lower concentration of siRNA could effectively rundown the endogenous of IKKα without effect on activation of NFκB pathways. This result agreed with another study in the Boyd laboratory, the results showed that 25-100nM siRNA for both IKKα and IKKβ effectively knockdown of IKKs without impact on activation of NFκB pathways in PC3M cell line (Khalid personal communication). There are very few studies examine the effect of IKKα siRNA on the non-canonical NFκB pathway. Li and co-workers demonstrated there was a reduce level of p52 formation during knockdown of IKKα in both HeLa cells and macrophage cells (Li et al., 2010). At present it is unclear if there is some experimental design problem or alternately IKKs are not essential to drive the non-canonical NFκB pathway. Given the vast range in characteristics outlined above, particularly at late stage in the pathway, there may be potential for an IKKα independent regulation.

There are a number of genes which regulated by IKKβ via the classical NFκB pathway activation such as, c-MYC, JUNB, cyclin D1 cyclin D2 (Lee and Hung, 2008). However the results in this study showed there were no significant changes in accumulation of cells in each phase following transfection with IKKβ siRNA. Similarly, Yemelyanov and co-workers found that inhibition of IKKβ using PS-1145 inhibitor had no effect on cell cycle progression in DU145 cells (prostate cancer cell line) (Yemelyanov et al., 2006).

Furthermore, IKKα is also thought to be involved in the regulation of a number of cell cycle genes such as cyclin D1 and Aurora-A (Cao, et al, 2001, Demicco, et al, 2005, Prajapati, et al, 2006). Again this study identified that IKKα silencing had no effect on cell cycle progression in Panc-1 cells. Other studies have found that knockdown of IKKα did not change the cell cycle stages in different cancer cell lines such as breast and prostate cancer cells (Mahato et al., 2011, Merkhofer et al., 2010). In contrast, other studies have demonstrated that inhibition of the activity of IKKα leads to alterations in cell cycle distribution in pancreatic, prostate and breast cancer cell lines (Schneider et al., 2006, Tu et al., 2006, Shukla et al., 2015). This suggests that the role of IKKs in regulation of the cell cycle is dependent on tumour and cell type, further examination using different techniques to inhibit the activity of IKKs are required.

The effect of IKKs on cell growth was also examined using a clonogenic assay. The results indicated that there was no significant change in survival fraction compared

with agonist treated control. As Panc-1 cells are differentiated from many other cancer cell lines such as PC3M and U2OS cells, Panc-1 cells usually grown as double layer, it is possible that not all the cells were transfected with siRNA during the experiment preparation.

Chapter Four

Characterisation of the inhibitory effect of IKKα selective inhibitors (SU compounds) on Non-Canonical and Canonical NFκB pathways

4.1 Introduction

The transcription factor NFκB has been implicated as a tumour promoter in a numbers of cancers (Pahl, 1999, Hanahan and Weinberg, 2011). Increased levels of NFκB proteins have been demonstrated in breast cancer (Chua et al., 2007), lung cancer (Tew et al., 2008), leukaemia (Vilimas et al., 2007), lymphoma (Zou et al., 2007) and in pancreatic cancer cell lines (Doppler et al., 2013). Furthermore, many studies have linked NFκB activation and resistance to chemotherapy in cancer cell lines (Chuang et al., 2002). In addition, inhibition of NFκB pathways, or knockout of NFκB subunits resulted in impaired tumour cell growth (Li et al., 2005, Meylan et al., 2009, Baldwin, 2001, Yamamoto and Gaynor, 2001).

A regulatory protein considered central to NFκB activation is IKKα. It has a critical role in activation of both NFκB pathways, but particularly in the non-canonical NFκB pathway. Phosphorylation of IKKα through cell stimulation with specific agonists leads to phosphorylation and processing of p100 and as a consequence the regulation of a number of genes (Liang et al., 2006, Luftig et al., 2004, Muller and Siebenlist, 2003). Furthermore, IKKα has a role in the phosphorylation of p65 NFκB which is essential for activation of the canonical NFκB pathway (Solt et al., 2009). Many studies suggest that IKKα may act as a tumour suppressor, Park and co-workers found that a reduction in the expression of IKKα promoted the development of squamous cell carcinomas (Park et al., 2007). Another study found that IKKα has a role in the prevention of skin cancer through maintainance of skin homeostasis (Liu et al., 2008). Several additional studies have implicated IKKα in cancer

development, for example, increased levels of IKKα are associated with the development of non-melanoma (Alameda et al., 2011), squamous carcinomas (Moreno-Maldonado et al., 2008), colorectal cancer (Fernandez-Majada et al., 2007b) and cancers of the pancreas (Shiah et al., 2006), prostate (Jain et al., 2012) and liver (Jiang et al., 2010).

Recently, many studies have examined the effect of IKKβ inhibitors in pancreatic cancer cell lines. For example, treatment of pancreatic cells with TPCA-1, an IKKβ inhibitor, reduced proliferation and resistance to chemotherapy (Cataldi et al., 2015). The same effect has been observed with Apigenin (Wu et al., 2014). Additionally, Ochia *et al* demonstrated that another IKKβ inhibitor, IMD-0354, suppressed the proliferation of pancreatic cell lines (Ochiai et al., 2008). However, to date, no IKKα selective inhibitors have been examined in similar studies due to the lack of available compounds.

A number of first class IKKα selective inhibitors have been produced, by the medicinal chemistry group at the University of Strathclyde, Glasgow. Therefore, the aims of chapter four were to determine the potency and selectivity of these novel SU compounds as IKKα inhibitors in pancreatic cancer cell lines. This was achieved through the assessment of the major markers of the non-canonical NFκB pathway; the phosphorylation of p100, the processing of p100 and the formation of p52 and subsequent translocation to the nucleus. Furthermore, selectivity was tested using the degradation of IκBα and phosphorylation of p65 NFκB as markers of the canonical NFκB pathway. Experiments in chapter three demonstrated that the non-

canonical NFκB pathway could be activated using $LT\alpha_1\beta_2$, whilst TNFα have the ability to active the canonical NFκB pathway. The hypothesis of this chapter was that if SU compounds could be revealed as selective IKKα inhibitors then their actions as inhibitors of cell proliferation could also be examined.

4.2 Determining the dissociation constant (K_i value) of the SU compounds

The effects of a number of prototypic IKKα inhibitor compounds on IKKα/β kinase activities were examined in-house by Mrs. Louise Young using an *in vitro* kinase assay using purified IKKα and IKKβ derived from commercial sources. The kinase reaction utilised a biotinylated peptide substrate containing the serine 32 residue of IκB-α. The extent of phosphorylation was assessed by dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA).

Table 4.1 The K_i value for SU compounds

SU compound	K_i Value IKKα/IKKβ (nM)
SU1087	3/5
SU1261	10/680
SU1349	21/2445
SU1392	24/7190
SU1399	11.1/1205
SU1411	54/838
SU1432	23/3100
SU1433	11/2295
SU1434	81/5029
SU1436	35/130
SU1438	4.5/135
SU1489	3.5/45.7
SU1497	3.6/36
SU1499	0.9/65

4.3 The effect of SU compounds on non-canonical NFκB pathway in Panc-1 cells

4.3.1 The effects of the IKKα inhibitor SU1087 on p100 expression and p52 formation in Panc-1 cells

The effects of SU compounds on the IKKα-dependent non-canonical NFκB pathway were investigated; initially the effects of these compounds on $LT\alpha_1\beta_2$ induced formation of p52 in Panc-1 cells were examined. Results in Figures 3.2 and 3.4 showed that $LT\alpha_1\beta_2$ induced a significant increase in level of p52 formation after 24h of stimulation. It was therefore decided to use this time point to examine the effect of SU compounds. In general compounds were selected as they became available althought compounds represented different scaffold structures.

Figure 4.1 shows pre-treatment of Panc-1 cells with SU1087 (K_i 3/5nM) and effects upon p52 formation. The compound had a small effect on the basal level of p52 which was not significant compared with untreated sample (Fold stimulation relative to control; 0.65 ± 0.06, n=3). Furthermore, $LT\alpha_1\beta_2$ stimulated a significant increase in p52 formation as expected, this response was not affected by the presence of DMSO, (fold stimulation: $LT\alpha_1\beta_2$ = 2.88 ± 0.16, $LT\alpha_1\beta_2$ plus DMSO = 2.80 ± 0.28, n=3). However, following incubation with increasing concentrations of SU1087, a significant concentration-dependent decrease in p52 formation was observed between 1-30μM (% maximum response at 30μM = 0.27 ± 0.05, n=3 (figure 4.1B). Furthermore, a reduction in p100 expression was also observed following treatment with SU1087, there was an approximate 80% reduction in levels at 30μM (0.17 ± 0.023, relative to control) (Figure 4.1C). Treatment with SU1087 had no effect on

cellular levels of p65 NFκB, confirming that the compound had no general effect on cellular integrity.

4.3.2 The effects of the IKKα inhibitor SU1261 on p52 and p100 levels

Another compound, SU1261 identified to be more selective for IKKα (K_i 10/680nM) compared with SU1087, was also tested as shown in Figure 4.2. Again $LT\alpha_1\beta_2$ stimulated a significant increase (4.6 ± 0.09 fold) this was decreased significantly and in a concentration-dependent manner by SU1261 over the low micromolar range (3-30μM) (Figure 4.2A). Likewise, over the same concentration range SU1261, also significantly reduced p100 levels reaching maximum inhibition at 30μM (0.23 ± 0.02 relative to control) (Figure 4.2C). Treatment of cells with SU1261 alone exhibited no change in the basal levels of either p52 or p100.

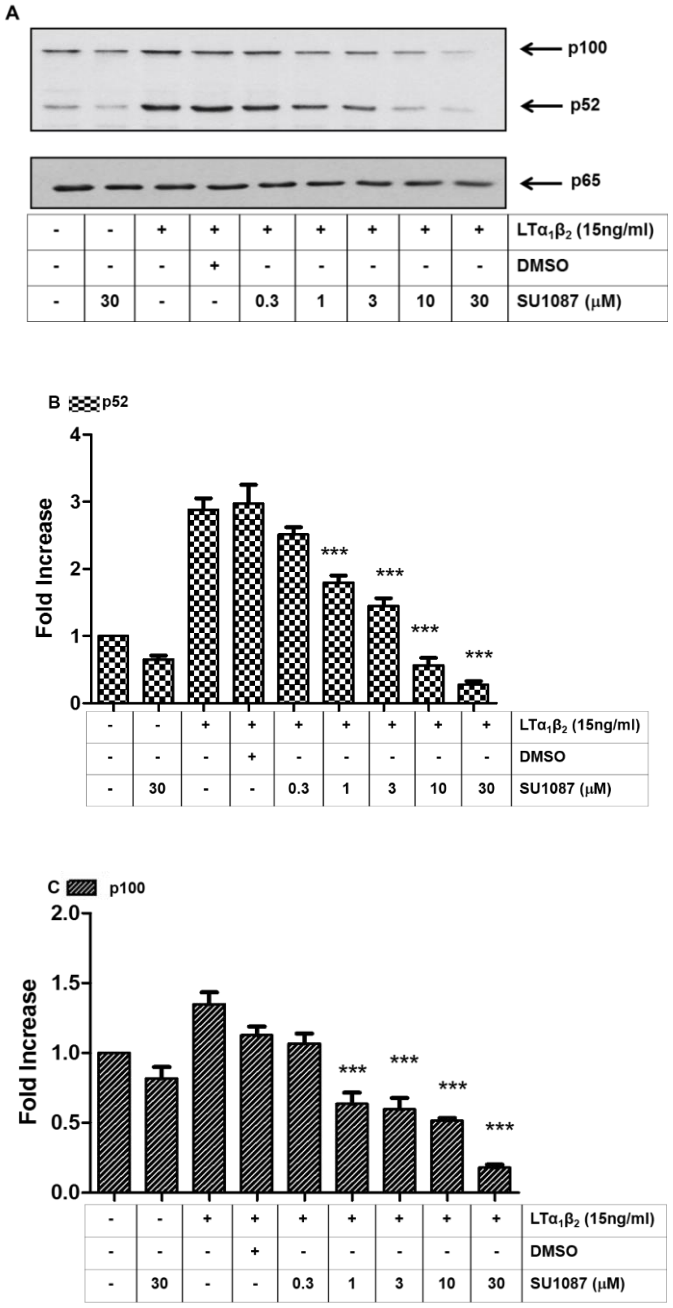


Figure 4.1 The effect of SU1087 on LTα$_1$β$_2$-induced p52 formation in Panc-1 cells

Panc-1 cells were pre-treated with increasing concentrations of SU1087 or 0.15% DMSO for 1h prior to stimulation with LTα$_1$β$_2$ (15ng/ml) for 24h. Whole cell extracts were prepared and proteins were analysis by Western blotting and assessed for p100 (100 kDa), p52 (52 kDa) and p65 NFκB (65 kDa) which was used as a loading control (A). Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p52 (panel B), p100 (panel C). Each value represents the mean ± S.E.M of three independent experiments, *** $p<0.001$ compared with agonist-stimulated control.

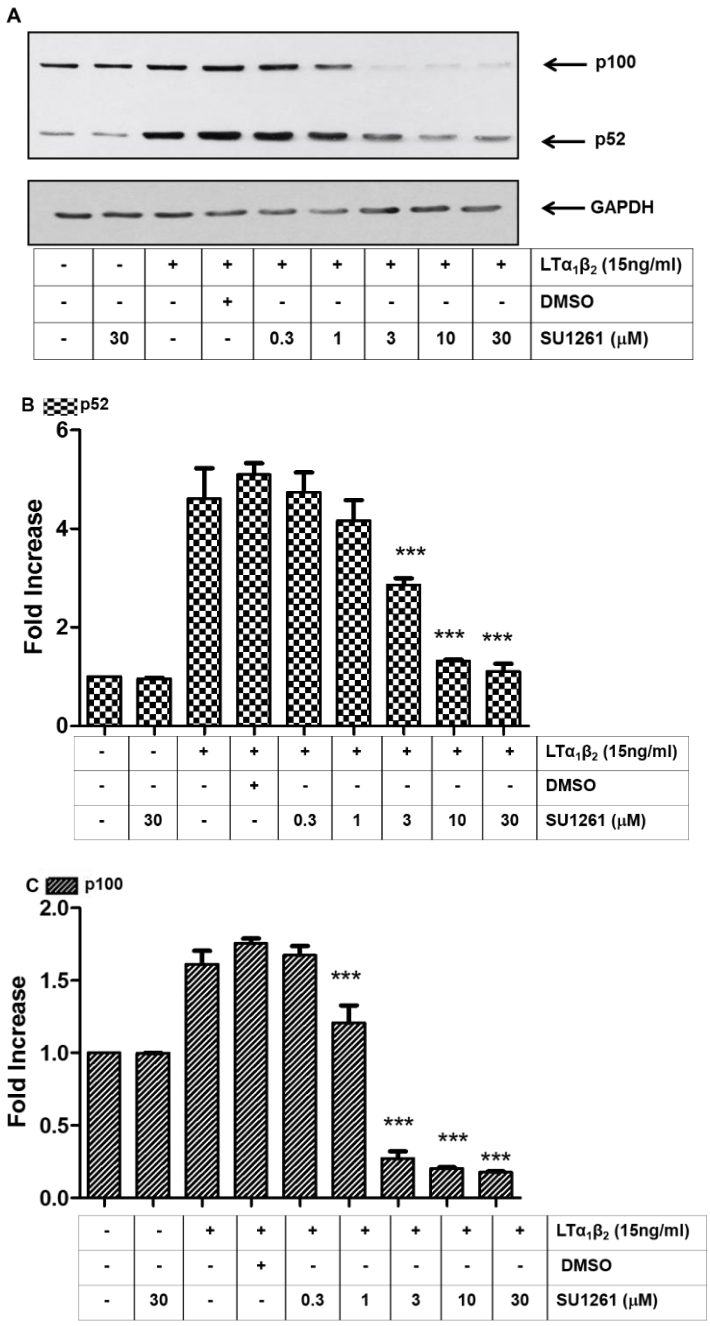


Figure 4.2 The effect of SU1261 on $LT\alpha_1\beta_2$-induced of p52 formation in Panc-1 cells

Panc-1 cells were pre-treated with increasing concentrations of SU1261 or 0.15% DMSO for 1h prior to stimulation with $LT\alpha_1\beta_2$ (15ng/ml) for 24h. Whole cell extracts were prepared and proteins were analysis by Western blotting and assessed for p100 (100 kDa), p52 (52 kDa) and GAPDH (37 kDa) which was used as a loading control panel A. Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p52: (panel B), p100 (panel C). Each value represents the mean ± S.E.M of three independent experiments, *** $p<0.001$ compared with agonist-stimulated control.

4.3.3 The effects of the IKKα inhibitor SU1411 on p52 and p100 levels

Another compound SU1411 (K_i 54/838nM), which was approximately 5 fold less potent than SU1261 in the kinase assay, was also tested as shown in Figure 4.3A. Pre-treatment of Panc-1 cells with 30μM of SU1411 alone had little effect on unstimulated levels of both p52 and p100 (Figure 4.3A). Following $LT\alpha_1\beta_2$ stimulation, the formation of p52 was significantly decreased by exposure to SU1411, but this effect was only observed following treatment with the highest concentrations, 10μM and 30μM (Figure 4.3, panel B). In addition, expression of p100 was decreased to 0.49 ± 0.37 and 0.39 ± 0.04 following treatment with 10μM and 30μM respectively, compared with agonist-stimulated control (fold stim 1.05 ± 0.045) (see Figure 4.3, panel C).

4.3.4 The effects of the IKKα inhibitor SU1432 on p52 and p100 levels

Another compound SU1432 (Ki 23/31000nM) was examined to study the effect of IKKα inhibitor on the p52 formation. The results has been showed there were inhibition of formation of p52 and expression of p100 and it was observed the highest concentration (10 and 30μM), as shown in Figure 4.4 (panels B and C).

4.3.5 The effects of the IKKα inhibitor SU1434 on p52 and p100 levels

Unlike the effects of SU compounds previously reported, SU1434 (K_i 81/5029nM) induced a significant decrease in the levels of p52 in unstimulated cells, compared to untreated controls. Furthermore, reduction in the expression of p100 was observed, p52 and p100 levels were significantly decreased following exposure to 10μM and 30μM SU1434 (Figure 4.5, panels B and C). Taken together, these experiments

showed a consistent pattern of results, IKKα inhibitors have the ability to block p100 expression and p52 formation in Panc-1 cells as well as MiaPaCa-2 cells (data not shown).

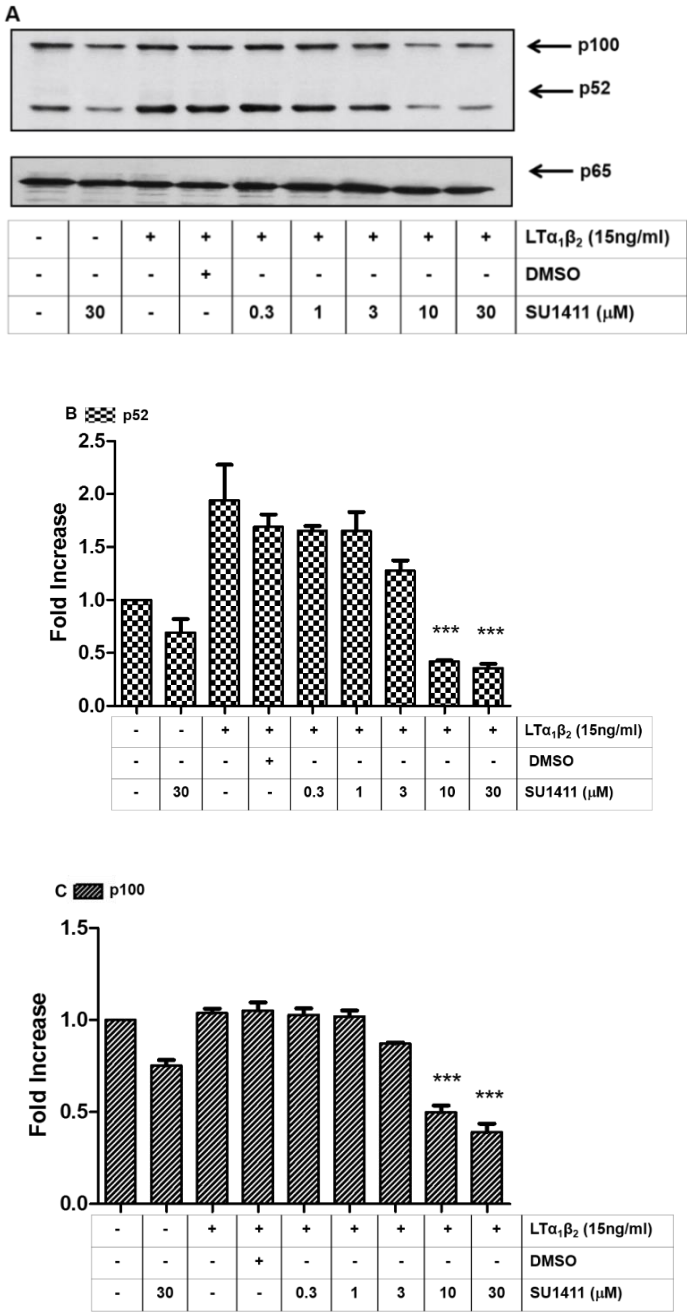


Figure 4.3 The effect of SU1411 upon LTα$_1$β$_2$- mediated p100 processing in Panc-1 cells

Panc-1 cells were pre-treated with treated with increasing concentrations of SU1411 or 0.15% DMSO for 1h prior to stimulated with LTα$_1$β$_2$ (15ng/ml) for 24h. Whole cell extracts were prepared and then separated by SDS-PAGE and assessed for p100 (100 kDa), p52 (52 kDa) and p65 NFκB (65 kDa) which was used as a loading control panel A. Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p52 (panel B), p100 (panel C). Each value represents the mean ± S.E.M of three independent experiments, *** $p<0.001$ compared with agonist-stimulated control.

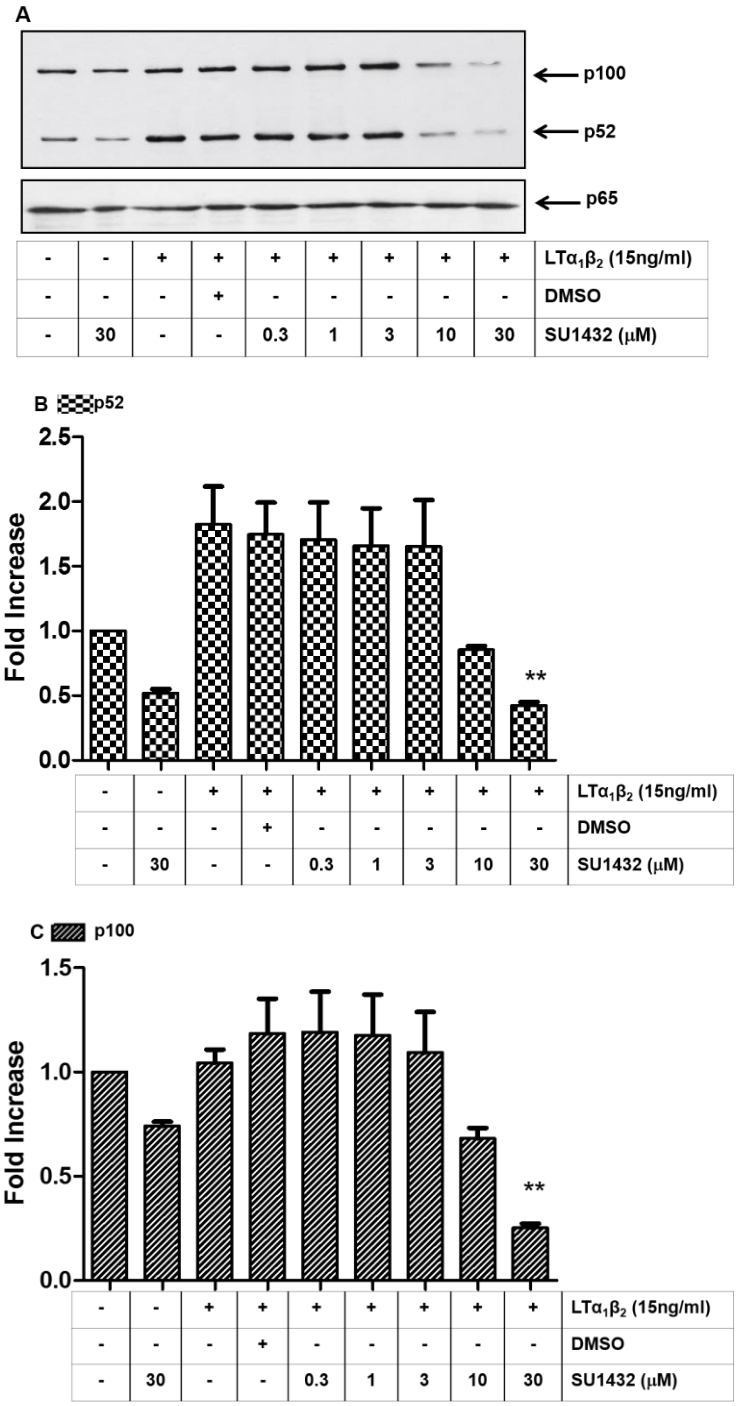


Figure 4.4 The effect of SU1432 upon LTα$_1$β$_2$-mediated p100 processing in Panc-1 cells

Panc-1 cells were pre-treated with treated with increasing concentrations of SU1432 or 0.15% DMSO for 1h prior to stimulated with LTα$_1$β$_2$ (15ng/ml) for 24h. Whole cell extracts were prepared and then separated by SDS-PAGE and assessed for p100 (100 kDa), p52 (52 kDa) and p65 NFκB (65 kDa) which was used as a loading control panel A. Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p52 (panel B), p100 (panel C). Each value represents the mean ± S.E.M of three independent experiments, *** $p<0.001$ compared with agonist-stimulated control.

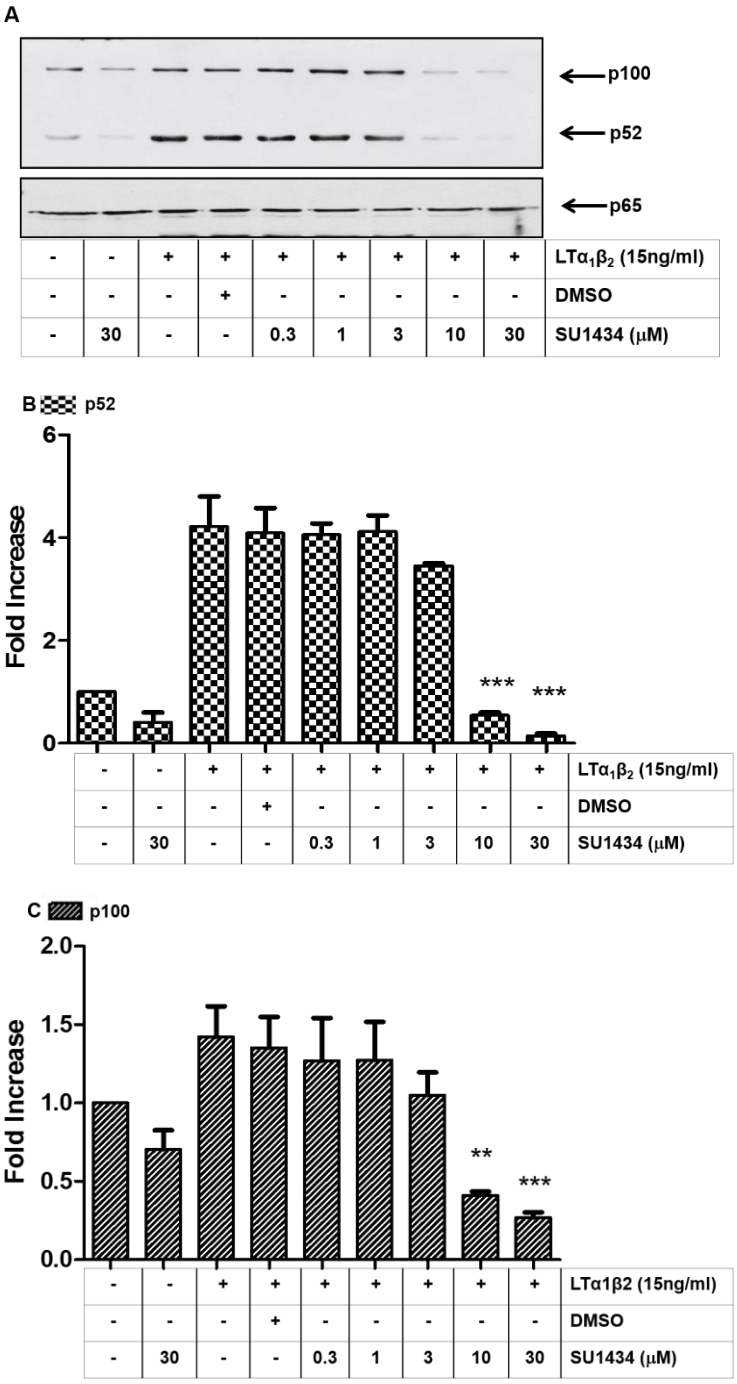


Figure 4.5 The effect of SU1434 upon LT$\alpha_1\beta_2$-mediated p100 processing in Panc-1 cells

Panc-1 cells were pre-treated with treated with increasing concentrations of SU1434 or 0.15% DMSO for 1h prior to stimulated with LT$\alpha_1\beta_2$ (15ng/ml) for 24h. Whole cell extracts were prepared and then separated by SDS-PAGE and assessed for p100 (100 kDa), p52 (52 kDa) and p65 NFκB (65 kDa) which was used as a loading control panel A. Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p52 (panel B), p100 (panel C). Each value represents the mean ± S.E.M of three independent experiments, *** $p<0.001$ compared with agonist-stimulated control.

4.4 Effect of SU compounds on $LT\alpha_1\beta_2$-induced nuclear translocation of p52 in Panc-1 cells

The results shown in Figure 4.1 to 4.5 demonstrated that SU compounds could inhibit the formation of p52 in whole cell extracts. However this does not necessarily indicate the effect of exposure to SU compounds on p52 mediated gene expression, thus nuclear translocation of p52 was assessed to determine whether the nuclear form of p52 would be also inhibited. SU1261 and SU1433 were used as a representative compound. The results in Figure 4.6 demonstrated that translocation of p52 into the nucleus was substantially increased following $LT\alpha_1\beta_2$ stimulation for 24 h. This response was substantially reduced following exposure to 3μM, 10μM and 30μM of SU1261 and 1μM, 3μM and 10μM SU1433.

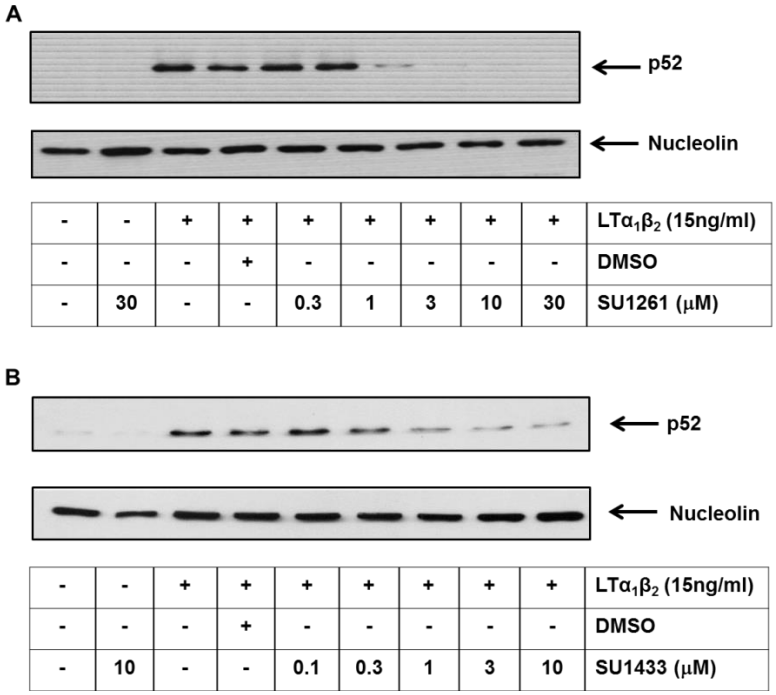


Figure 4.6 The effect of SU1261 and SU1433 upon $LT\alpha_1\beta_2$ induced translocation p52 in Panc-1 cells

Panc-1 cells were pre-treated with SU1261 or 0.15% DMSO panel A or SU1433 or 0.05% DMSO panel B for 1h prior to stimulated with $LT\alpha_1\beta_2$ (15ng/ml) for 24h. Nuclear extracts were prepared as outlined in section 2.4. The samples were separated by SDS-PAGE and assessed for p52 (52 kDa) and nucleolin (100 kDa) which was used as loading. The results are representative two independent experiments.

4.5 The effect of SU compounds on $LT\alpha_1\beta_2$-induced phosphorylation of p100 in Panc-1 cells

As previously discussed in Chapter Three, $LT\alpha_1\beta_2$ can also induce the phosphorylation of p100 in Panc-1 cells (Figure 3.1.). The results in Figures 4.1 to 4.6 demonstrated that SU compounds could inhibit the formation of p52 and nuclear translocation of p52 in Panc-1 cells. Therefore, the effects of exposure to SU compounds on the phosphorylation of p100 were also tested below using a 4h stimulation period, previously shown to be the maximal response to $LT\alpha_1\beta_2$.

4.5.1 Effects of IKKα inhibitors SU1087 and SU1261 on $LT\alpha_1\beta_2$-induced phosphorylation of p100 in Panc-1 cells

The results in Figure 4.7 and 4.8 show the effect of both SU1087 and SU1261 on the phosphorylation of p100. $LT\alpha_1\beta_2$ stimulated a marked increase in p-p100 levels (35.47± 2.6 fold stim, n=3), which was reduced by approximately 25% following pre-treated with DMSO (28.45 ± 2.78). Nevertheless, SU1087 was found to be a potent inhibitor of phosphorylation p100, as little as 0.3μM reduced phosphorylation levels from 28.45 ± 2.78 to 9.4 ± 1.78 fold whilst at 30μM stimulation was reduced further to 1.7 ± 0.51 fold, approximately equivalent to 95% inhibition (Figure 4.7). Likewise, pre-treatment with SU1261 also resulted in a significant inhibition of p100 phosphorylation which was observed at the lowest concentration used, 0.3μM. Over 85% inhibition was achieved at 1μM of SU1261 (7.23 ± 2.08 fold) (see Figure 4.8).

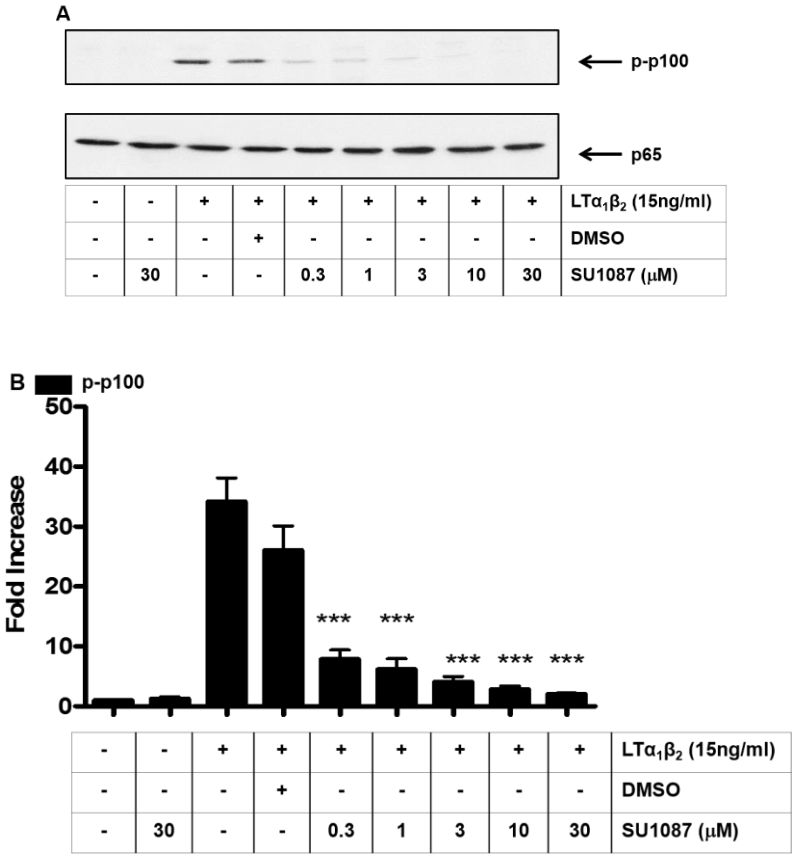


Figure 4.7 The effect of SU1087 on LT$\alpha_1\beta_2$-induced phosphorylation of p100 in Panc-1 cells

Panc-1 cells were pre-treated with increasing concentrations of SU1087 or 0.15% DMSO for 1h prior to stimulation with LT$\alpha_1\beta_2$ (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by SDS-PAGE and assessed for p-p100 (100 kDa) and p65 NFκB (65 kDa) which was used as a loading control panel A. In panel B blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p- p100. Each value represents the mean ± S.E.M of three independent experiments, *** $p<0.001$ compared with agonist-stimulated control.

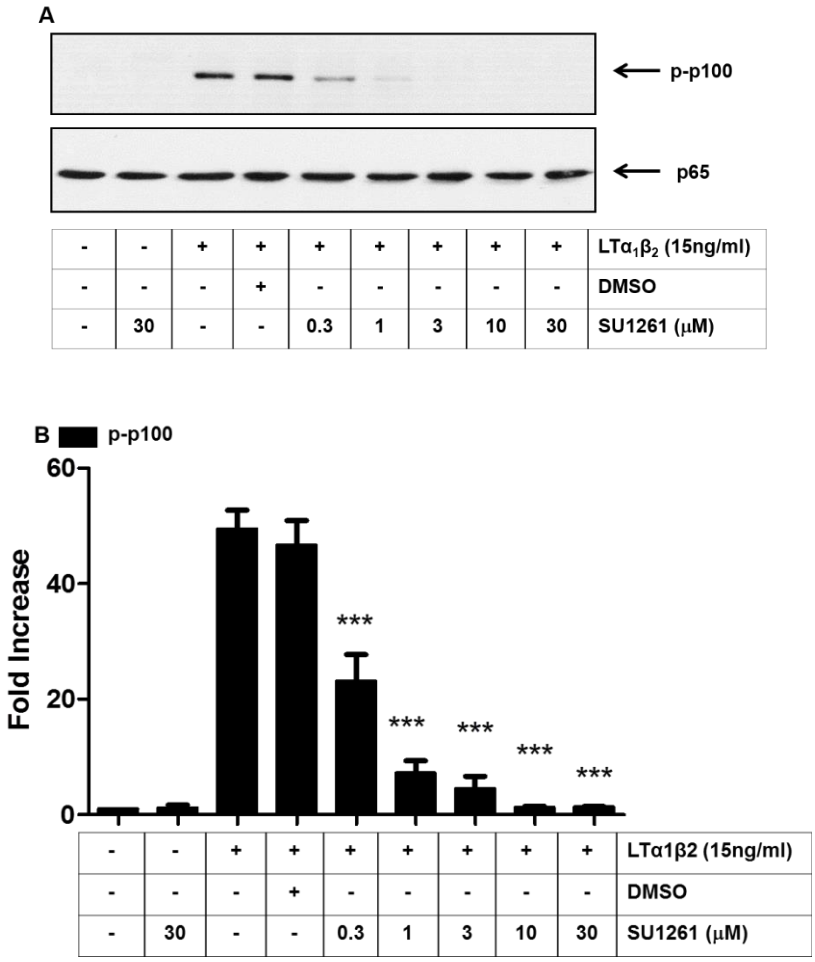


Figure 4.8 The effect of SU1261 on LT$\alpha_1\beta_2$-induced phosphorylation of p100 in Panc-1 cells

Panc-1 cells were pre-treated with increasing concentration of SU1261 or 0.15% DMSO for 1h prior to stimulation with LT$\alpha_1\beta_2$ (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by SDS-PAGE and assessed for p-p100 (100 kDa) and p65 NFκB (65 kDa) which was used as a loading control panel A. in panel B blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p- p100. Each value represents the mean ± S.E.M of three independent experiments, *** $p<0.001$ compared with agonist-stimulated control.

4.5.2 The effects of the IKKα inhibitor SU1438 on LT$\alpha_1\beta_2$-induced phosphorylation of p100 in Panc-1 cells

Furthermore, in LT$\alpha_1\beta_2$-stimulated Panc-1 cells, SU1438 (K_i 4.5/135nM) exposure (Figure 4.9) induced significant inhibition of p100 phosphorylation from 1-30 μM in a concentration-dependent manner, the maximum inhibition observed at 30μM (1.64 ± 0.53 fold) compared to 33.52 ± 2.15 in agonist-stimulated control.

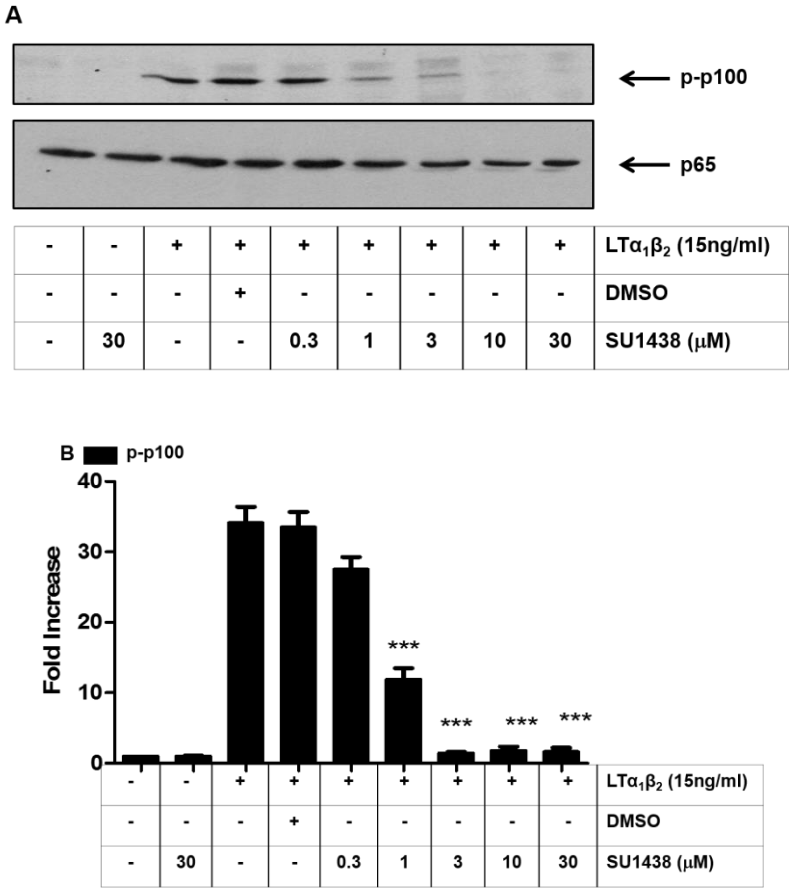


Figure 4.9 The effect of SU1438 on LTα$_1$β2 induced phosphorylation of p100 in Panc-1 cells

Panc-1 cells were pre-treated with increasing concentrations of SU1438 or 0.15% DMSO for 1h prior to stimulation with LT$\alpha_1\beta_2$ (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by SDS-PAGE and assessed for p-p100 (100 kDa) and p65 NFκB (65 kDa) which was used as a loading control (A). In panel B blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p-p100. Each value represents the mean ± S.E.M of three independent experiments, *** $p<0.001$ compared with agonist-stimulated control.

4.5.3 The effects of the IKKα inhibitors SU1349 and SU1433 on phosphorylation of p100 and p52 formation in Panc-1 cells

The results in Figure 4.7 to 4.9 showed there was a significant reduction in level of phosphorylation of p100 from the lowest concentration used (0.3μM). It was decided to develop a fuller concentrations curve and to re-assess the effects upon p52 formation and p100 levels at the 4h time point. This was exemplified using SU1349 and SU1433 compounds that were readily available. Both Figures 4.10 and 4.11 show that whilst in response to $LT\alpha_1\beta_2$ shorter time sitmulation at 4 h, there was increased levels of p-p100 as expected, interestingly there was an increase in p52 formation which was acompanied by a loss in p100 experssion. Figure 4.10 demonstrates that increasing concentrations of SU1349 (0.1-10μM) significantly reduced the level of p100 over all concentrations that were used in the experiment (see Figure 4.10 panel B). Furthermore, formation of p52 was also significantly inhibited by SU1349 and the loss of p100 also reversed. A very similar result was observed for SU1433 (see Figure 4.11).

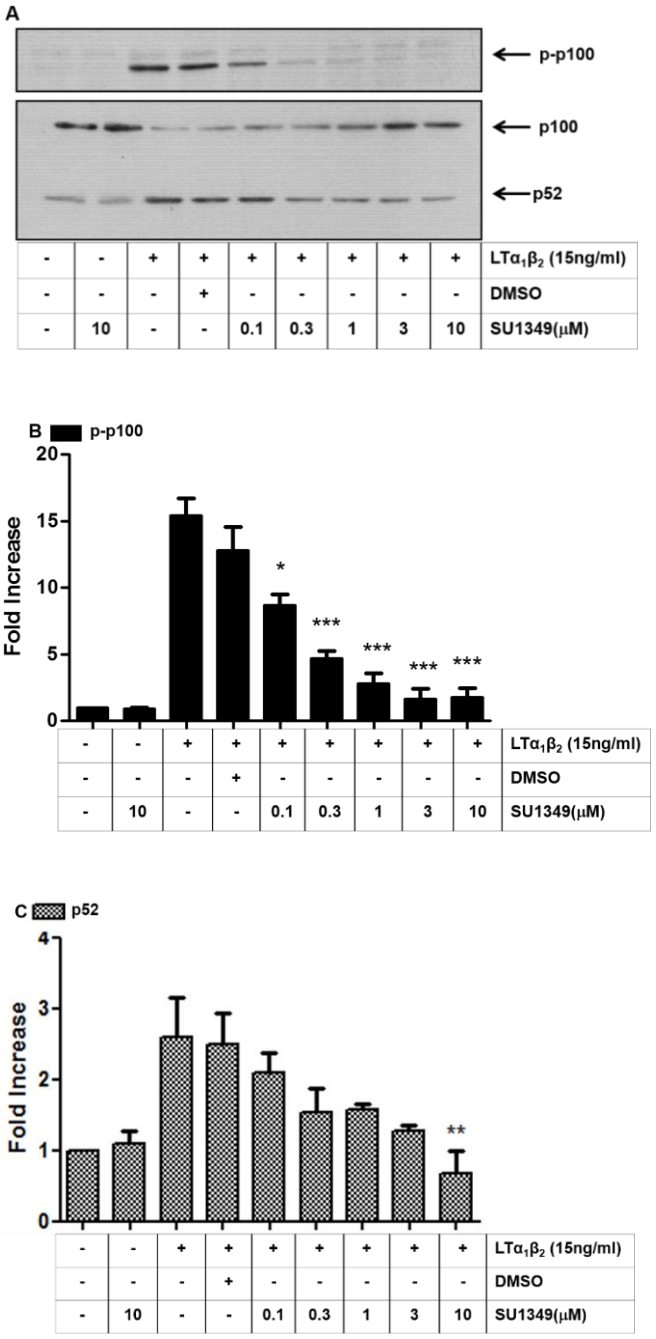


Figure 4.10 The effect of SU1349 on LT$\alpha_1\beta_2$-induced phosphorylation of p100 and formation of p52 in Panc-1 cells

Panc-1 cells were pre-treated with increasing concentrations of SU1349 or 0.05% DMSO for 1h prior to stimulation with LT$\alpha_1\beta_2$ (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by SDS-PAGE and assessed for p-p100 (100 kDa) and p52 (52 kDa) panel A. Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p-p100 (panel B) and p52 (panel C). Each value represents the mean ± S.E.M of three independent experiments, *** $p<0.001$ compared with agonist-stimulated control.

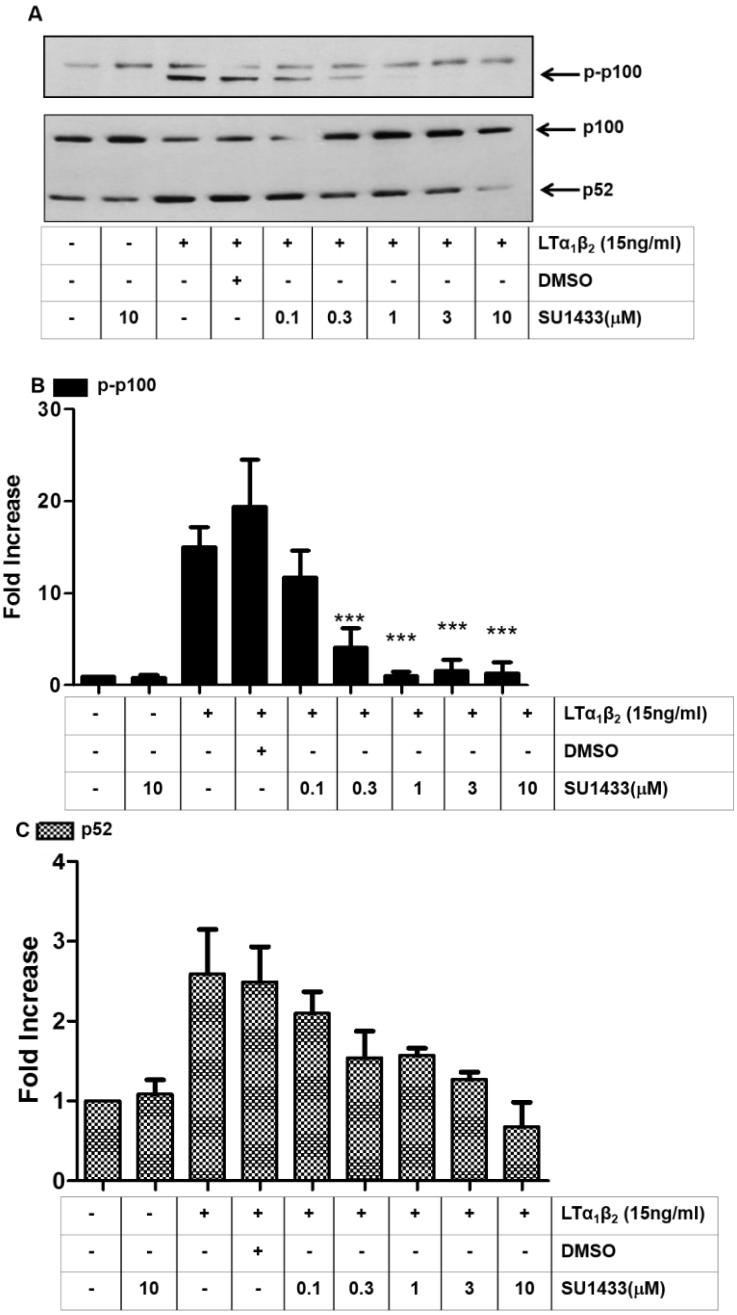


Figure 4.11 The effect of SU1433 on LTα₁β2 induced phosphorylation of p100 and formation of p52 in Panc-1 cells

Panc-1 cells were pre-treated with increasing concentration of SU1433 or 0.05% DMSO for 1h prior to stimulation with $LT\alpha_1\beta_2$ (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by SDS-PAGE and assessed for p-p100 (100 kDa) and p52 (52 kDa) (panel A). Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p-p100 (panel B) and p52 (panel C). Each value represents the mean ± S.E.M of three independent experiments, *** $p<0.001$ compared with agonist-stimulated control.

4.6 The effect of SU compounds on the canonical NFκB pathway in Panc-1 cells

4.6.1 The effects of SU compounds on TNFα-induced IκBα degradation and phosphorylation of p65 NFκB in Panc-1 cells

In order to determine whether the SU compounds were selective for IKKα their effects were assessed against TNFα-stimulated the IκBα degradation and phosphorylation of p65 NFκB as markers of the canonical NFκB pathway. SU1087 inhibited both TNFα-stimulated IκBα degradation and p65 phosphorylation in a concentration-dependent manner (Figure 4.12). The levels of IκBα degradation retuned to basal levels with increasing concentrations of SU1087 (40.58 % ± 4.3%, 87.08 ± 7.2%, 100 ± 1.14% and 101 ±0.54%, following exposure to 1μM, 3μM, 10μM and 30μM respectively, compared with agonist-stimulated control and the phosphorylation of p65 NFκB was also inhibited (fold stim was 4.84 ± 0.043, 1.75± 0.046, 1.89 ± 0.56 and 0.99 ± 0.018 following exposure to 1μM, 3μM, 10μM and 30μM respectively, compared to TNFα-stimulated control (14.93 ± 0.03). These observations suggest that SU1087 could inhibit both the canonical and non-canonical NFκB pathway and are consistent with the lack of selectivity at the level of kinase activity.

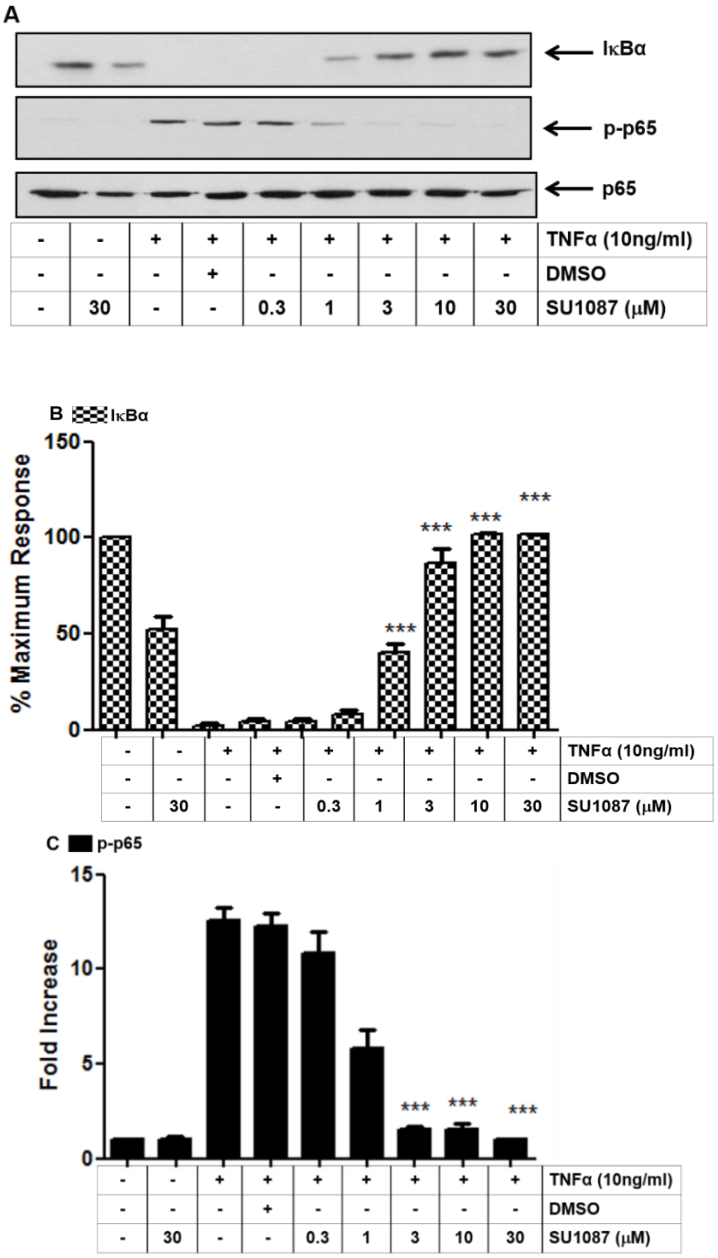


Figure 4.12 Effect of SU1087 on TNFα-mediated IκBα degradation and phosphorylation of p65 NFκB in Panc-1 cells

Cells were pre-treated with SU1087 and 0.15% DMSO for 1h before stimulation with TNFα for 15 min. Whole cells extracts were prepared, and then blotted for IκBα (37 kDa) (B), p-p65 NFκB (65 kDa) (C) and p65 NFκB (65 kDa) which was used as a loading control. Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for IκBα (panel B) and p-p65 NFκB (panel C). Each value represents the mean ± S.E.M of three independent experiments, *** $p<0.001$ compared with agonist-stimulated control.

A number of other SU compounds were examined. Exposure of Panc-1 cells to SU1432 (Figure 4.13A) and SU1436 (Figure 4.13B) induced partial inhibition of phosphorylation of p65 NFκB and IκBα degradation however, these responses were only observed following pre-treatment with the highest concentrations of compound, 10 μM and 30 μM.

The results shown in Figures 4.14 demonstrate that of the SU compounds investigated, namely SU1349, SU1261, SU1433 had no effect on either IκBα loss or phosphorylation of p65 NFκB confirming their selectivity as IKKα inhibitors at this level.

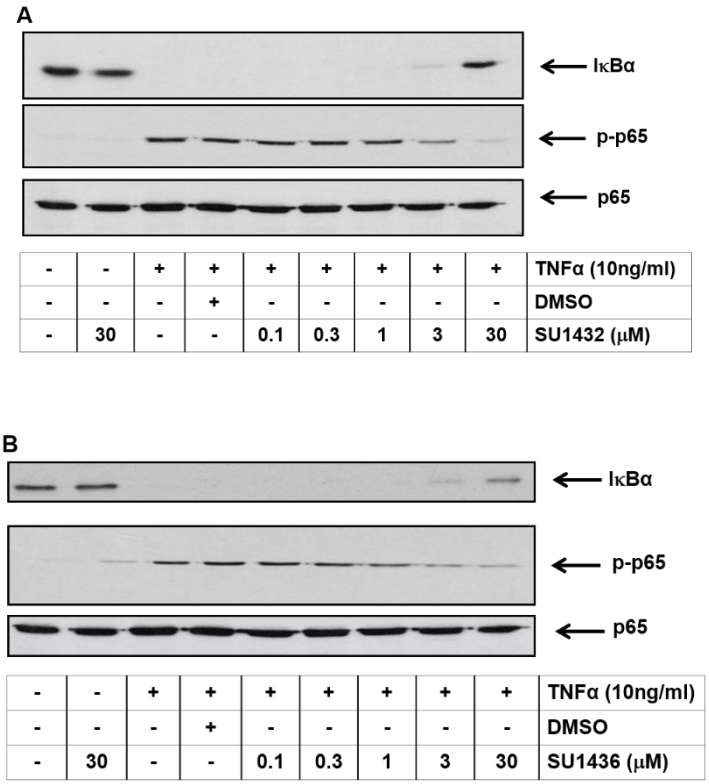


Figure 4.13 The effects of SU1436 andSU1432 on TNFα-mediated IκBα degradation and phosphorylation of p65 NFκB in Panc-1 cells

Cells were pre-treated with SU1432 (panel A) and SU1436 (panel B) or 0.15% DMSO for 1h before stimulation with TNFα for 15 min. Whole cells extracts were prepared, and then blotted for IκBα (37 kDa), p-p65 NFκB (65 kDa) and p65 NFκB (65 kDa) which was used as a loading control. Each blot is representative of three experiments.

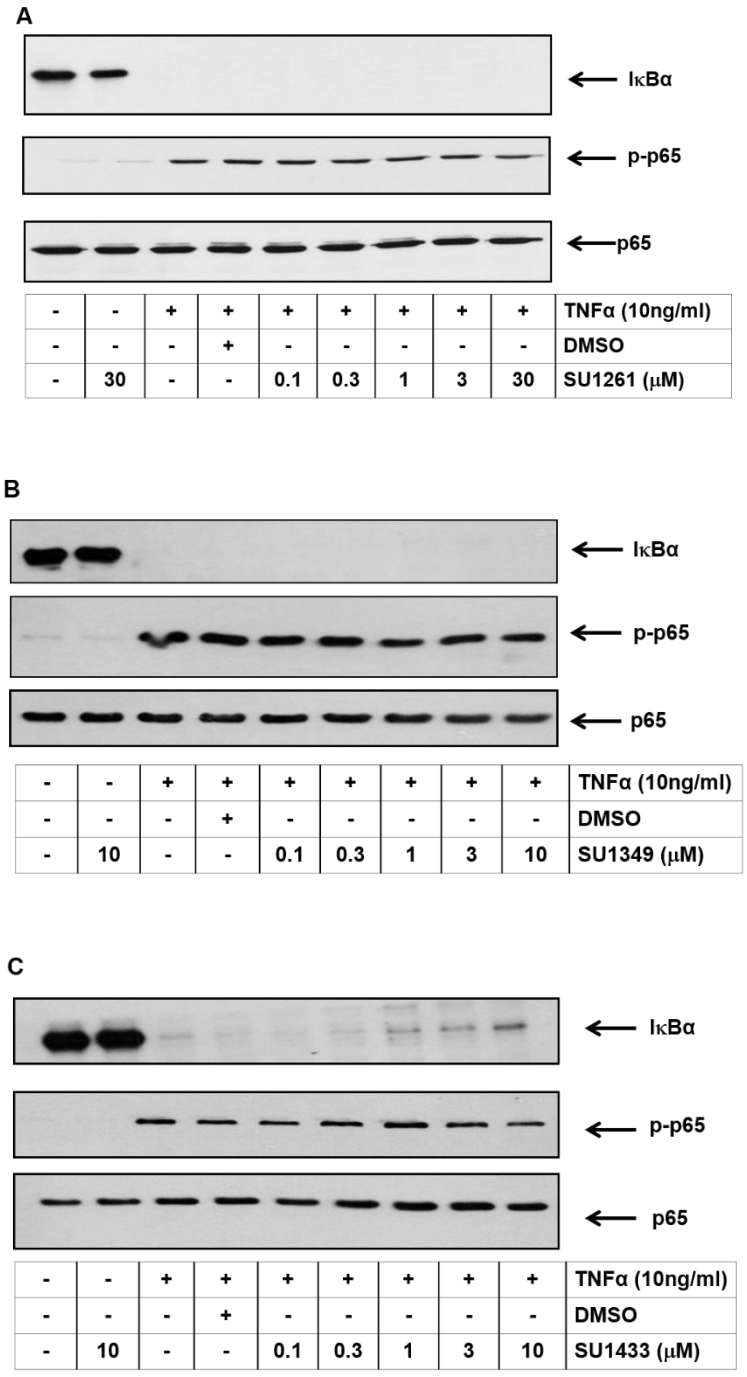


Figure 4.14 The Effects of SU1261, SU1349 and SU1433 on TNFα-mediated IκBα degradation and phosphorylation of p65 NFκB in Panc-1 cells

Cells were pre-treated with SU1261 or 0.15% DMSO (panel A), SU1349 or 0.05% DMSO (Panel B) and SU1433 or 0.5% DMSO (panel C) for 1h before stimulation with TNFα for 15 min. Whole cells extracts were prepared, and then blotted for IκBα (37 kDa), p-p65 NFκB (65 kDa) and p65 NFκB (65 kDa) which was used as a loading control. Each blot is representative of three experiments.

4.6.2 The effects of SU compound on LTα$_1$β$_2$-induced IκBα degradation and phosphorylation of p65 NFκB in Panc-1 cells

Chapter Three demonstrated that LTα$_1$β$_2$ could activate both canonical and non-canonical NFκB pathways. In order to confirm selectivity further the effects of two SU compounds, SU1087 and SU1261, on LTα$_1$β$_2$-induced IκBα degradation and phosphorylation of p65 NFκB were examined (Figure 4.15A). The results showed that following SU1087 exposure there was a concentration-dependent inhibition of p65 phosphorylation and reversal of IκBα degradation. However, following treatment with either SU1261, no effect on degradation of IκBα or phosphorylation of p65 NFκB was observed in Panc-1 cells (Figures 4.15B). This further confirmed the selectivity profile of SU1261 against LTα$_1$β$_2$.

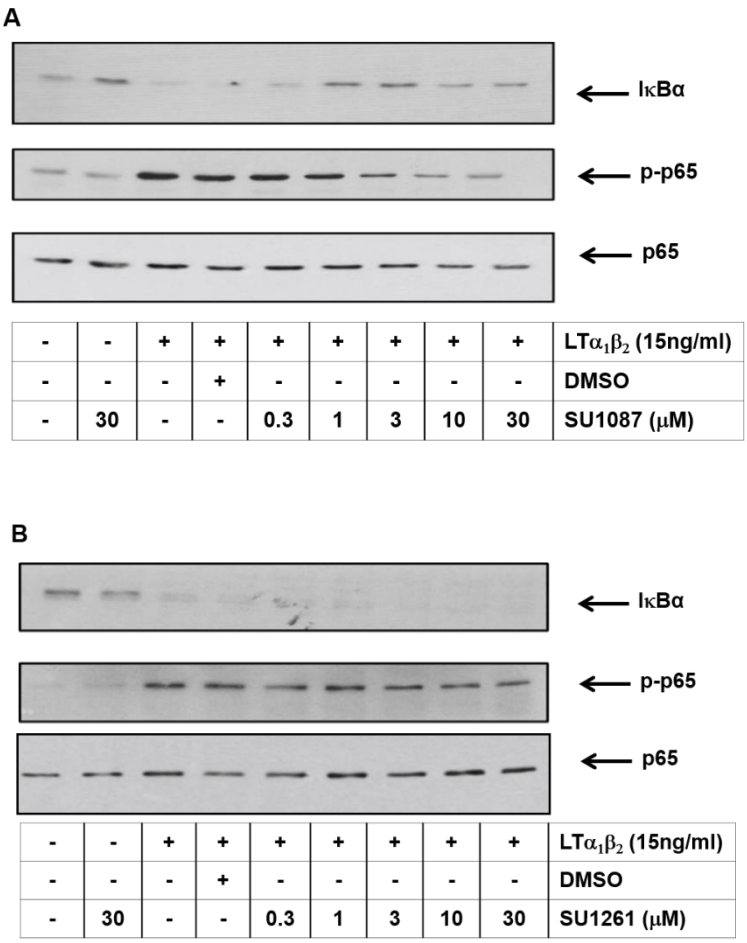


Figure 4.15 The effect of SU1087 and SU1261 on $LT\alpha_1\beta_2$-mediated IκBα degradation and phosphorylation of p65 NFκB in Panc-1 cells

Cells were pre-treated with SU1087 (panel A), SU12161 (panel B) or 0.15 %DMSO for 1h before stimulation with $LT\alpha_1\beta_2$ for 1h. Whole cells extracts were prepared, and then blotted for IκBα (37 kDa), p-p65 NFκB (65 kDa) and p65 NFκB (65 kDa) which was used as a loading control. Each blot is representative two experiments.

4.7. IC_{50} values for SU compounds against $LT\alpha_1\beta_2$-induced p52 formation, p100 expression and phosphorylation of p100

Next, IC_{50} values for the SU compounds were calculated and directly compared, through analysis of the Western blotting data and fitting to a sigmoidal concentration response curve using GraphPad Prism software (version 5), see Table 4.2. The IC_{50} values for p52 formation and p100 expression were determined after stimulation with $LT\alpha_1\beta_2$ (15ng/ml) for 24 h (Figure 4.16). There are some caveats, on occasions there were not enough points in the dynamic part of the curve and the range of compound tested was not always optimal. Nevertheless, there were two SU compounds that showed IC_{50} for both p52 and p100 less than 1μM; SU1433 and SU1349. All the other compounds were less potent with between values 1 and 10μM. In addition, for 2 compounds (SU1349 and in particular SU1261) there seemed to be some dissociation between inhibition of p100 and p52, see Figure 4.16.

The IC_{50} values for the inhibition of the phosphorylation of p100 were also calculated as outlined previously (Figure 4.17). Again the curve fitting procedure was not optimal there were not enough points to cover the full curve and these can only be estimations. However what can be ascertained is that in general the IC_{50} values for p-p100 at 4 h were of an order of magnitude less than the IC_{50} values estimated for the inhibition of p52 formation at 4 h. The IC_{50} of SU1349 and SU1433 for p52 formation at 4 h stimulation, p100 expression and phosphorylation of p100 were less than 1μM in comparison with other SU compounds that suggest these compounds are most signifcant compared with other SU compounds.

Table 4.2 IC_{50} values SU compounds against both non-canonical and canonical NFκB pathways intermediates

SU compounds	p100 (at 24 h)	p52 (at 24 h)	p-p100 (at 4 h)	p-p65 NFκB (at 30 min)	Reversal of IκBα (at 30 min)
SU1087	5.47 ± 2.31 μM	p52 2.08 ± 0.22 μM	> 0.3 μM	0. 83 ± 0.03 μM	0. 83 ± 0.03 μM
SU1261	1.22 ± 0.11 μM)	3.08 ± 0.25 μM)	0.75 ± 0.25μM	> 30 μM	> 30 μM
SU1349	0.29 ± 0.06μM	0.48 ± 0.15μM	0.24 ± 0.01μM	> 10 μM	> 10 μM
SU1399	Between 3-10 μM (N=1)	Between 3-10 μM (N=1)	Between 3-10 μM N=3	Between 3-10 μM (N=2)	Between 3-10 μM (N=2)
SU1411	5.09 ±0.09μM)	3.88 ± 0.27 μM	0.85 ± 0.16μM	> 30 μM	> 30 μM
SU1432	9.8 ± 1.13 μM	10.67 ± 1.01 μM	Between 3-10 μM N=2	Between 10-30 μM (N=3)	Between 10-30 μM (N=3)
SU1433	0.65 ± 0.15μM)	0.45 ± 0.11μM	0.39 ± 0.08μM	> 10 μM (N=3)	> 10 μM (N=3)
SU1434	5.66 ± 1.28 μM	5.19 ± 0.51 μM	Between 0.3-1.0 μM (N=1)	> 10 μM (N=3)	> 10 μM (N=3)
SU1436	58.25 ± 15.25	14.25 ± 2.75 μM		7.1 ± 0. 3 μM (N=3)	> 10 μM (N=3)
SU1438	Between 0.3-1 μM (N=1)	Between 0.3-1 μM (N=1)	0.94 ± 0.01μM)	> 10 μM (N=1)	> 10 μM (N=1)
SU1497	Between 3-10μM	Between 3-10μM	Between 3-10 μM (N=1)	Between 3-10 μM (N=1)	Between 3-10 μM (N=1)
SU1499	Between 1-3 μM (N=3)	Between 1-3 μM (N=3)	Between 0.3-1 μM (N=3)	Between 0.3-3 μM (N=3)	Between 0.3-3 μM (N=3)

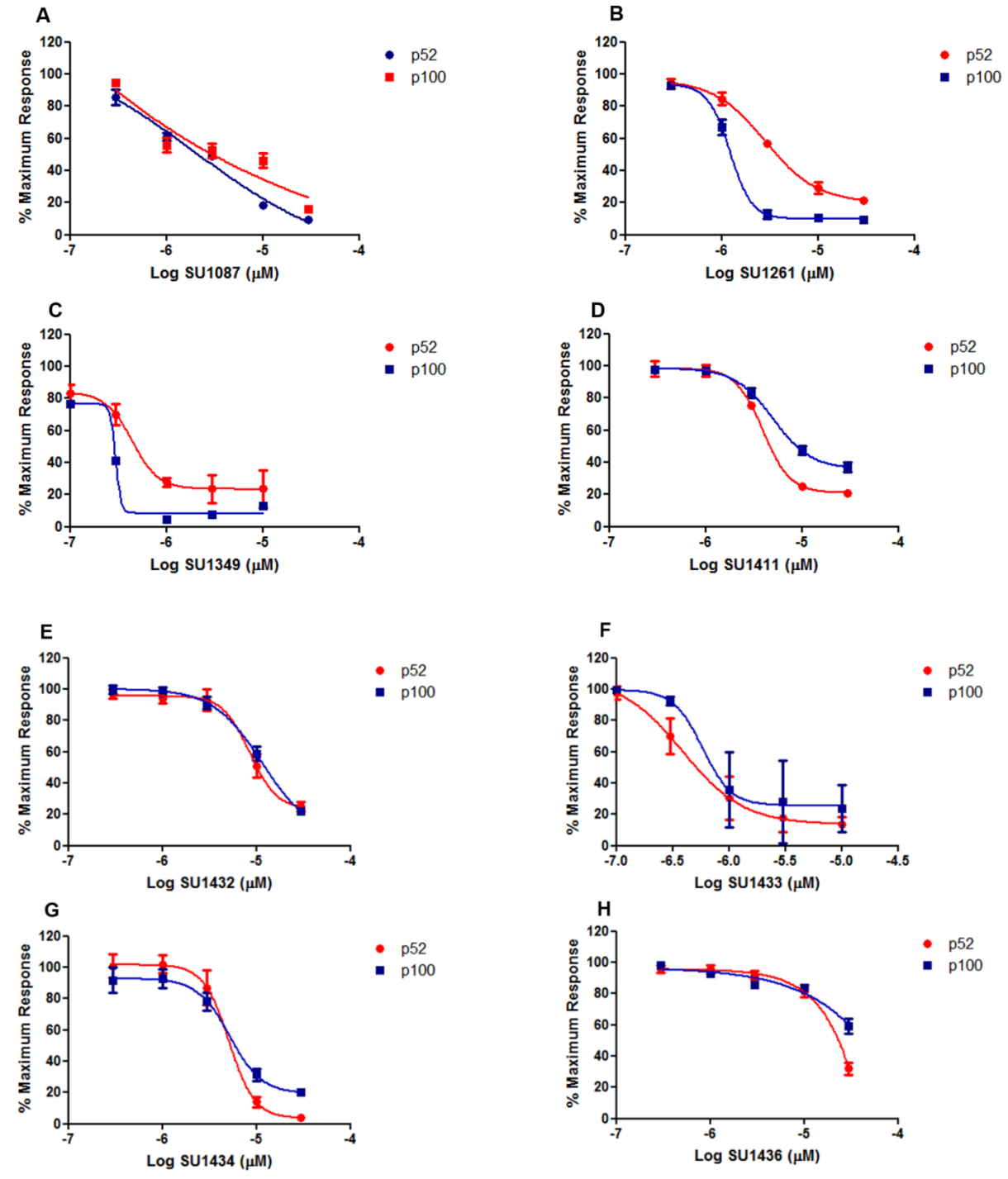


Figure 4.16 The IC_{50} values of SU compounds aganist p100 expression and p52 formation following $LT\alpha_1\beta_2$ stimulation of Panc-1 cells

The data obtained from Western blotting of SU compounds. Results of IC_{50} of p100 expression and p52 formation were semi-quantified by densitometric analysis then data was fitted to a sigmoidal dose response curve. Each value represented the mean ± SEM of three independent experiments for (A) SU1087, (B) SU1261, (C) SU1349, (D) SU1411, (E) SU1432, (F) SU1433, (G) SU1434 and (H) SU1436.

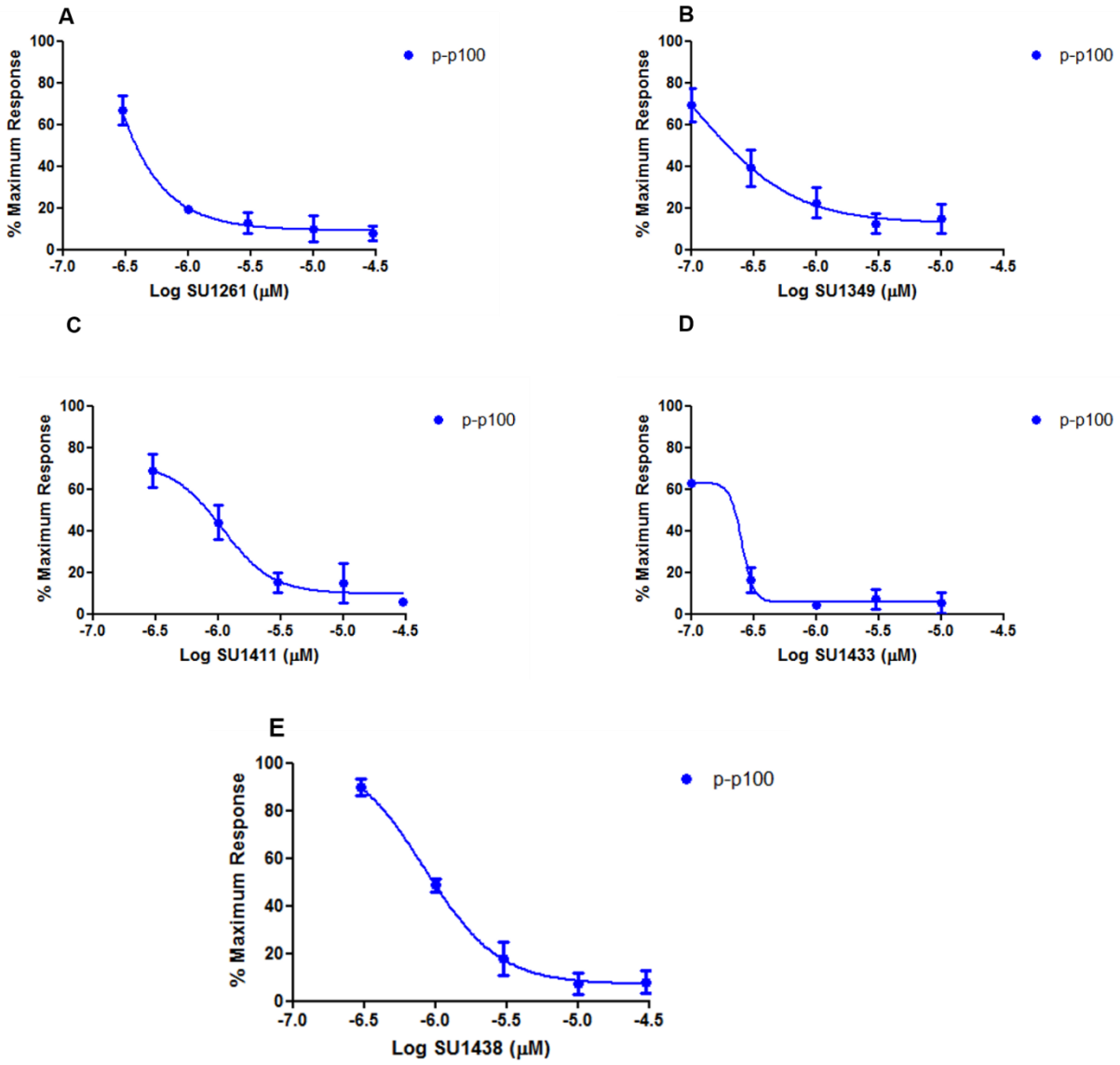


Figure 4.17 The IC_{50} values of SU compounds against phosphorylation of p100 following $LT\alpha_1\beta_2$ stimulation of Panc-1 cells

The data obtained from Western blotting of SU compounds. Results of IC_{50} of p-p100 were semi-quantified by densitometric analysis then data was fitted to a sigmoidal dose response curve. Each value represented the mean ± SEM of three independent experiments for (A) SU1261, (B) SU1349, (C) SU1411, (D) SU1433 and SU1438.

4.8 The effect of SU compounds on cell proliferation

4.8.1 The effect of SU compounds on cell cycle progression in Panc-1 cells

Following the assessment of the effect of SU compounds on the non-canonical and canonical NFκB pathways, the SU compounds that act as selective IKKα inhibitors (SU1261, SU1349 and SU1433) were chosen to investigate whether these compounds have effects on cell cycle progression in Panc-1 cells. This was examined through assessing the spread of cells within cell cycle stages using flow cytometry following propidium iodide (PI) staining. Panc-1 cells were treated with various concentrations (0.1- 30μM) of SU1261, SU1349 and SU1433 separately for 48 h and cell cycle analysis carried out using FACS (Becton Dickinson System, Cowely, UK). Three independent experiments were carried out and results presented a percentage of cells in each cell cycle stage (mean ± SEM).

As shown in Figure 4.18 following incubation of Panc-1 cells with increasing concentrations (0.1- 30μM) of SU1261 for 48h, cells were arrested in G_2/M (were cells prepared cells for mitosis) most markedly after treatment with 3 and 10μM of compound. The percentage of cells increased to (32 ± 1.02% and 26.36 ± 4.04%) respectively compared with cells treated with 0.15% DMSO (24.26 ± 1.33%). However, this arrest was consistent with a change in the percentage of cells in other stages such as G_0/G_1 (growth phase) and S (replication of DNA phase). Cells were treated with 30μM of SU1261 showed only change in G_0 (post-mitotic phase). This suggests that treatment cells with of high concentration of SU1261 caused cell damage.

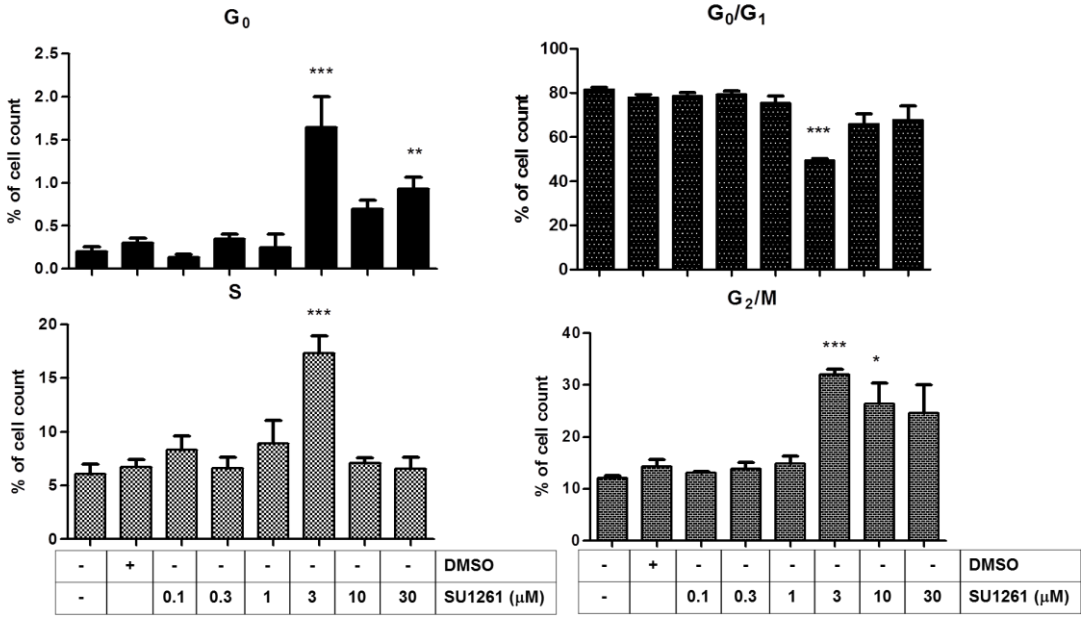


Figure 4.18 The Effect of SU1261 on cell cycle progression in Panc-1 cells

Cells were treated with increasing concentrations of SU1261 or 0.15% DMSO for 48 h, and assessed flow cytometry using propidium iodide (PI) for determination of total of DNA content. The four charts demonstrated the distribution of cells in different phases of cell cycle following treatment with SU1261. Statistical analysis was carried out using a one-way ANOVA with Dunnett post-test. Each value represents the means ± SEM of three independent experiments. ***$p<0.001$ compared with control.

Next, another IKKα inhibitor which showed more selectivity compared to SU1261 was examined. Cells were treated with SU1349 at various concentrations (0.1 - 10μM) for 48 h. (see Figure 4.19). Analysis found that cells pre-treatment with SU1349 were clearly arrested in over the 1-10μM concentrations range in G_2/M phase. Accumulation of cells in this phase were increased (24.2 ± 3.33%, 26.92 ± 2.57% and 24.02 ± 3.97% n=3) respectively compared with control group (10.01 ± 0.55%). In addition there were fewer cells in G_0/G_1 phase over the same range of concentrations. The percentage of cells significantly decreased to 62.42 ± 3.68%, 57.25 ± 1.32% and 63.52 ± 4.48% at 1, 3 and 10μM respectively compared with control group 83.82 ± 1.37%. The highest concentration (10μM) also caused increasing cells number in the G_0 phase, again the higher concentration showed toxic effects in Panc-1 cells.

Similar to SU1261 and SU1349, SU1433 also caused alterations in the cell cycle distribution in Panc-1 cells. The results in Figure 4.20 showed there was accumulation of cells in G_2/M phase following pre-treatment of cells with 3 and 10μM of SU1433. The maximum increase was at 3μM, the percentage of cells was increased to (29.7 ± 3.89%) compared with control group (10.47 ± 0.69%). This was related to a significant decreased in percentage of cells in the G_0/G_1 phase, and there was no significant change in S phase. With the selective IKKα inhibitors examined above, pre-treatment of Panc-1 cells with higher concentrations were implicated with significant increases in G_0 phase.

In conclusion, treatment of cells with selective IKKα inhibitors (SU1261, SU1349 and SU1433), resulted delayed in cell cycle through a significant arrest cells in G_2/M

phase of the cell cycle compared with control group and this increase was also accompanied by changes in percentage of cells in other cell cycle phases. That confirmed the role of IKKα in cell cycle distribution in Panc-1 cells.

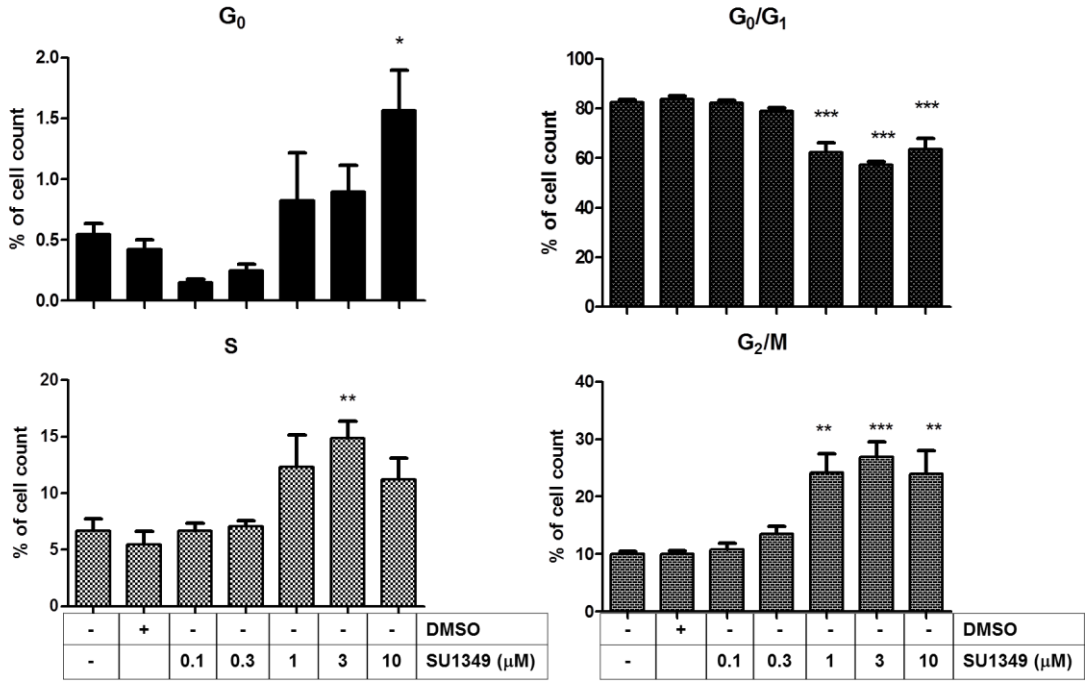


Figure 4.19 The Effect of SU1349 on cell cycle progression in Panc-1 cells

Cells were treated with increasing concentration of SU1349 or 0.05% DMSO for 48h, and samples were assessed by flow cytometry using propidium iodide (PI) for determination of total of DNA content. The four charts demonstrated the distribution of cells in different phases of cell cycle following treatment with SU1349. Statistical analysis was carried out using a one-way ANOVA with Dunnett post-test. Each value represents the means ± SEM of three independent experiments. ***$p<0.001$ compared with control.

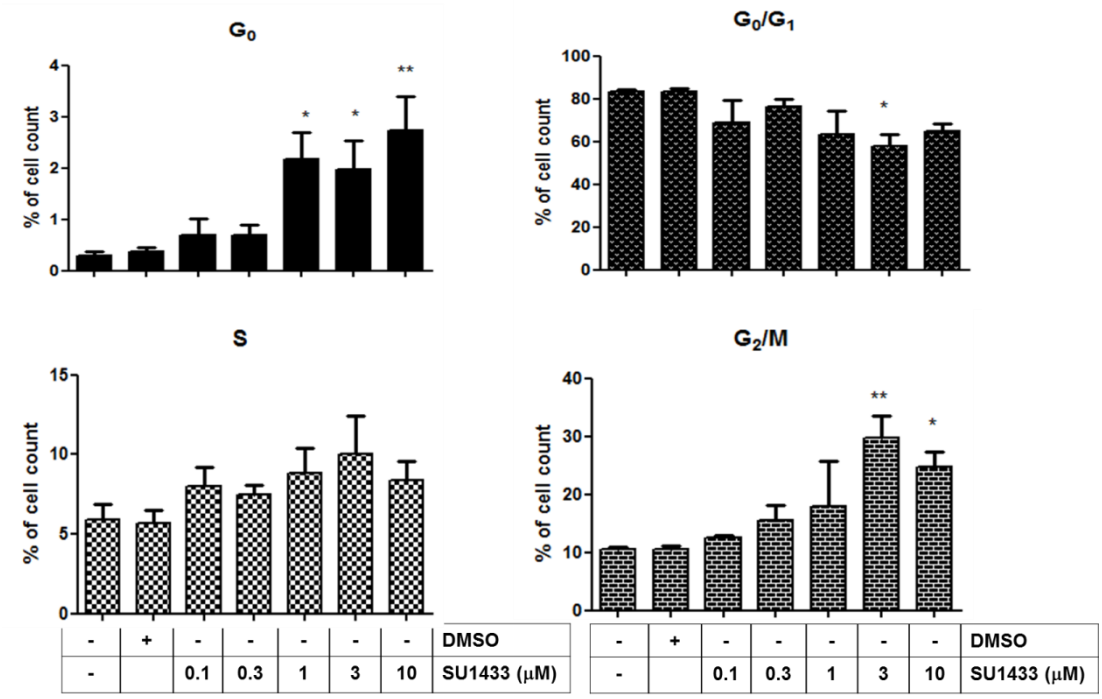


Figure 4.20 The Effect of SU1433 on cell cycle progression in Panc-1 cells

Cells were treated with increasing concentrations of SU1433 for 48 h or 0.05% DMSO, and samples assessed by flow cytometry using propidium iodide (PI) for determination of total of DNA content. The four charts demonstrated the distribution of cells in different phases of cell cycle following treatment with SU1433. Statistical analysis was carried out using a one-way ANOVA with Dunnett post-test. Each value represents the means ± SEM of three independent experiments. ***$p<0.001$ compared with control.

4.8.2 Clonogenic survival of Panc-1 following treatment with SU1261, SU1349 and SU1433

The results above showed that selective IKKα inhibitors could cause change in the accumulation of cells in the different stages of the cell cycle. Next, the effect of the same compounds (SU1261, SU1349 and SU1433) on clonogenic survival was investigated by using the same concentrations that were used in the previous experiments. Exposure of cells to agents could affect their division and ability to form colonies. Panc-1 cells were treated with SU compound (0.1- 30μM) for 24 h and their ability to produce colonies was then assessed by accounting the number of colonies that formed after 7 and 14 days of incubation. Results were presented as concentration response curves as the mean of survival fraction (SF) ± SEM on Y-axis, and logarithmic concentrations of compounds on X-axis for three independent experiments. As shown in Figure 4.21, it was observed that 0.1 and 0.3μM of SU1261 showed no significant inhibition of SF, however, it was noted a significant decreased within higher concentration (1-30μM) of SU1261 either by incubation for 7 day or 14 day period compared with control (cells treated with 0.05% DMSO). Then the IC_{50} values were calculated for both incubation periods; the IC_{50} for the 7 days of incubation was 2.69 ± 1.31μM while the IC_{50} for 14 days of incubation was more than double compared with first period of incubation, the value of the IC_{50} was 6.42 ± 2.54μM.

Moreover, Panc-1 cells treated with different concentrations of SU1349 (0.1-10μM) also showed a significant inhibition of SF between (1-10μM) during 7 days or 14 days of incubation. The survival fraction was significantly decreased reaching a

maximum at 10μM (0.3 ± 0.14) compared with control sample, see Figure 4.22. The IC_{50} for the two periods of incubation were 0.62 ± 0.23 μM (7 days of incubation) respectively, 3.12 ± 1.17μM (14 days of incubation). In contrast, for SU1433 there was only a slight difference in the SF two-period of incubation. For example, the SF at 1μM was 0.2 ± 0.16 at 7 days of incubation vs 0.3 ± 0.09 at 14 days of incubation, Figure 4.22. In addition, there was a slight different in IC_{50} value between 7 and 14 days incubation; 0.63 ± 0.23μM vs 0.81 ± 0.25μM respectively. The finding of this chapter demonstrated that there are a number of SU compounds which act as selective IKKα inhibitors, and these inhibitors confirmed the role of IKKα in regulation of Panc-1 cell cycle progression and cell growth.

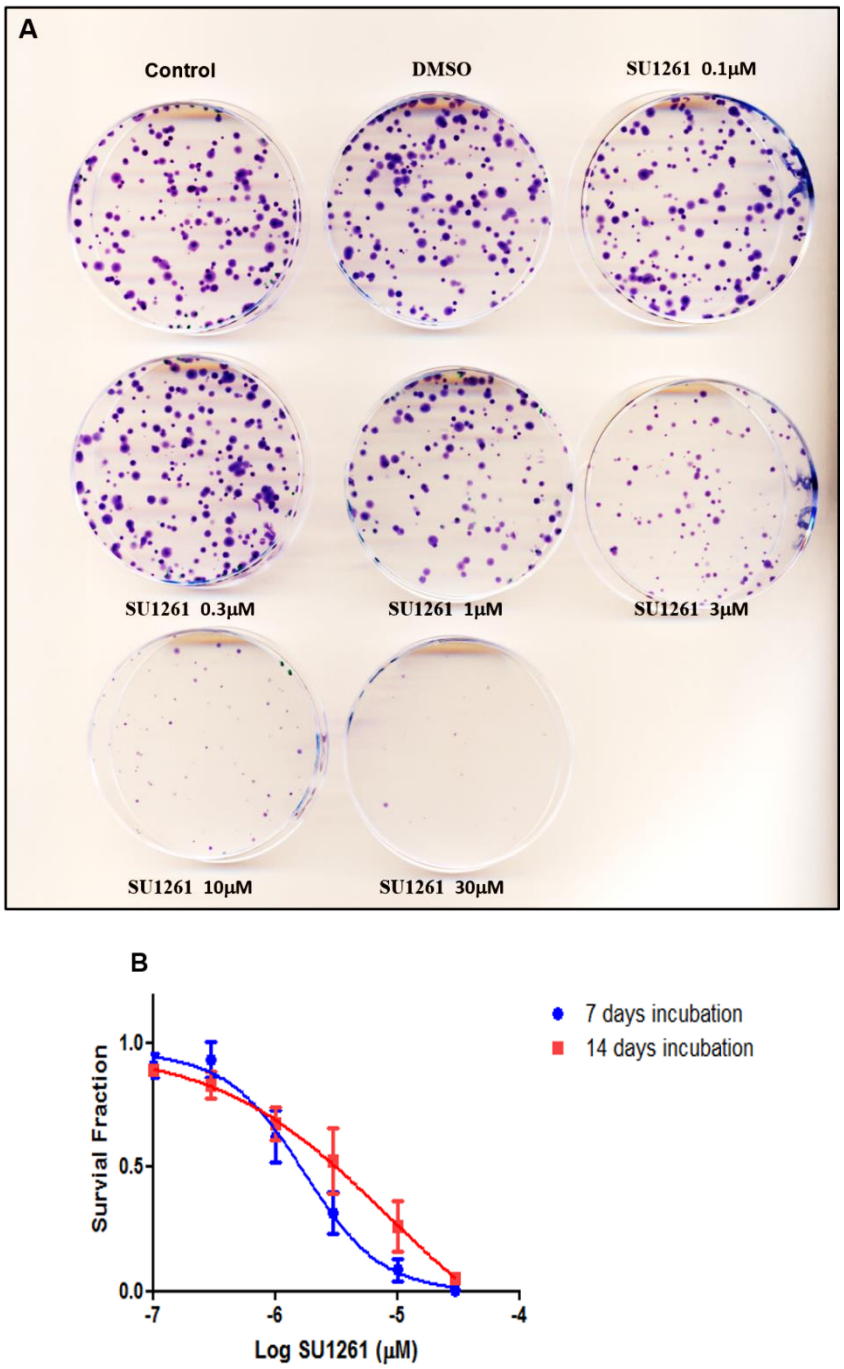


Figure 4.21 The effect of SU1261 on Panc-1 cells clonogenicity

Panc-1 cells were treated with increasing concentrations of SU1261 (0.1-30µM) or 0.15% DMSO for 24 h and then clonogenic survival assay was performed using two incubation periods 7 day and 14 days. Panel A cells that incubated for 14 days. Data was fitted as outlined in Section 2.11 (Panel B), results were expressed as the average of survival fraction (SF) compared with control group (cells that treated with 0.15% DMSO). Data was fitted to a sigmoidal dose response curve. Each value represented the mean ± SEM of three independent experiments.

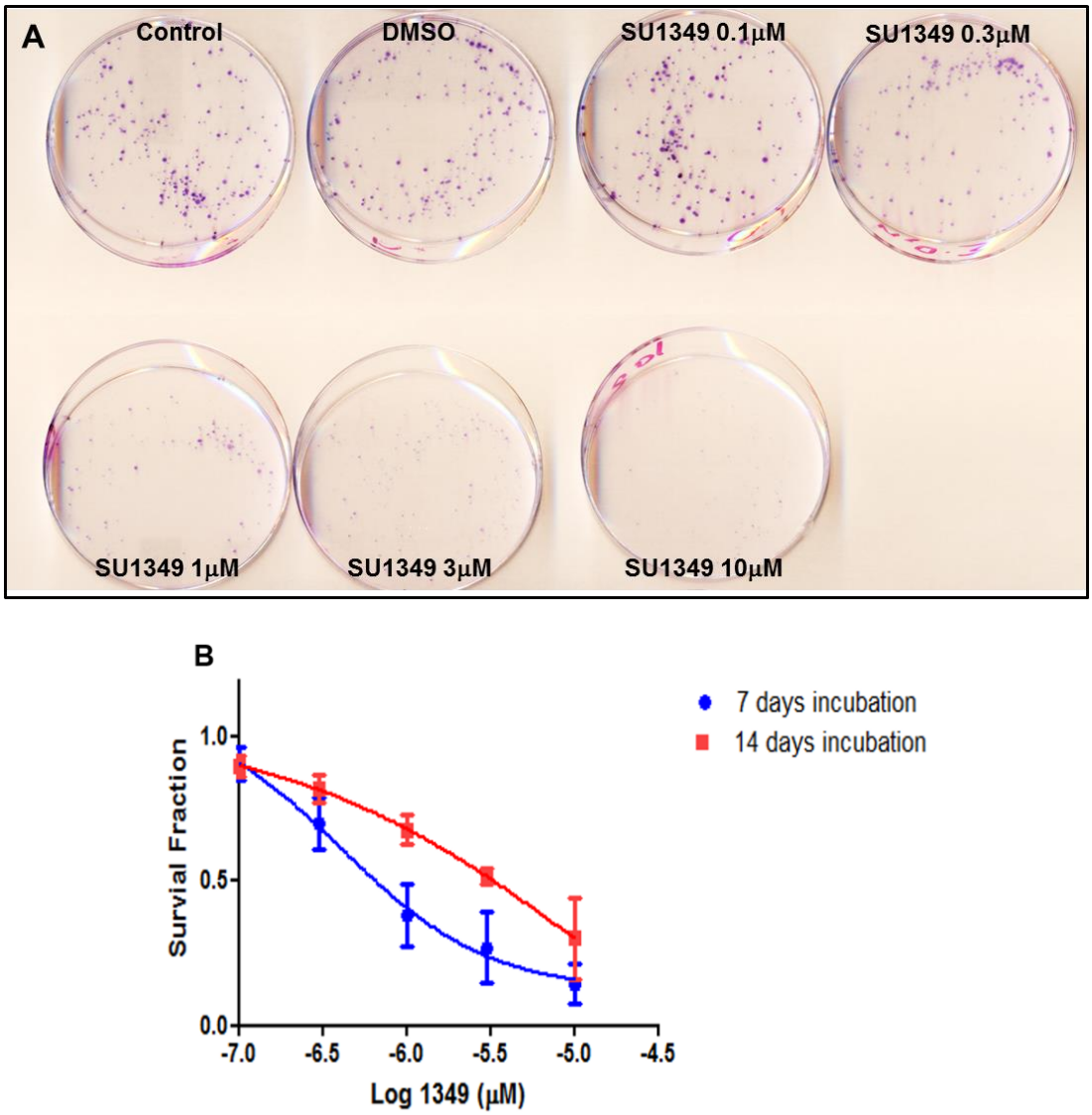


Figure 4.22 The effect of SU1349 on Panc-1 cells clonogenicity

Panc-1 cells were treated with increasing concentrations of SU1349 (0.1-10µM) or 0.05% DMSO for 24h and then clonogenic survival assay was performed using two incubation periods 7 day and 14 days. Panel A cells that incubated for 7 days. Data was fitted as outlined in Section 2.11 (Panel B), results were expressed as the average of survival fraction (SF) compared with control group (cells that treated with 0.05% DMSO). Data was fitted to a sigmoidal dose response curve. Each value represented the mean ± SEM of three independent experiments.

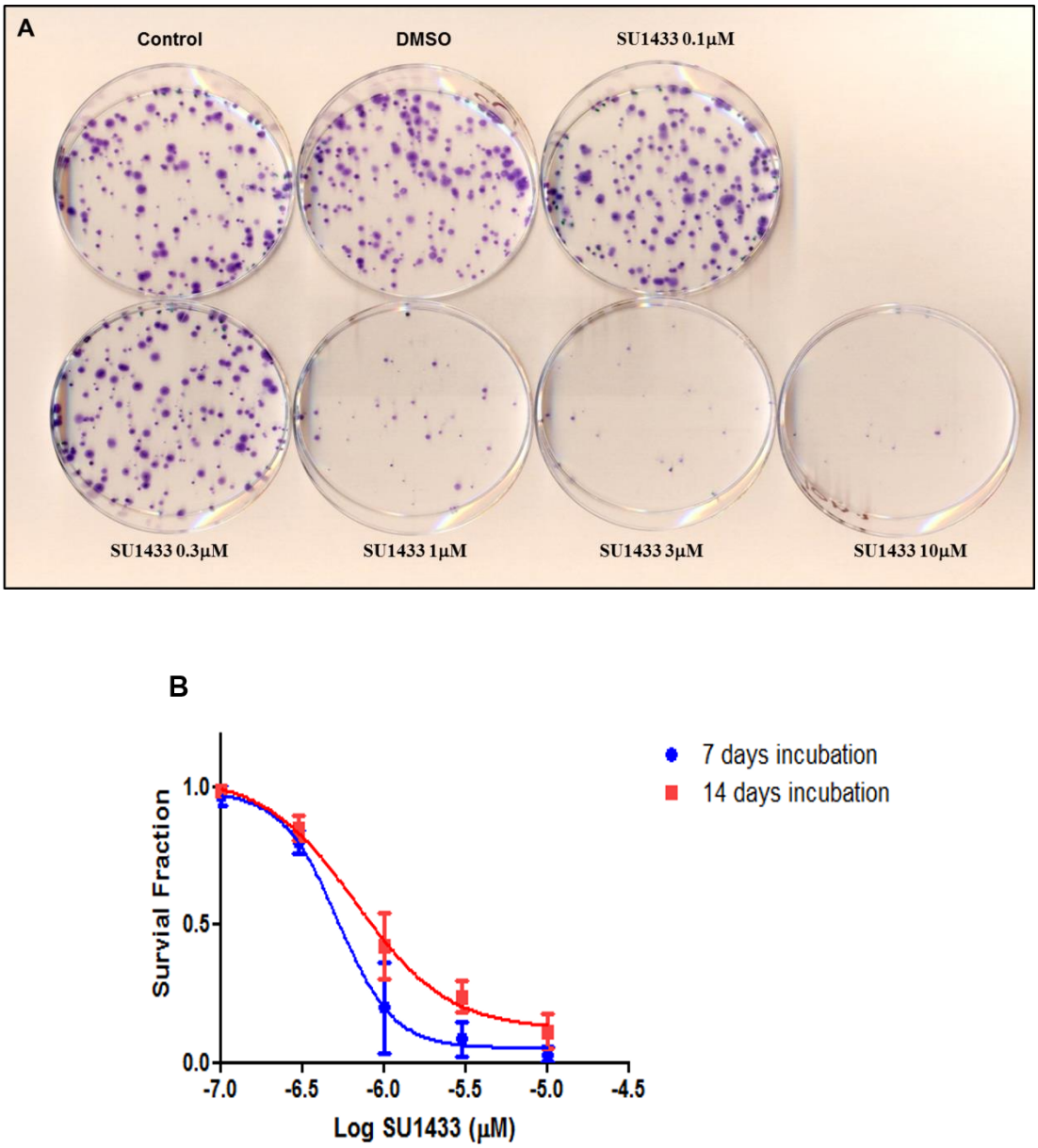


Figure 4.23 The effect of SU1433 on Panc-1 cells clonogenicity

Panc-1 cells were treated with increasing concentrations of SU1433 (0.1 -10µM) or 0.05% DMSO for 24h and then clonogenic survival assay was performed using two incubation periods 7 day and 14 days. Panel A indicates cells that were incubated for 14 days. Data was fitted as outlined in section 2.11 (Panel B), results were expressed as the average of survival fraction (SF) compared with control group (cells that treated with 0.05% DMSO). Data was fitted to a sigmoidal dose response curve. Each value represented the mean ± SEM of three independent experiments.

4.9 Effect of NFκB-inducing kinase (NIK) inhibitors in Panc-1 cell line

4.9.1 Effect of NIK inhibitors on LT$\alpha_1\beta_2$-induced phosphorylation of p100 and p100 processing in Panc-1 cells

In order to further determine if the cellular effect of the IKKα inhibitors where due to *bonafide* effects upon the non-canonical NFκB pathway, we utilised two novel NIK inhibitors, CW15407 (K_i 15nM) and SU15420 (K_i 80nM) which were recently synthesised in-house. Figures 4.24 shows that CW15407 caused a concentration dependent decrease in the phosphorylation of p100 stimulated in response to LT$\alpha_1\beta_2$ (15ng/ml) at 4 h. This effect was observed in the high nanomolar range, inhibition was substantial at 1μM. Again, a similar effect was observed for the formation of p52 and the reversal in p100 loss, again consistent with what was observed for the SU compounds.

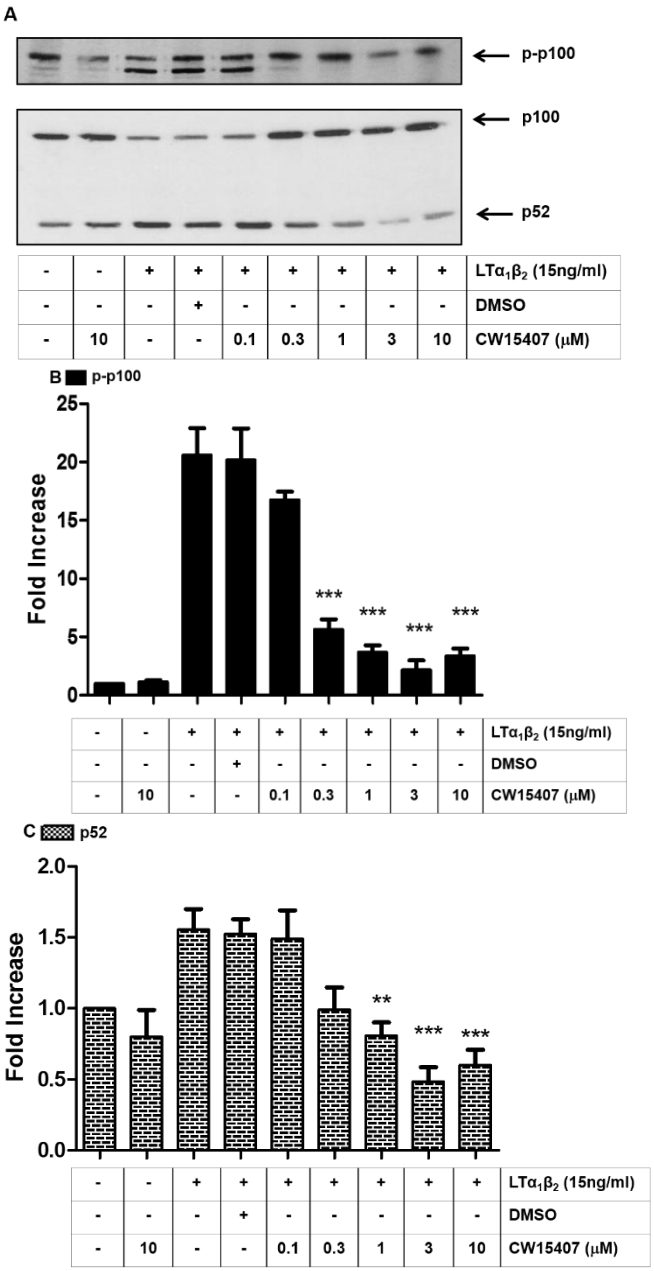


Figure 4.24 The effect of CW15407 on LTα$_1$β$_2$-induced phosphorylation of p100 and formation of p52 in Panc-1 cells

Panc-1 cells were pre-treated with CW15407 or 0.05% DMSO for 1h prior to stimulation with LTα$_1$β$_2$ (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by SDS-PAGE and assessed for p-p100 (100 kDa) and p52 (52 kDa) (panel A). Blots were assessed by semi-quantitative densitometry and results expressed as fold stimulation relative to control for p-p100 (panel B) and p52 (panel C). Each value represents the mean ± S.E.M. The results are representative 3 independent experiments. ***p< 0.001 compared with agonist-stimulated control.

4.9.2 Effect of NIK inhibitors on TNFα–induced degradation of IκBα and phosphorylation of p65 NFκB in Panc-1 cells

Again to confirm the selectivity of the compounds within the non-canonical NFκB pathway the effects upon canonical NFκB pathway markers were examined. As shown in Figure 4.25A, TNFα stimulated IκBα degradation and phosphorylation of p65 NFκB did not change with following pre-treatment with increasing concentrations of CW15407. However, in cells treated with different concentrations of CW15420 prior to stimulation with TNFα, the levels of IκBα degradation returned to the basal expression with increasing concentrations range (1-10μM), without inhibition of phosphorylation of p65 NFκB, see Figure 4.25B.

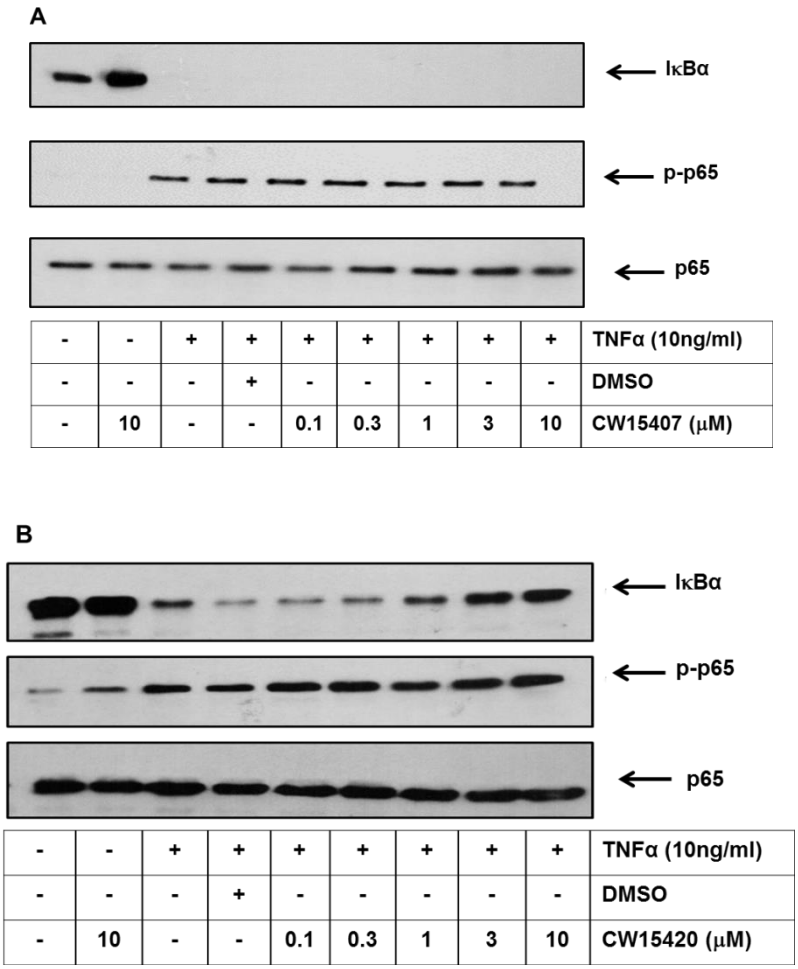


Figure 4.25The effect of CW15407 and CW15420 on TNFα-mediated IκBα degradation and phosphorylation of p65 NFκB in Panc-1 cells

Cells were pre-treated with CW15407 (panel A), and CW15420 (panel C) or 0.05% DMSO for 1h before stimulation with TNFα for 15 min.Whole cells extracts were prepared, and then blotted for IκBα (37 kDa), p-p65 NFκB (65 kDa) and p65 NFκB (65 kDa) which was used as a loading control. Each blot is representative three experiments.

4.10 The effect of NIK inhibitors on cell proliferation of Panc-1 cells

4.10.1 The effect of CW15407 treatment on cell cycle progression in Panc-1 cells

In order to investigate the potential effect of CW15407 on cell cycle distribution of Panc-1 cells, cells were treated with various concentrations of CW15407 for 48h and the data examined to identify whether CW15407 could affect cell cycle distribution in Panc-1 cells. The results in Figure 4.26 represented the effect of CW15407 on the cell cycle; there were significant accumulation of cells in G_2/M phase following treatment with 1μM CW15407, the percentage of cells were increased to 28.0 ± 4.88% in comparison cells treated with 0.05% DMSO only; 13.96 ± 2.9%. Furthermore, there was no change in percentage of cells in cell cycle stages between the untreated cells and cell that treated with DMSO.

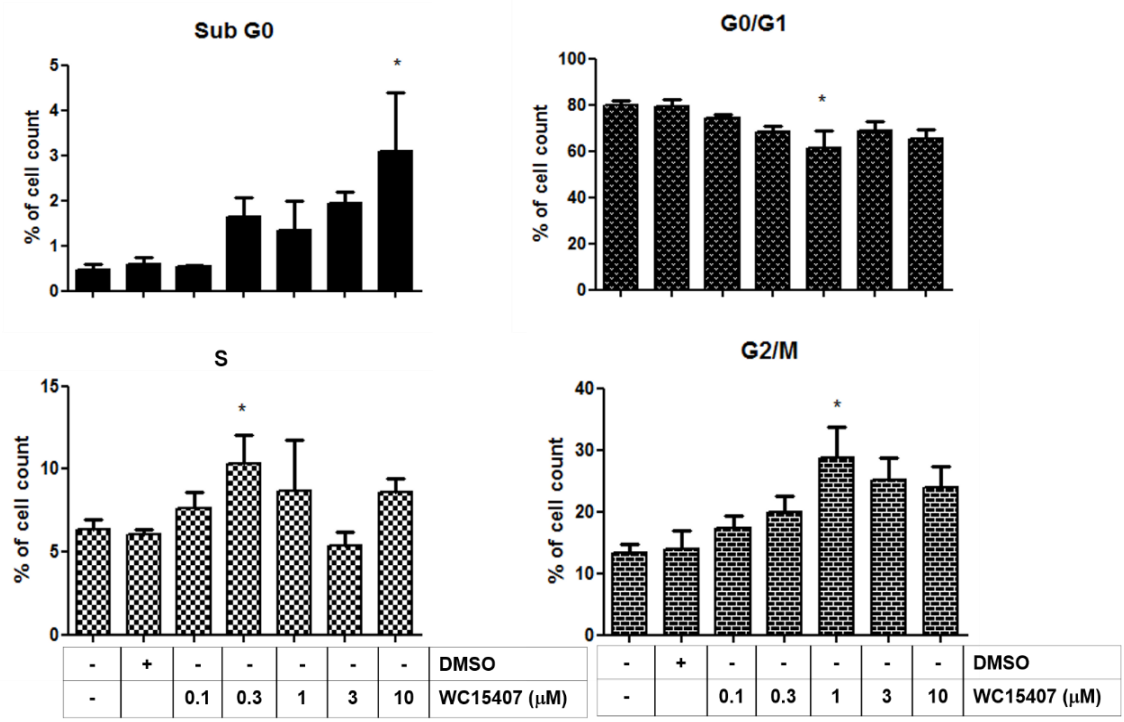


Figure 4.26 The Effect of CW15407 on cell cycle distribution in Panc-1 cells

Cells were treated with increasing concentrations of CW15407 for 48h, then the cell cycle assessed by flow cytometry using propidium iodide (PI) for determination of total of DNA content. The four charts demonstrated the distribution of cells in different phases of cell cycle following treatment with SU15407. Statistical analysis was carried out using a one-way ANOVA with Dunnett post-test to compare to control. Each value represents the means ± SEM of three independent experiments. ***$p<0.001$ compared with control.

4.10.2 The effect of CW15407 treatment on clonogenicity cell growth of Panc-1 cells

Next, the effect of CW15407 on cell survival was examined by using clonogenic assay as explained in Chapter Two Sections 2.10. Cells were treated with various concentrations of CW15407 for 24h then incubated for 7 and 14 days. As shown in Figure 4.27, the survival fraction (SF) of cells was significant reduced followed treatment with 0.3μM of CW15407 and it increased with increasing concentration of CW15407. The IC_{50} for CW15407 was 0.39 ± 0.25μM (7 days incubation) vs 1.72± 1.05μM (14 days incubation). Data in Figures 4.24 to 4.27 suggest that NIK has a role in the regulation of the non-canonical NFκB pathway, could be inhibited using a NIK inhibitor and is linked to cell cycle progression and clonogenicity in Panc-1 cells.

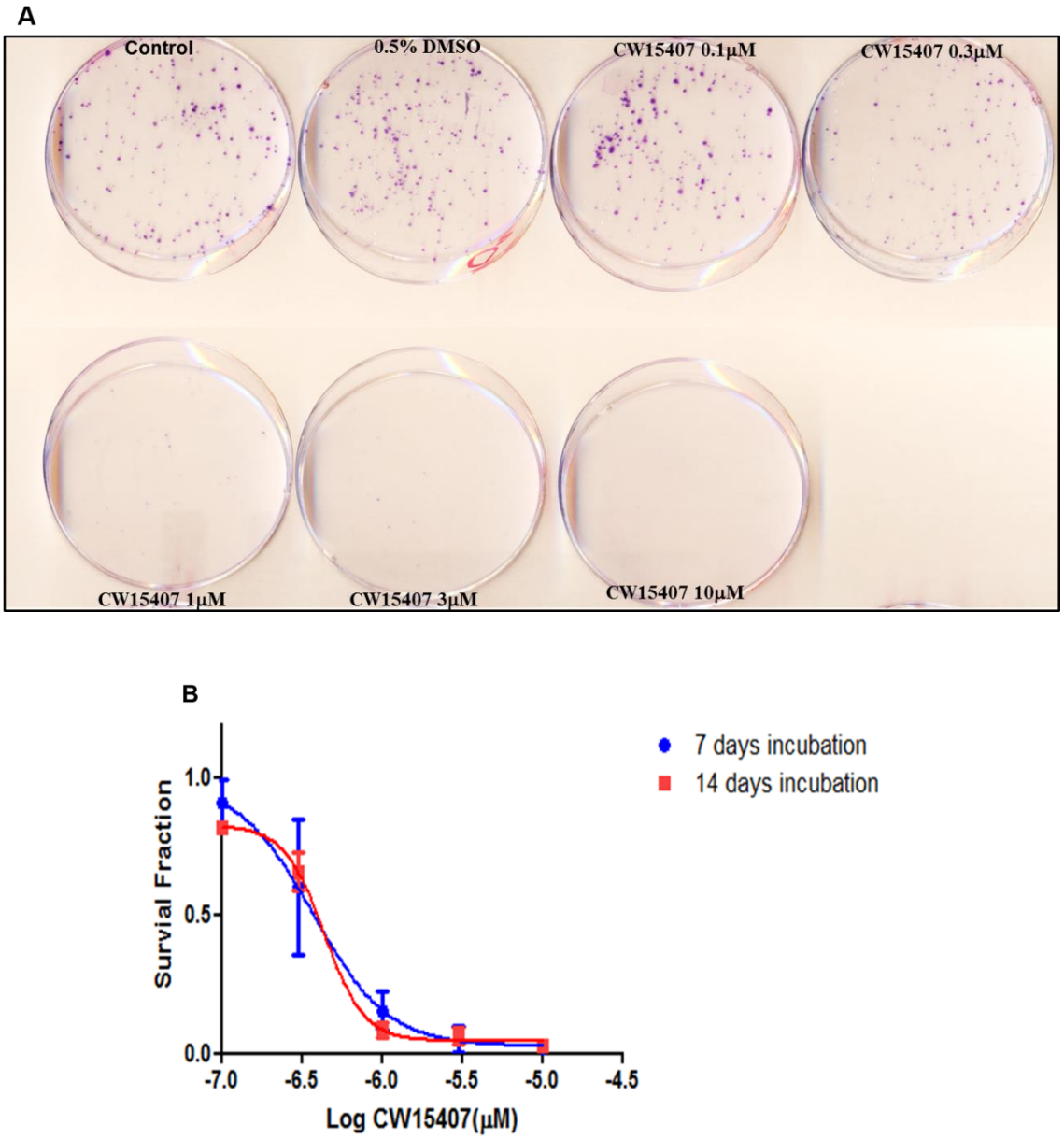


Figure 4.27 The effect of CW15407 on Panc-1 cells clonogenicity

Panc-1 cells were treated with increasing concentrations of CW15407 (0.1 -10µM) for 24h and then clonogenic survival assay was performed using two incubation periods 7 day and 14 days. In panel A cells were that incubated for 7 days. Data shown in Panel B were espressed as the average of survival fraction (SF) compared with control group (cells that treated with 0.05% DMSO). Data was fitted to a sigmoidal dose response curve. Each value represented the mean ± SEM of three independent experiments.

4.11 Discussion

Pancreatic cancer is difficult to treat, in part due to the high incidence of recurrence following surgery (Thota et al., 2014). Furthermore, the majority of patients receive little or no benefit from chemotherapy, due to the presence of chemo-resistant tumours cells or the emergence of a therapy-resistant phenotype (Whatcott et al., 2013). Recently many studies have investigated the therapeutic potential of agents which selectively inhibit the kinase activity of IKKs and which have been demonstrated to suppress the growth of tumour cells. Therefore, developing IKKs inhibitors that block the activity of NFκB pathways has become an interesting area for a number of studies in the cancer field. For example, Yang *et al,* showed that treatment of melanoma tumours with IKK inhibitors such as BMS-345541 led to the suppression of tumours cell growth (Yang et al., 2012). Many IKKβ inhibitors have been developed, such as ML120B (Catley et al., 2006), PS-1145 (Castro et al., 2003) and TPCA-1 (Birrell et al., 2005). However, for these and other IKKs inhibitors their potential effect as anti-cancer drug was limited because their non-selectivity against IKKα and IKKβ lead to some harmful effect to normal cells. Furthermore, compounds which are selectively inhibitors of IKKα are less common. For example, peptidyl glucosamine has been shown to inhibit IKKα nuclear translocation in human chondrocytes (Scotto d'Abusco et al., 2010) and Asamitsu *et al* found that noraristeromycin selectively inhibited IKKα in HEK293 (Asamitsu et al., 2008). Furthermore, it has been shown that knockdown of IKKα in HeLa cells led to the reduced the metastatic potential of these cells (Prajapati et al., 2006). However, to

date, no selective IKKα inhibitor has been developed or interrogated in pancreatic cancer.

As discussed in Chapter one, the non-canonical NFκB pathway is dependent on IKKα activity. In addition, knockdown of IKKα by siRNA confirmed this, through inhibition phosphorylation of p100 and p100 processing in Panc-1 cells. The initial objective in this chapter was to examine the effects of exposure to SU compounds on IKKα–dependent NFκB signalling through inhibition of p52 formation. The results demonstrate that all SU compounds with the exception SU1392 showed the ability to impair the formation of p52 in Panc-1 cells and phosphorylation of p100.

Interestingly, while SU compounds such as SU1261, SU1434, SU1433, SU1349 and SU1411 have been shown to inhibit the expression of p100 in Panc-1 cells, these results could be explained by three different hypothesises. The first involves regulation of the expression of p100 by IKKα. This hypothesis is supported by IKKα knockdown by siRNA, which also resulted in a decrease in p100 expression. Senftleben *et al* has demonstrated that IKKα can regulate the p100 subunit (Senftleben et al., 2001). However, the regulation of p100 could also be due to IKKβ activation through regulation of the p65 NFκB subunit, (Ishimaru et al. 2006; Basak et al. 2007), in a study by Basak and co-workers study, overexpression of RelA using rotavirus transgene was resulted in increased levels of p100, p65 NFκB and p50 (Basak et al., 2007), and, as the agonist used in this study ($LT\alpha_1\beta_2$) has the ability to activate both NFκB pathways (Chapter Three), this hypothesis cannot be discounted. The medicinal chemistry group has reported that some of these compounds have

activity against cyclin-dependent kinase 9 (CDK9). CDK9 plays an important role in the regulation of transcription, cell cycle progression and DNA repair (Storch and Cordes, 2015). CDK9 inhibitors work by disruption of S-phase during the cell cycle, and, additionally, inhibit mRNA synthesis (Shan et al., 2005, Krystof et al., 2006). This suggests that the ability number of SU compounds to inhibit CDK9 linked to the inhibition of p100 expression.

Another IKKα– dependent marker was examined, namely phosphorylation of p100. As shown in Figure 4.6-4.10; there were significant concentration-dependent decreases in p100 phosphorylation. It was noted that the IC_{50} values for p-p100 were less than p52 formation following treatment of Panc-1 cells with same compounds. For example, the IC_{50} value of SU1261 for p52 formation was 3.08 ± 0.25μM, whilst the IC_{50} for p-p100 was 0.75 ±0.25 μM, only SU1432 showed IC_{50} values were nearly the same for both p52 formation and p-p100. That suggests these compounds are more important during a short time of treatment. Since the stimulation time for p-p100 was 4h compared with 24h for p52 formation, this was encouraging to reduce the chance for out of target but also multiple signalling pathways have a role in forming p52, in addition, $LT\alpha_1\beta_2$ has the ability to activate other pathways. In Chapter Three Figure 3.18 confirmed that the canonical NFκB pathway could be activated through $LT\alpha_1\beta_2$ stimulation, and in addition a number of other signalling pathways could be activated during binding to the lymphotoxin β receptor such as, activation of the JNK pathway and the MAPK pathway (Mikami et al., 2014, Baud et al., 1999).

Since IKKα has no role in the IKKβ-dependent NFκB pathway (Dejardin et al., 2002, Claudio et al., 2002) the effects of the SU compounds on inhibition of degradation of IκBα and phosphorylation of p65 NFκB were investigated, to determine if these compounds were, indeed, selective IKKα inhibitors or could also inhibit IKKβ–dependent processing. The results discussed in this chapter demonstrated that six of the SU compounds (SU1261, SU1411, SU1433, SU1349, SU1438 and SU1434) were selective IKKα inhibitors, whereas other SU compounds induced inhibition of both the canonical and non-canonical NFκB pathways. One compound, SU1392, had no effect on either NFκB pathways.

In chapter three, it was demonstrated that $LT\alpha_1\beta_2$ could also activate the canonical NFκB pathway, therefore the effect of SU compounds on $LT\alpha_1\beta_2$ activation of IKKβ-dependent NFκB induction was also examined. The results shown in this chapter confirm that SU1261 was an IKKα selective inhibitor, SU1087 was a dual IKKα/IKKβ inhibitor, and SU1392 had no inhibitory function in either pathway (data not shown).

Three different effects were observed; inhibition of IKKα (by SU1261, SU1411, SU1433, SU1349, SU1438 and 1434), inhibition of both IKKα and IKKβ (by SU1087, SU1432, SU1499 and SU1436) and no inhibition of either pathway (by SU1392). Moreover, some SU compounds were examined using MiaPaC-2 cells such as SU1261, SU1411, SU1087, SU1349 and SU1433 and similar results were noted in MiaPaCa-2 cells (data not shown).

Next, the potential effect of selective IKKα inhibitors; (SU1349, SU1261 and SU1433) upon cell proliferation were also investigated through examining the effect of these inhibitors on cell cycle and cell growth. The three IKKα inhibitors indicated as significant change in cell cycle stages (Figure 4.21 -4.23). These results suggest these compounds could arrest of Panc-1 cell cycle. Another study in the Boyd lab (SIPBS), found that treatment of PC3M cells (prostate cancer cell line) with various concentration of SU1261 (0. 1-30μM), induced no change in the various phase of cell cycle, only the higher concentration (10μM) lead to change the percentage of cells in G_0 without effect other cell cycle phases. Many studies have demonstrated IKKα has a role in regulation a number of cell cycle protein, for instance, Aurora-A and Cyclin D1 (Cao, et al, 2001, Demicco, et al,2005, Prajapati, et al, 2006), and the results in this study are in agreement with finding of Schneider et al. They found that knockdown of IKKα using siRNA in MiaPaCa-2 cells lead to a significant arrest G_0/G_1 (Schneider et al, 2006). In addition, another study by Shukla et al found that rundown IKKα in prostate cancer cell line; PC3M and 22Rv1, cells have alterations in cell cycle distribution through accumulation of cells in G_0/G_1 phase (Shukla, et al, 2015). Furthermore, the cells were arrested in S phase after knockdown of IKKα in breast cancer using siRNA (Tu, et al, 2006). In contrast, our results disagree with other studies such as Mahato and co-workers finding that silencing of IKKα in PC3M and DU145 did not affect the cell cycle progression (Mahato, et al, 2011). Moreover, the accumulation of cells in various cell cycle phases did not change followed silencing of IKKα in breast cancer cell lines. It seems to be that the function of IKKα in regulating the cell cycle is dependent on tumour and cell type.

Many studies have demonstrated that NIK has a role in the regulation of the non-canonical NFκB pathway or both NFκB pathways and this is dependent on tumour cell types (Xiao et al., 2001b). The results in Figures 4.24 and 4.25A showed the selective effect of NIK inhibitor (CW15407) on non-canonical NFκB pathway this suggest that NIK have a role in activation this pathway and the finding of this study was agreed with other studies found that NIK have a role in regulation the non-canonical NFκB pathway (Nishina, et al, 2009, Xiao, et al, 2001b). In contrast, the results of the second NIK inhibitor was used in this study (CW1420) showed that the effect of TNFα-inducing IκBα degradation was blocked using CW15420 (Figure 4.25B) and same inhibitor was blocked $LT\alpha_1\beta_2$-inducing phosphorylation of p100 (data not shown), that suggest that NIK have a role in both NFκB pathways, and this results agreed with other studies that reported NIK is involved in activation of both NFκB pathways in breast cancer (Yamaguchi, et al, 2009) or lung cancer cells (Saitoh, et al, 2008).

To date there is no successful NIK inhibitors because a number of issues, for example selectivity in cell lines or not active *in vivo* (Nishina et al., 2009, Doppler et al., 2013), for example, Pyrazolo 4,3c isoquinoline is a NIK inhibitor but not selective since it could be inhibited both NFκB pathways (Storz, 2013). The results in this chapter was the first study reported a selective NIK inhibitor through inhibition non-canonical NFκB pathway without any effect on canonical NFκB pathway, investigate the effect on other signalling pathways and *in vivo* study are required to confirmed the selectivity of NIK inhibitor.

One of the mechanisms that leads to cancer development is anti-apoptosis, through overexpression of number of survival proteins such as BCL2, survivin and cIAPI and these proteins could be regulated through NIK overexpression or activation of the non-canonical NFκB pathway (Baldwin, 2001). Down-regulation of these proteins have been linked to impaired cancer cells survival or growth (Grossman et al., 2001, Hilmi et al., 2008, Lecis et al., 2010). Yin and co-workers have reported that NIK deficient melanoma tumour cells linked to down-regulation of the non-canonical NFκB pathway, is related to tumour cells growth and regulation of survival proteins and indirectly associated with activity of the non-canonical NFκB pathway and not related to the canonical NFκB pathway (Yin et al., 2001). Furthuremore, Baldwin has investigated the role of NIK in regulation of the proteins mentioned above in melanoma cells and the results showed that the expression of these genes were signifacntly decreased during blockade of the activty of NIK (Baldwin, 2001), however, NIK too has NFκB-independent function like IKKβ and IKKα (Chariot, 2009).

Many studies have reported that NIK plays a role in the regulation of cell proliferation in different cancer types such as. Myeloma, lymphoma and leukaemia (Annunziata et al., 2007 Conze et al., 2010 Demchenko et al., 2010). CW15407 has confirmed that NIK has a role in cell proliferation, it showed there was arrest in the cell cycle and inhibited cell growth. These data agreed with the finding of Dopper and co-workers, that documented that of blockade NIK expression, using shRNA NIK, resulted in decreased cell proliferation in Panc-1 and MiaPaCa-2 cells (Doppler et al., 2013). Another study found that inhibiting the activity of NIK, by siRNA, lead

to reduce processing of p100 and cell growth and proliferation in pancreatic cancer (Nishina et al., 2009).

The results in Chapter Four clearly indicated that both IKKα and NIK are involved in regulation the non-canonical NFκB pathway activity and associated with cell proliferation, therefore, represent targets for cancer treatment.

Chapter Five

Characterisation of the inhibitory effect of IKKα selective inhibitors (SU compounds) on Non-Canonical NFκB pathway target genes

5.1 Introduction

In response to cellular activation, the NFκB complexes, RelA/p65 or RelB/p52, are translocated to the nucleus and bind to the promoter regions of a substantial number of genes. Studies suggest that the expression of as many as 150 genes are regulated, the proteins encoded by NFκB include chemokines and growth factors, cell adhesion molecules and cytokines (Birbach et al., 2002). The majority of these proteins are involved in the host immune response, as many as 27 different cytokines and chemokines and receptors are required for this process (Pahl, 1999).

A number of studies have shown that NFκB has a critical role in development of cancers (Lu et al., 2004, Lu and Stark, 2004, Senegas et al., 2015). However whilst activation of NFκB during cancer development is not related to a specific mutation resulting in alteration in any of the NFκB components, exaggerated production of a number of pro-inflammatory cytokines such as IL-6, IL8 and CXCL is consistently observed (DiDonato et al., 2012). NFκB activation also leads to upregulation of genes involved in cell survival which increases resistance to apoptosis and cell cycle genes such as cyclin D1 and cyclin D2 (Tysnes, 2010). Santos and co-workers found that constitutive activation of the non-canonical NFκB pathway and increased nuclear translocation of the RelB complex is linked to the development of resistance of leukemic cells to radiation therapy (dos Santos et al., 2008).

In pancreatic cancer, several studies have reported that increased NFκB plays a role in resistance to apoptosis, proliferation, chemo-resistance and metastasis (Wharry et al., 2009, Chandler et al., 2004). Other groups have established a link between elevated levels of NIK and upregulation of proliferation genes (Wharry et al., 2009,

Nishina et al., 2009, Schneider et al., 2006), in addition to a number of pro-inflammatory markers such as CXCL12, CXCL13, BAFF, CCL19 and CCL21 (Wharry et al., 2009). Taken together these studies suggest that the growth of pancreatic cancer may be underpinned by the activation of the non-canonical NFκB pathway. In this chapter the consequence of activating the IKKα-dependent, non-canonical NFκB pathway on expression of NFκB target genes was examined. In addition, the effect of IKKα inhibitors upon expression of these genes was tested. The hypothesis of this chapter is that selective pharmacological inhibition of the IKKα-dependent NFκB pathways leads to down regulation of a specific subset of IKKα-dependent target genes.

5.2 Characterision of target genes expressed through activation of the non-canonical NFκB pathway

In order to study the induction of target genes in pancreatic cancer cells, Panc-1 cells were stimulated with $LT\alpha_1\beta_2$ (15ng/ml) between 4 to 48h and total RNA was extracted for quantitative real-time polymerase chain reaction (qPCR) analysis. Three independent experiments were carried out for genes initially identified as changing significantly in a selected gene array through another study in our group; Interleukin-6 (IL-6), BCL2, Binding Component 3 (BBC3), The Enhancer Zeste homolog2 (EZH2), Mitogen activated protein kinase 14 (MAP3K14), serpin family B member 6 (SERPINB6), TNF alpha induced protein 3 (TNFAIP3) and (VCAM-1). An additional control experiment was run parallel to the qPCR; whole cell extracts were prepared for Western blot analysis to confirm that $LT\alpha_1\beta_2$ stimulated the non-canonical NFκB pathway through the phosphorylation of p100 and formation p52 (see Figure 5.1).

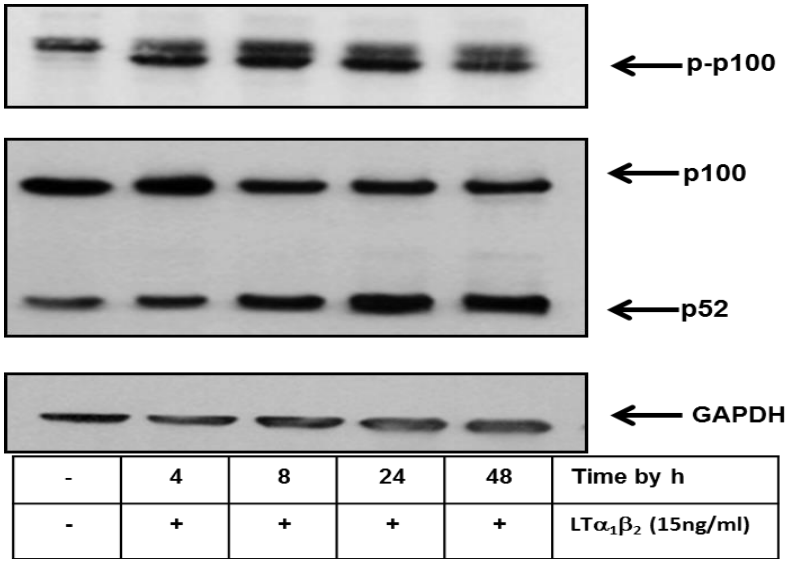


Figure 5.1 LT$\alpha_1\beta_2$- induced phosphorylation of p100 and formation of p52 in Panc-1 cells

Cells were stimulated with LT$\alpha_1\beta_2$ over the times indicated and whole cells lysates prepared as outlined in the Section 2.3.1. Cell extracts were separated by SDS- PAGE and blotted for p-p100 then reprobed for p100/p52 and GAPDH, which was used as a loading control. The blot is representative of three independent experiments.

5.2.1 LT$\alpha_1\beta_2$ induced expression of BBC3, EZH2, TNFAIP3 and VCAM genes

For each of the genes studied three independent experiments were performed. The results were inconsistent with a big different in fold increase in gene expression in each experiment, and each experiment was run in triplicate. For this reason it was decided to deal with each experiment individually and do statistical analysis for each experiment. There were four genes which shared the same results profile; BBC3 (apoptosis), EZH2 (cell adhesion and inflammation), VCAM (cell adhesion molecule) and TNFAIP3 (anti-apoptosis). The first experiment was showed there was increasing expression which was time-dependent. In the second experiment, the expression of genes was increased over three time points; 4, 8 and 24h followed by a decrease at 48h. In the third experiment, the expression of genes was increased only at 4h and 24h of stimulation (in third experiment the expression of housekeeping genes were increased at 8h and 48h of stimulation compared with unstimulated cells).

For example, as shown in Figure 5.2A, in the first experiment, expression of BBC3 increased after 4h stimulation with LT$\alpha_1\beta_2$ continued to and thereafter reached a maximum at 48h (13.04 ± 1.73 fold), compared with untreated cells. However, the results of the second experiment also showed increased expression of BBC3 at 4h of stimulation reaching a maximum by 8h (4.03 ± 0.62 fold) then gradually decreasing before returning to basal level at 48h (see Figure 5.2B). The expression of BBC3 in the third experiment (Figure 5.2C) was increased up to 177± 1.46 fold at 4h of stimulation then rapidly returned to basal level at the next time point 8h followed by a sharp increase at 24h of stimulation (808.22 ± 177.32 fold) before decreasing again

to lower than basal level by 48h. Taken together these results do suggest, despite the obvious variability, that the non-canonical NFκB pathway may play a role in the upregulation of these genes.

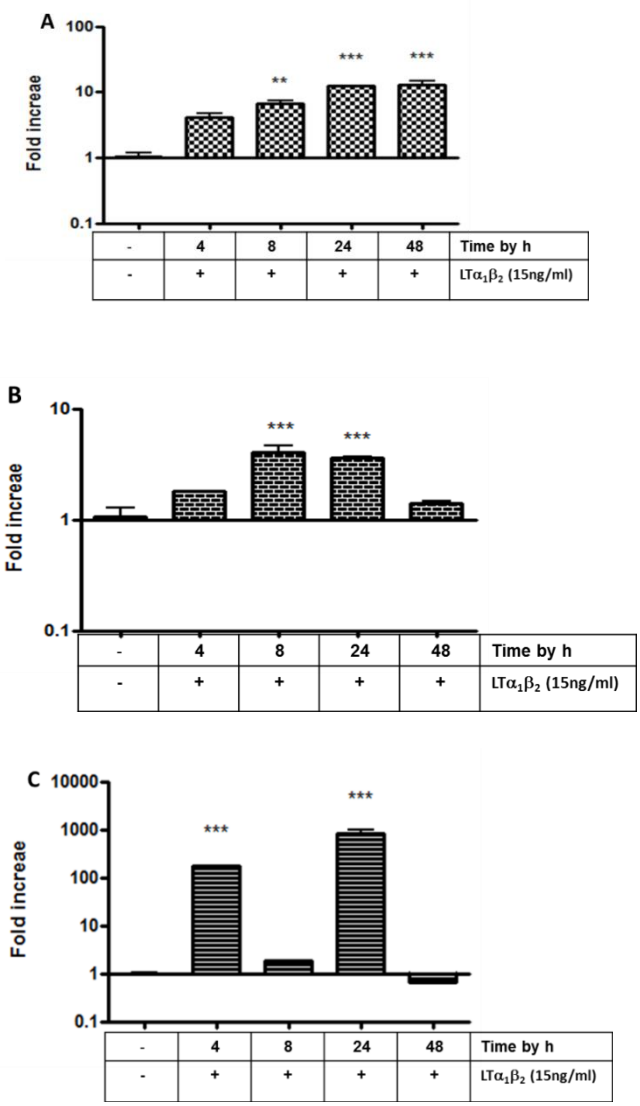


Figure 5.2 LTα$_1$β$_2$-induced BBC3 mRNA expression in Panc-1 cells

Cells were treated with LTα$_1$β$_2$ (15ng/ml) for the times indicated, total RNA was extracted as outlined in Section 2.9.1. QPCR analysis was performed on the cDNA using primers designed to detect BBC3 as described in Methods Section 2.9.4. Relative expression levels of BBC3 mRNA transcripts were normalised to reference genes GAPDH, NCL and 18s using delta-delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean ± SEM of triplicate determinations from a single experiment. ***P< 0.001, **P< 0.01 compared with untreated cells.

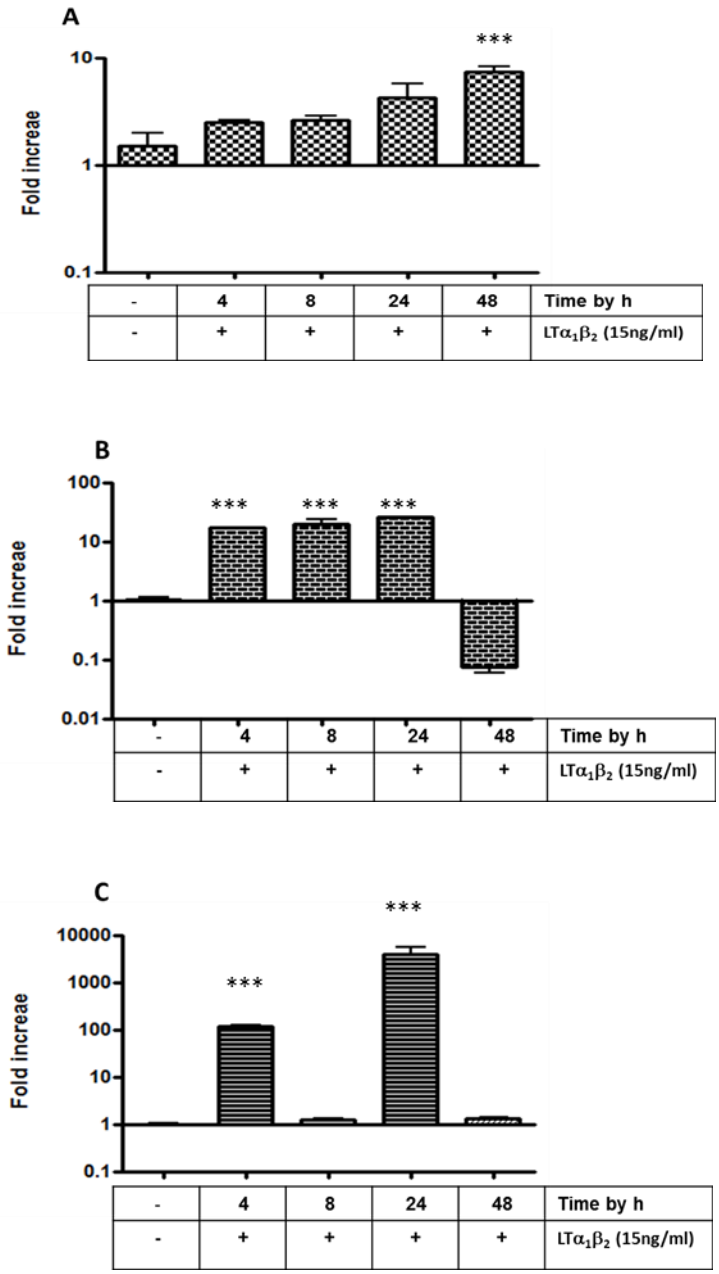


Figure 5.3 LT$\alpha_1\beta_2$-induced EZH2 mRNA expression in Panc-1 cells

Cells were treated with LT$\alpha_1\beta_2$ (15ng/ml) for the times indicated, total RNA was extracted as outlined in Section 2.9.1. QPCR analysis was performed on the cDNA using primers designed to detect BBC3 as described in Methods Section 2.9.4. Relative expression levels of EZH2 mRNA transcripts were normalised to reference genes GAPDH, NCL and 18s using the delta-delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean ± SEM of triplicate determinations from a single experiment. ***P< 0.001, **P< 0.01 compared with untreated cells.

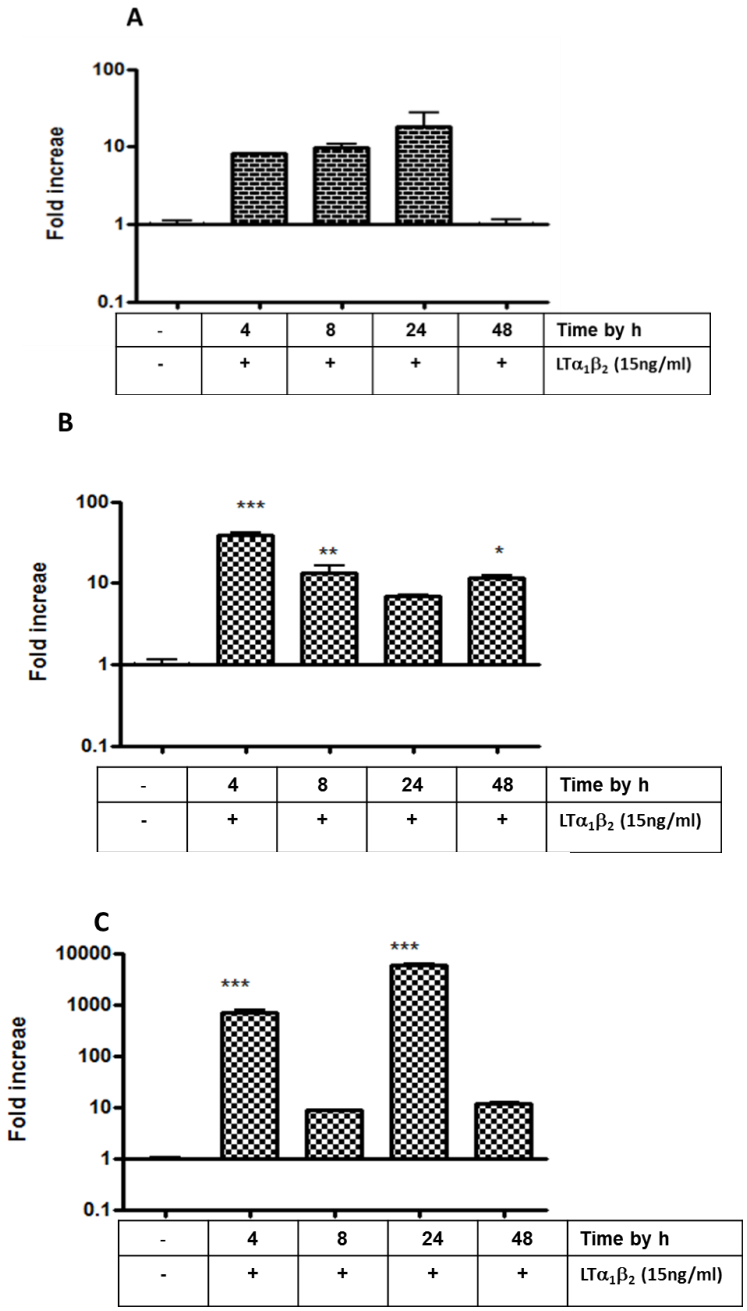


Figure 5.4 LTα$_1$β$_2$-induced TNFAIP3 mRNA expression in Panc-1 cells

Cells were treated with LTα$_1$β$_2$ (15ng/ml) for the times indicated, total RNA was extracted as outlined in Section 2.9.1. QPCR analysis was performed on the cDNA using primers designed to detect BBC3 as described in Methods Section 2.9.4. Relative expression levels of TNFAIP3 mRNA transcripts were normalized to reference genes GAPDH, NCL and 18s using delta-delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean ± SEM of triplicate determinations from a single experiment. ***P< 0.001, **P< 0.01 compared with untreated cells.

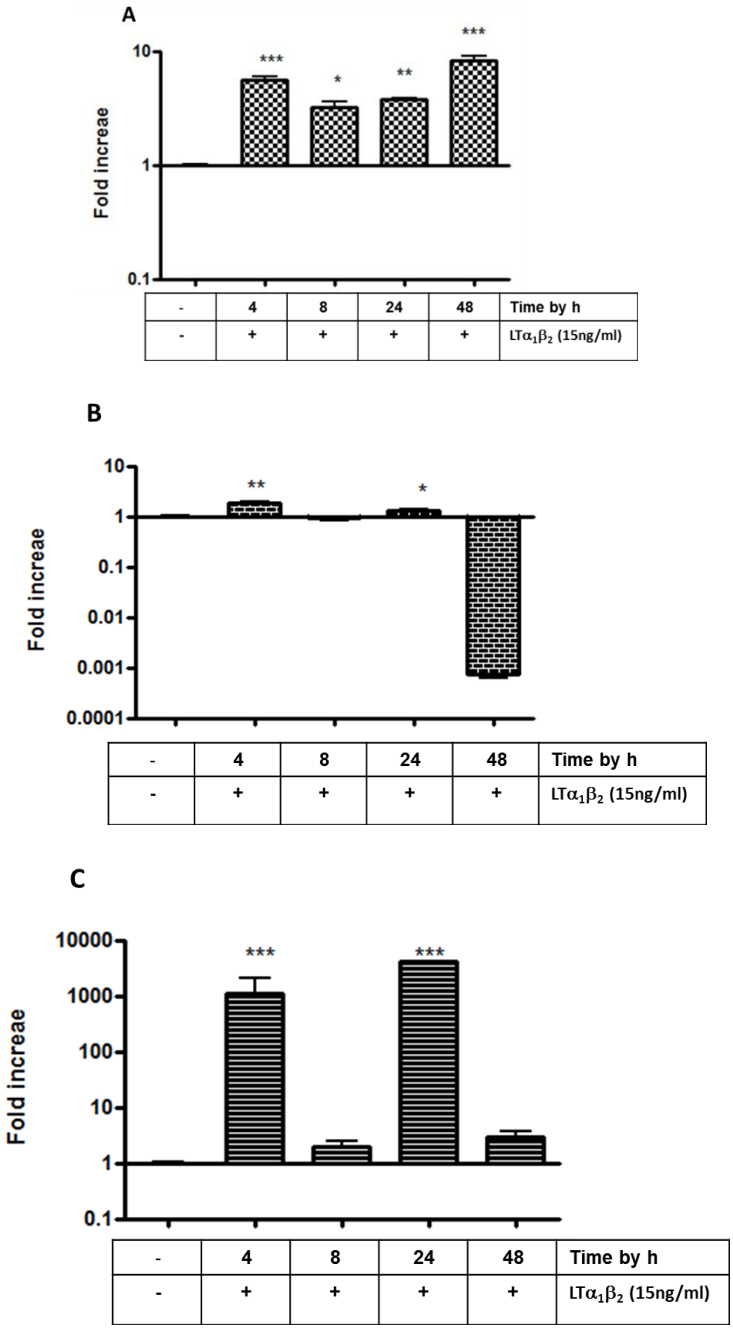


Figure 5.5 LTα$_1$β$_2$-induced VCAM-1 mRNA expression in Panc-1 cells

Cells were treated with LTα$_1$β$_2$ (15ng/ml) for the times indicated, total RNA was extracted as outlined in section 2.9.1. QPCR analysis was performed on the cDNA using primers designed to detect BBC3 as described in Methods Section 2.9.4. Relative expression levels of VCAM-1 mRNA transcripts were normalized to reference genes GAPDH, NCL and 18s using delta-delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean ± SEM of triplicate determinations from a single experiment. ***P< 0.001, **P< 0.01 compared with untreated cells.

5.2.3 Characterisation of the role of non-canonical NFκB pathway activation in the expression of MAP3K14 (NIK) and SERPINB6 genes

As described in chapter one NIK plays a critical role in the activation of IKKα and the non-canonical NFκB pathway (Nishina, et al, 2009). Interestingly the gene responsible for encoding NIK protein was also enhanced in the gene array and therefore examined here. In the first experiment (Figure 5.4A), the expression of MAP3K14 was significantly increased at 24 and 48h of stimulation, in the second (Figure 5.4B), the expression of MAP3K14 was increased only at 24h of stimulation (not significantly), whilst, the third experiment results shows a significant increase in MAP3K14 at 24h of stimulation (4.22 ± 0.07 fold increase) Figure 5.4C.

Expression of another gene, SERPINB6 was examined, this gene encode protein called maspin which have an important role in prevention number of processes that implicate in cancer development, for example, invasion, resistance to apoptosis and metastasis (Jiang et al., 2002, Sheng et al., 1996, Zhang et al., 2000). in the first two experiments, there was a delay in response of $LT\alpha_1\beta_2$ stimulation, and the level of SERPINB6 mRNA expression was significantly increased after 48h of stimulation in both experiments compared with untreated cells, see Figure 5.5A and B. In contrast, the third experiment showed there was a significant increase in expression of SERPINB6 mRNA after 24h of stimulation (Figure 5.5C).

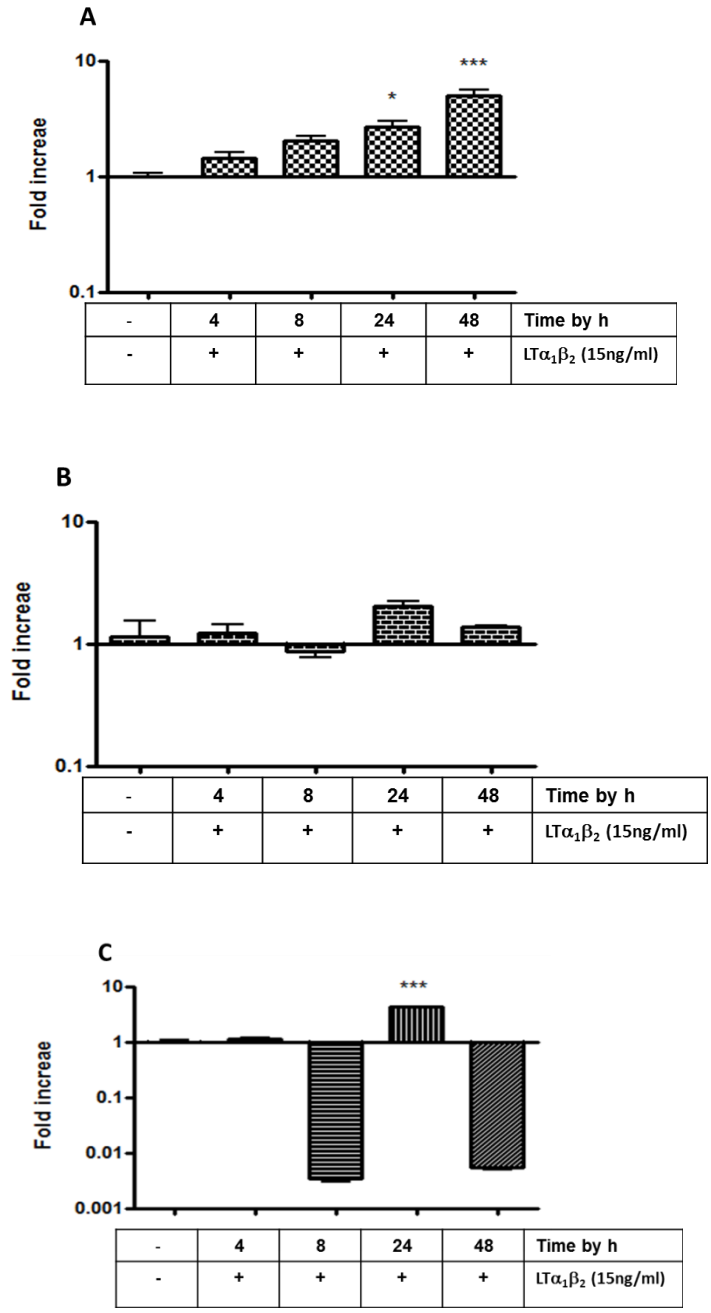


Figure 5.6 LT$\alpha_1\beta_2$-induced MAP3K14 mRNA expression in Panc-1 cells

Cells were treated with LT$\alpha_1\beta_2$ (15ng/ml) for times indicated, total RNA was extracted as outlined in section 2.9.1. QPCR analysis was performed on the cDNA using primers designed to detect MAP3K14 as described in Methods Section 2.9.4. Relative expression levels of MAP3K14 mRNA transcripts were normalized to reference genes GAPDH, NCL and 18s using delta-delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean ± SEM of triplicate determinations from a single experiment. ***P< 0.001, *P< 0.05 compared with untreated cells.

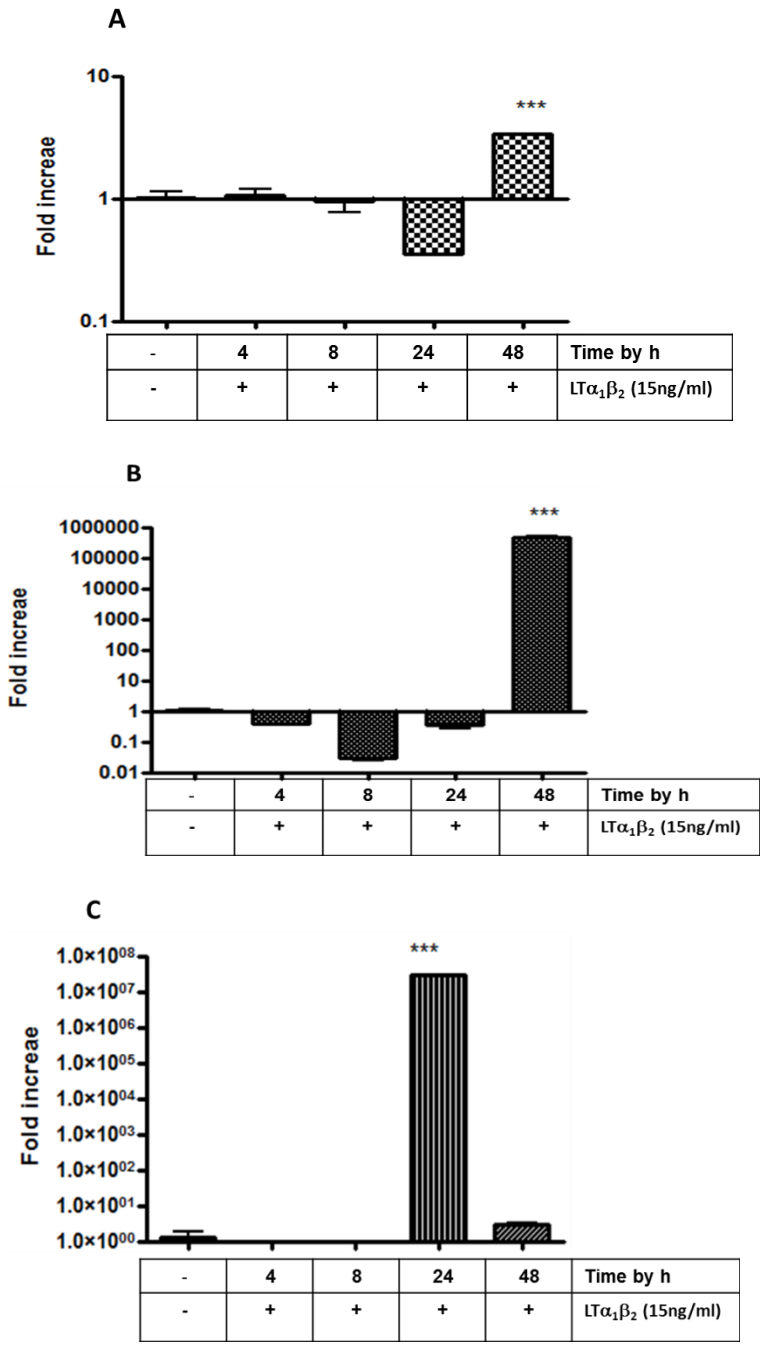


Figure 5.7 LT$\alpha_1\beta_2$-induced mRNA SERPINB6 expression in Panc-1 cells

Cells were treated with LT$\alpha_1\beta_2$ (15ng/ml) for time indicated, total of RNA were extracted as outlined in section 2.9.1. QPCR analysis was performed on the cDNA using primers designed to detect SERPINB6 as described in Methods Section 2.9.4 Relative expression levels of SERPINB6 mRNA transcripts were normalized to reference genes GAPDH, NCL and 18s using delta-delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean ± SEM of triplicate determinations from a single experiment. ***P< 0.001, compared with untreated cells.

5.3 The effect of IKKα inhibitors on expression of target genes

In order to investigate the effects of IKKα inhibitors on the regulation of the expression of genes identified from $LT\alpha_1\beta_2$ time course experiments, SU1349 and SU1433 were utilised in expression experiments. As both compounds showed good functional selectively as IKKα inhibitors in Panc-1 cells, it was decided to use these to examine the role of IKKα in the regulation of these genes. Figures 5.2-5.7 indicated that the expression of most of target genes mRNA were increased after 24h of stimulation with $LT\alpha_1\beta_2$ (15ng/ml), therefore this time point was chosen. Two experiments were conducted in parallel. Panc-1 cells were pre-treated separately with 1μM of either SU1349 and SU1433 for 1h then stimulated with $LT\alpha_1\beta_2$ (15ng/ml) for 24h. A first set of samples were prepared for qPCR, whilst a second set were blotted for p52 and p-p100. These control experiments demonstrated a consistent and significant inhibition in the phosphorylation of p100 and p52 formation in Panc-1 cells indicating that SU1349 and SU1433 treatment had been effective in targeting IKKα and the non-canonical NFκB pathway (see Figure 5.8.).

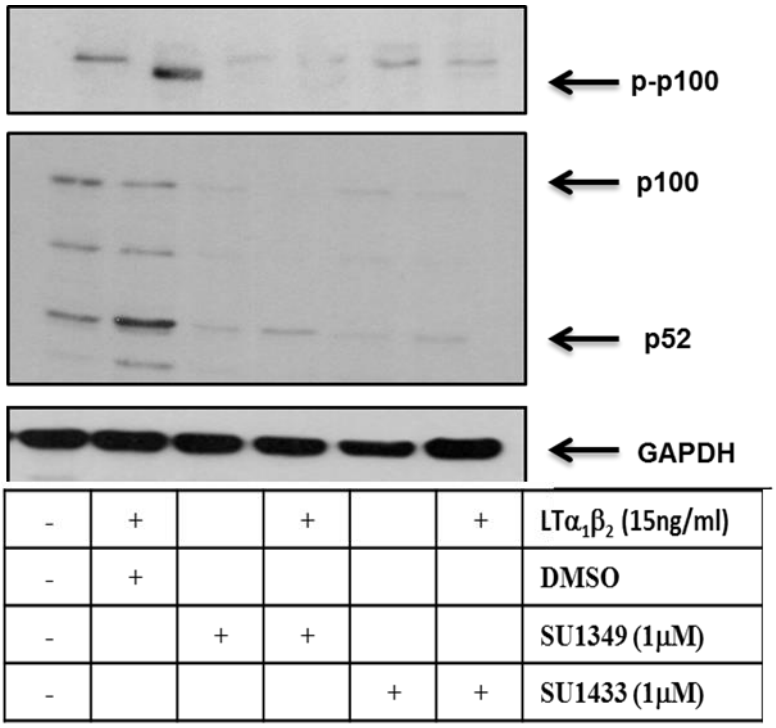


Figure 5.8 The effect of SU1349 and SU1433 on LT$\alpha_1\beta_2$-induced phosphorylation of p100 and formation p52 in Panc-1 cells

Cells were pre-treated with 0.005% DMSO or 1μM of SU1349 or SU1433 or 0.005% DMSO for 1h and then exposed to LT$\alpha_1\beta_2$ for 24 h. Whole cells lysates were prepared as outlined in Methods Section 2.3.1. Cell extracts were separated by SDS-PAGE blotted for p-p100 then stripped for p100/p52 and GAPDH, which was used as a loading control. The blot is representative of three independent experiments.

5.3.1. The effect of SU1349 and SU1433 in the expression of VCAM-1, TNFAIP3 MAP3K14, BBC3 and EZH2 genes

As shown in Figure 5.9 $LT\alpha_1\beta_2$ stimulated a significant increase in the expression of the VCAM-1 gene compared to untreated cells (29.96 ± 12.17 fold increase), however, pre-treating the cells with 1μM of either selective inhibitor prior to $LT\alpha_1\beta_2$ significantly inhibited the expression of VCAM-1 (0.11 ± 0.007 or 0.01 ± 0.002 fold). Interestingly, SU1349 alone mediated a consistent and substantial inhibition of basal gene expression (0.03 ± 0.003 fold).

A similar result was noted for TNFAIP3 as shown in Figure 5.10. The IKKα inhibitors used in the study also significantly inhibited $LT\alpha_1\beta_2$-induced mRNA expression of TNFAIP3 in Panc-1 cells (fold stim 1.5 ± 0.21, 4.5 ± 0.85) compared with stimulated control (144.04 ± 2.31 fold). In addition, treatment of cells with SU1349 or SU1433 alone increased the basal level of TNFAIP3 in two experiments, in contrast in the third experiment the IKKα inhibitors significantly decreased expression (SU10.004 ± 0.0003, 0.02 ± 0.02 fold) vs untreated cells (1.2 ± 0.49 fold increase) similar results were reported in the second and third experiments.

In Figure 5.11, $LT\alpha_1\beta_2$ stimulated a substantial increase in the expression of MAP3PK14 (265.41 ± 32.71 fold) compared with untreated cells (1.0 ± 0.01 fold). However, both SU1349 and SU1433 caused a significant decrease in expression of MAP3PK14, in comparison to agonist-stimulated control. Furthermore, treatment of cells with SU1349 and SU1433 alone resulted in a decrease in the expression of MAP3K14 in two independent experiments, compared with controls (see Figures 5.10A and C).

The expression of another target gene, BBC3, was also examined as shown in Figure 5.11A. $LT\alpha_1\beta_2$ induced a significant increase in the mRNA expression of BBC3 (5039.79 ± 1929 fold) compared with untreated cells (1.08 ± 0.13 fold). Pre-treatment with SU1433 and SU1349 reduced the response to $LT\alpha_1\beta_2$ by over 95% in each case to (fold stimulation =1.57 ± 0.1, 0.2 ± 0.002, n=3 $p<0.005$) respectively compared with agonist-stimulated control. When Panc-1 cells were treated with inhibitors alone, only SU1433 decrease the expression of BBC3 (fold stim 0.18 ± 0.02 fold) compared with untreated sample, SU1349 was without effect.

Similarly, cells stimulated with $LT\alpha_1\beta_2$ prompted a significant increase in EZH2 mRNA expression (5.74 ± 0.78 fold n=1 $P<0.001$) in comparison with untreated sample (1± 0.16 fold). The ability of $LT\alpha_1\beta_2$ induce the expression of EZH2 was significantly inhibited following pre-treatment of cells with SU1439 and SU1433 (0.14 ± 0.01, 1.04 ± 0.01 fold) respectively, compared with agonist-stimulated control. In contrast with the results obtained for BBC3, cells pre-treated with SU1439 alone resulted in a significant decrease in the basal level of EZH2 mRNA compared with untreated cells. As with some of the other genes above (Figure 5.10B, Figure 5.11A and 5.11B), SU1433 alone caused a small increase in expression (1.73 ± 0.51 fold) compared with untreated cells (see Figure 5.11B).

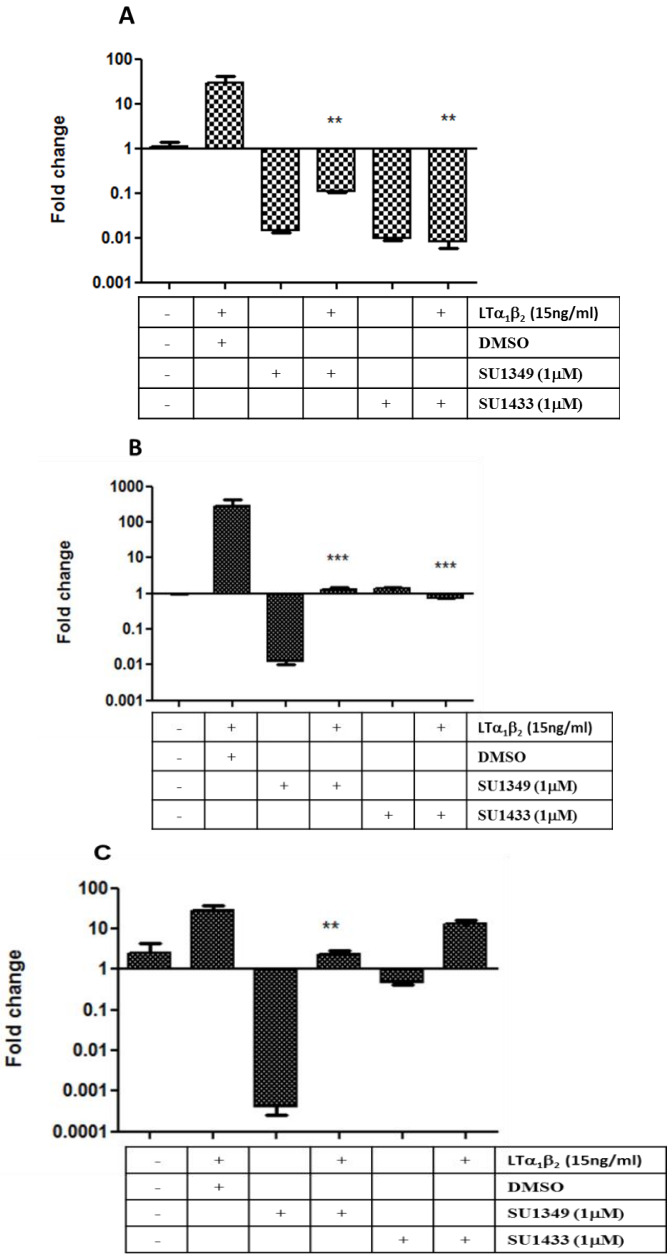


Figure 5.9 The effect of SU1349 and SU1433 on LTα$_1$β$_2$-induced expression of VCAM-1 mRNA in Panc-1 cells

Cells were treated with SU1349 and SU1433 or 0.005% DMSO for 1h and then stimulated with LTα$_1$β$_2$ (15ng/ml) for 24h. Total RNA was extracted as outlined in Section 2.9.1. QPCR analysis was performed on the cDNA using primers designed to detect VCAM-1 as described in Methods Section 2.9.4. Relative expression levels of VCAM mRNA transcripts were normalized to reference genes GAPDH, NCL and 18s using Delta-Delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean ± SEM of triplicate determinations from a single experiment. ***P< 0.001, **P< 0.01 compared with stimulated control.

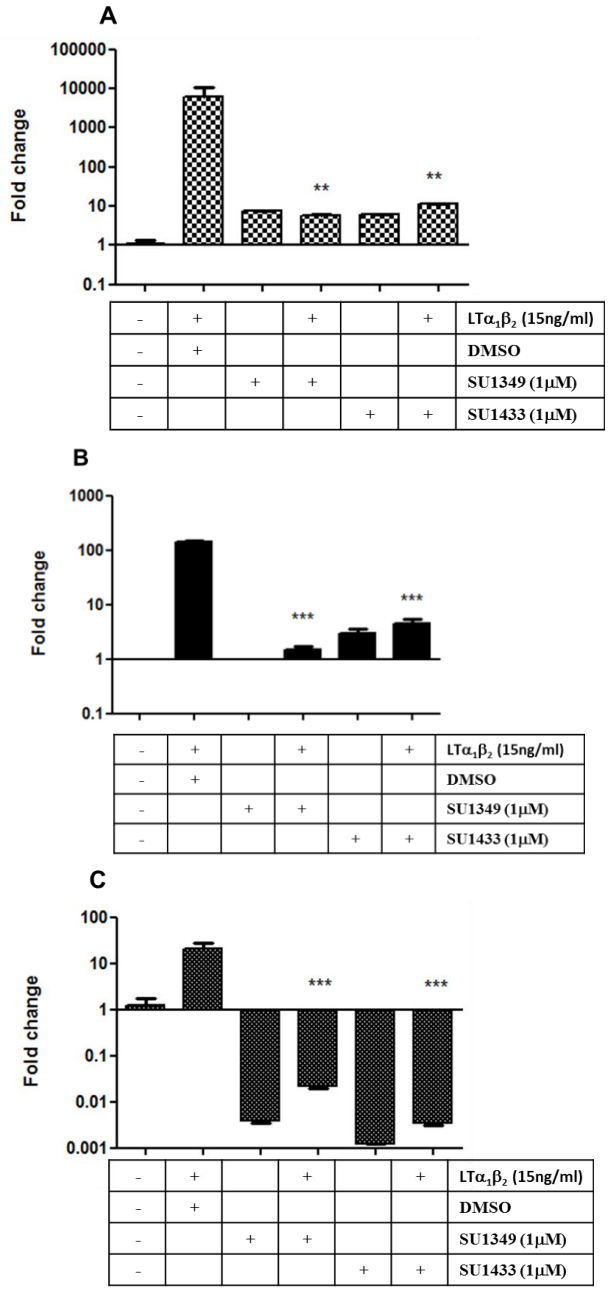


Figure 5.10 The effect of SU1349 and SU1433 on LT$\alpha_1\beta_2$-induced expression of TNFAIP3mRNA in Panc-1 cells

Cells were treated with SU1349 and SU1433 or 0.005% DMSO for 1h and then stimulated with LT$\alpha_1\beta_2$ (15ng/ml) for 24h, total RNA was extracted as outlined in Section 2.9.1. QPCR analysis was performed on the cDNA using primers designed to detect TNFAIP3 as described in Methods Section 2.9.4. Relative expression levels of TNFAIP3 mRNA transcripts were normalized to reference genes GAPDH, NCL and 18s using Delta-Delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean ± SEM of triplicate determinations from a single experiment. ***P< 0.001, **P< 0.01 compared with stimulated control.

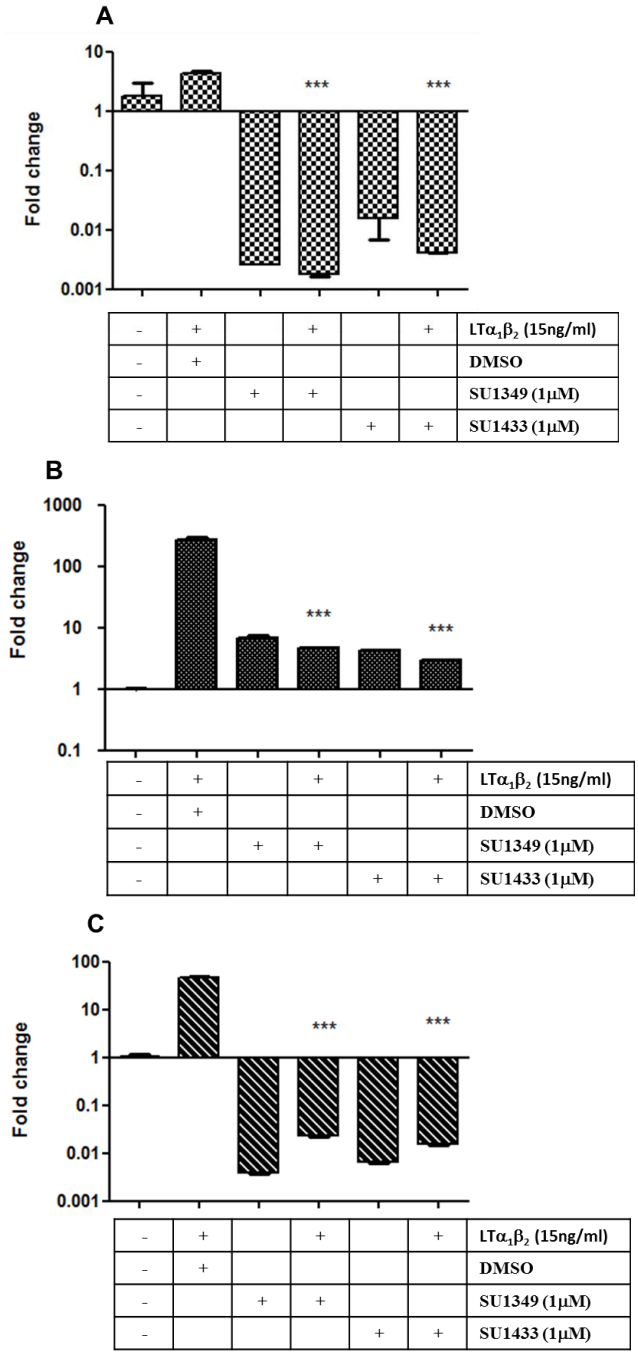


Figure 5.11 The effect of SU1349 and SU1433 on LT$\alpha_1\beta_2$-induced expression of MAP3K14 mRNA in Panc-1 cells

Cells were treated with SU1349 and SU1433 or 0.005% DMSO for 1h and then stimulated with LT$\alpha_1\beta_2$ (15ng/ml) for 24h, total RNA was extracted as outlined in Section 2.9.1. QPCR analysis was performed on the cDNA using primers designed to detect MAP3K14 as described in Methods Section 2.9.4. Relative expression levels of VCAM mRNA transcripts were normalized to reference genes GAPDH, NCL and 18s using Delta-Delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean ± SEM of triplicate determinations from a single experiment. ***P< 0.001compared with stimulated control.

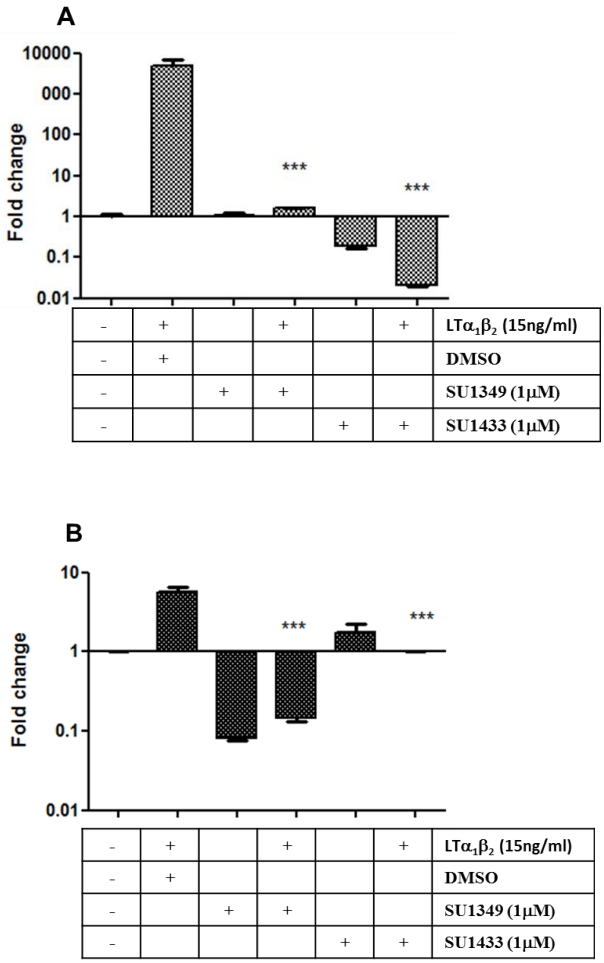


Figure 5.12 The effect of SU1349 and SU1433 on LT$\alpha_1\beta_2$-induced expression of BBC2 and EZH2 mRNA in Panc-1 cells

Cells were treated with SU1349, SU1433 or 0.005% DMSO 1h and then stimulated with LT$\alpha_1\beta_2$ (15ng/ml) for 24h. Total RNA was extracted as outlined in Section 2.9.1. QPCR analysis performed on the cDNA using primers designed to detect BBC3 panel A and EZH2 panel B as described in Methods Section 2.9.4. Relative expression levels of BBC3 and EZH2 mRNA transcripts were normalized to reference genes GAPDH, NCL and 18s using Delta-Delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean ± SEM of triplicate determinations from a single experiment. ***P< 0.001compared with stimulated control.

5.4 Discussion

Emerging literature has established an important role for the non-canonical NFκB pathway in the regulation of a number of genes including CCL19, CXCL13, CCL21, CXCL12, BAFF and COX-2. These genes play key roles in a number of biological processes, as well as cellular events implicated in tumour development such as resistance to both chemotherapy and apoptosis (Wharry et al., 2009). Wharry and co-workers have identified overexpression of these genes in both Panc-1 and MiaPaCa-2 cells due to constitutive activation of the non-canonical NFκB pathway (Wharry et al., 2009). In this chapter a number of IKKα-dependent genes were examined by; firstly, assessing which genes were upregulated by the activation of the non-canonical pathway, and secondly, pharmacological regulation of their expression. This builds on a rather limited literature assessing their regulation.

This chapter has demonstrated that a number of genes are regulated by the non-canonical NFκB pathway. We initially investigated IL-6 as this cytokine has been strongly implicated in pancreatic cancer development and would be an attractive target for regulation. For example, studies have shown that IL6 have a role in the initiation of pancreatic cancer development (Lesina et al., 2011, Fukuda et al., 2011, Corcoran et al., 2011). Another study reported that IL6 is essential for regulation of the inflammatory microenvironment through each stage of pancreatic cancer progression (Zhang et al., 2013).

The promoter region of IL6 does contain a binding site for p65 NFκB but no other studies have identified sites for any other isoforms (Ray et al., 1988, Deng et al., 2002). Indeed, Yoon and co-workers confirmed that in their study, treatment of Hela

cells with NFκB inhibitors (JSH-23) or shRNA for p65 NFκB under starvation conditions inhibited classical NFκB activity and reduced IL6 mRNA expression (Yoon et al., 2012). These and other studies (Chou et al., 2005, McFarland et al., 2013) implicate the classical NFκB activation in IL6 expression; the results in this chapter demonstrate that activation the non-canonical of NFκB pathway using $LT\alpha_1\beta_2$ was unable to promote an increase in IL6 expression.

A number of further variations apply for other genes and give clues to the mode of regulation. For example EZH2, a protein which modulates many cellular events in cancer development, including, cell adhesion and inflammation (Dreger et al., 2012). In this chapter it was found that $LT\alpha_1\beta_2$ stimulated a strong and significant increase in EZH2 expression which was abolished by IKKα inhibition clearly implicating a role for IKKα. However, there are very limited studies to confirm these findings, one study has shown that there is a correlation between activation of the non-canonical NFκB pathway and EZH2 expression in a breast cancer cell lines such as MDA-MB 231 (Lee et al., 2011), whilst in SLK and BOEC cells, inhibition the classical NFκB pathways using BAY-11 led to a 80% reduction in expression of EZH2 (He et al., 2012). A further study demonstrated a negative correlation between EZH2 and the IKKα gene; decreased expression of EZH2 in nasopharyngeal tumour cell lines such as CNE2 cells was accompanied by a high level of IKKα mRNA, whilst knock-down of IKKα using siRNA resulted in the opposite outcome (Yan et al., 2014). Furthermore, EZH2 can also regulate the binding of RelB and p52 heterodimers to gene promoters enhancing the expression NFκB target genes, an

effect dependent on ER status (Lee et al., 2011). This makes interpretation of the role of IKKα in the regulation of EZH2 expression and function difficult.

Despite these issues the results in this chapter still suggests that targeting of EZH2 via pharmacological inhibition of IKKα may be beneficial. Many studies have found that resistance to cisplatin, a chemotherapy for ovarian cancer, is related to an increase in EZH2 expression (Lee et al., 2011). Similarly, progression of bladder (Yun et al., 2011), lung, gastric (Srimongkolpithak et al., 2014) and renal cancers have been linked with elevated expression of EZH2. It is likely that in some of these cancers the non-canonical NFκB pathway is also over active making targeting of EZH2 a more effective strategy.

One gene which seems to be regulated by both canonical and non-canonical NFκB pathways is VCAM-1. The VCAM-1 promoter has a number of binding sites for different signalling pathways such as GATA, AP-1 and NFκB, (Cybulsky et al., 1991, Neish et al., 1992). Indeed many studies have reported a role for the classical NFκB pathway in VCAM-1 expression (Chen et al., 1998, Chen and Ghosh, 1999, Shu et al., 1993). In this chapter it was found $LT\alpha_1\beta_2$ which strongly activates the non-canonical NFκB pathway, has the ability to induce the expression of VCAM-1 in Panc-1 cells. Furthermore, selective inhibition of IKKα resulted in a marked reduction in mRNA expression suggesting a key role for this pathway. However, it is noted that $LT\alpha_1\beta_2$ stimulated a delayed activation of the canonical NFκB pathway (Chapter 3) and therefore this activation may also be required to increase VCAM-1 expression in these cells. This possibility could have been tested, if time allowed,

using siRNA or IKKβ selective compounds. Further studies are required to fully characterise the role of IKKα in VCAM-1 expression including the use of more selective agonists, siRNA and the use of KO mouse models.

Other genes examined in this chapter gave a similar interpretation. For example, whilst in this study IKKα was strongly implicated in Maspin expression, in other studies both pathway seems to be implicated (Guo et al., 2011). Guo and co-workers have reported that PC-3 cells stimulated with TNFα led to increase the level of both NFκB pathway proteins such as, RelA, RelB, p100 and p52 this was accompanied by a decrease in the expression of Maspin. From the results in this chapter $LT\alpha_1\beta_2$ was found to induce Maspin expression in Panc-1 cells but this was after 48 hours, rather than 24h as shown in the Guo study, and lack of strong IKKβ-canonical medtated NFκB activation may slow expression down. A similar interpretation is also noted for BBC3, the induction of which has again been demonstrated to involve both pathways (Kanduri et al., 2011). Again the results may suggest a sufficient level of activation of both NFκB pathways in Panc-1 by $LT\alpha_1\beta_2$ is required to drive maximal expression. For each gene a role in pancreatic cancer is implicated (Maass et al., 2001, Chen et al., 2012) thus inhibition of each gene through potentially achievable blockage of IKKα may be worthwhile.

An additional finding of interest involved TNFAIP3, or A20. TNFAIP3 functions as inhibitor of classical NFκB signalling pathways (Pujari et al., 2013, Shembade and Harhaj, 2012). However, it has also been found that overexpression of TNFAIP3 in HEK293T cells using retrovirus mediated gene transfer resulted in increased NIK

and p52 formation, similar results were also obtained in MDA-AB-231 cells (breast cancer cells) (Yamaguchi et al., 2013). Similarly it was reported that overexpression of TNFAIP3 reduced the activity of NFκB pathways in 293T cells (Sakakibara et al., 2013). Taken together, these results suggest that TNFAIP3 has a role in the negative regulation of the canonical NFκB pathway, whilst enhancing the activation of the non-canonical NFκB cascade. The finding that A20 expression can be increased through $LT\alpha_1\beta_2$ stimulation suggests the potential of a negative feedback loop; activation of the non-canonical NFκB pathway over the longer term could prevent the activation of the classical NFκB pathway. This has the potential to temper the potential of supra-maximal activation of the canonical pathway which is more strongly implicated in inflammation. Future studies could involve treatment of cells with $LT\alpha_1\beta_2$ followed by activation of the canonical NFκB pathway to determine if this form of regulation applied to Panc-1 cells.

Another interesting finding involved the expression of NIK. NIK is essential for the activation of the non-canonical NFκB pathway, blocking the expression NIK in Panc-1 using siRNA inhibited processing of p100 to p52 whilst proliferation was reduced (Nishina et al., 2009). However, a number of studies have demonstrated that NIK is normally constitutively expressed and that activation involves rapidly accumulation of the NIK protein through inhibition of proteosomal degredation (Doppler et al., 2013, Pan et al., 2008a). In this chapter, it was found that the NIK gene, MAP3K14 was significantly increased by activation of the non-canonical NFκB pathway via $LT\alpha_1\beta_2$ stimulation, results confirmed using the selective IKKα inhibitors, and suggesting increased expression of NIK as a route to pathway

activation. However, in additional Western blot studies it was found that NIK expression was low and not significantly increased by long term $LT\alpha_1\beta_2$ treatment (Chapter 3). These Western blots results did not agree with other studies which show very high levels of NIK expression in Panc-1 cell lines and patients samples, associated with increased activation of non-canonical NFκB signalling (Doppler et al., 2013). Taken together it does suggest that increased NIK expression mediated by IKKα signalling may lead to sustained or hyper-activation of the non-canonical NFκB pathway itself and thus may be relevant in cancer.

In conclusion this chapter demonstrated the potential for IKKα via the non-canonical NFκB pathway to play a significant role in the regulation of a number of genes implicated in pancreatic cancer development. Further studies looking at protein expression levels and subsequent effects upon the relevant pathways or functional outcomes are required to confirm these findings.

Chapter Six

General Discussion

6.1 General discussion

Pancreatic cancer is one with a high mortality; it is currently the fourth most common cancer and is expected to be the second within a decade (Bailey et al., 2016, Waddell et al., 2015). There are a number of significant issues with this cancer, for example, it is hard to diagnosis in early stages, it is a very aggressive cancer, survival rates are very low and there are very limited options for treatment (Prabhu et al., 2014, Wang et al., 2013). Success in the development of new treatments is essential to combat the negative outcomes of such a cancer. In this thesis I examined the potential use of IKKα inhibitors in the treatment of pancreatic cancer by, in the first instance, analysing a number of selective IKK inhibitors against a number of parameters linked to proliferation outputs including cell cycle progession and cell growth.

In actual fact most cases of pancreatic cancer display a high frequency of mutations in a number of genes, in particular the Kirsten rat sarcoma viral oncogene homolog (KRAS). This mutation leads to modification and activation of a number of signalling pathways which participate in the development of resistance to treatment (Prabhu et al., 2014). The oncogenic potential of KRAS is linked to the regulation growth factor mediated signalling. Mutations of KRAS are detected in 90% of early grade pancreatic cancer (pancreatic intraepitheilia neoplasis lesions) (Kanda et al., 2012). The KRAS protein and itis variants activate a number of signalling pathways implicated in pancreatic cancer development, for example, ERK, Glycogen synthase kinase-3 (GSK3) and NFκB itself. A number of studies have demonstrated that KRAS mutations may be involved in constitutive activation of both NFκB pathways

in pancreatic cancer (Rachagani et al., 2011, Maier et al., 2013, Ling et al., 2012, Chiao and Ling, 2011, Pak and Miyamoto, 2013).

These data suggest that targeting KRAS is good strategy for the development of pancreatic cancer treatments, however, to date there are no successful therapies developed as a result (Oliveira-Cunha et al., 2011, Collins and Pasca di Magliano, 2013). Recently, alternative approaches targeting kinases downstream of RAS have become a topic of interest for many research groups, including ERK, MEK-1 and RAF-1 (Collins and Pasca di Magliano, 2013). Whilst some progress has been made there has been no discovery of significant clinical impact and new targets are required.

The studies examining the role of the ERK signalling pathway in pancreatic cancer nevertheless provide an exemplar for this thesis which examines the role of IKKα. As long ago as 1999, NFκB was found to be constitutively active in most cases of pancreatic cancer (Wang et al., 1999, Prabhu et al., 2014), and a large body of evidence has demonstrated that both NFκB pathways play an important role in the development and progression of pancreatic cancer and other cancers (Aggarwal and Shishodia, 2006, Lu et al., 2004, Lu and Stark, 2004, Cogswell et al., 2000, Prabhu et al., 2014). In addition, NFκB activation is a significant factor in the development chemo-resistance and eventual metastasis (Oeckinghaus and Ghosh, 2009, Ling et al., 2012).

The majority of studies have focussed on targeting IKKβ, the key upstream activator of the canonical NFκB pathway as an anti-cancer treatment strategy. There are

several IKKβ inhibitors such as PS-1145, ML120B and TPCA-1, currently in use in experimental setting. Experimentally, these inhibitors have been investigated in different cancer cell lines and *in vivo* cancer models, and found to be effective however, there are a number of serious side effects and toxicities in normal cells reported when using these inhibitors clinically (Nomura et al., 2016, Castro et al., 2003, Chariot, 2009, Gamble et al., 2012b). Initial findings in chapter 3 (Figures 3.4-3.8) confirmed activation of the non-canonical NFκB pathway could be prolonged in both Panc-1 and MiaPaCa-2 cells. Therefore, reducing the activity of the non-canonical NFκB pathway via IKKα inhibition could be a good approach for the treatment of pancreatic cancer.

There are a number of issues to be considered in the development of selective IKKα inhibitors. Firstly IKKα and IKKβ share more than 50% sequence homology within N-terminal kinase domains, thus generating selective ATP binding site inhibitors is a big challenge (Israel, 2010, DiDonato et al., 1997, Gamble et al., 2012b). Secondly, at the time of writing this project the crystal structure of IKKα has not been resolved, but a recent study has found the structural basis for activation of IKKα (Polley et al., 2016), thus new compounds have had to be developed by standard determination of structure activity relationships (SAR). Thirdly, all new compounds need to have appropriate physiochemical properties including solubility and resistance to degradation and clearance. The small molecule drug discovery group at the University of Strathclyde provided a series of chemical compounds for assessment in Panc-1 cells.

The results in this study demonstrated for the first time that these compounds were selective inhibitors of IKKα dependent non-canonical NFκB pathway signalling in Panc-1 cells as measured by reduction in phosphorylation of p100 and formation and nuclear translocation of p52 NFκB. In addition, it was demonstrated that these inhibitors could arrest cell cycle progression, inhibit cell growth and colony formation. To confirm and extend these findings a number of other cellular parameters could be tested including effects upon apoptosis, inflammatory gene expression, key components of the tumour environment, and initial cellular events associated with angiogenesis such as cell migration and VEGF formation.

Despite this breakthrough there is clearly further optimisation and testing required. For example, there is the potential of off-target effects, commercial kinase profiling of compounds showed the inhibition of a number of others kinases (personal Communication, A. Paul). Off targets effects may for example explain why IC_{50} values are much lower for some compounds following a shorter stimulation time compared with 24 h incubation (Chapter 4). Effects upon the expression of p100 itself could be a factor or the potential of regulation by another pathway as yet uncharacterised. In this regard it was significant that siRNA, whilst abrogating the expression of IKKα really effectively had no inhibitory effect on p52 formation.

How could the findings in this thesis be extended to other aspects of the cancer drug discovery process? It is now accepted that combining different anti-cancer drugs is more effective than using them separately, through increasing the efficacy against cancer cells whilst simultaneously reducing the chance of developing drug resistance (Komarova and Boland, 2013). A number of studies have shown that blocking the

activity of NFκB in combination with chemotherapy or irradiation induces a more favourable outcome (Baldwin, 2001, Yamamoto and Gaynor, 2001, Nakanishi and Toi, 2005). For example, targeting the EGFR using the HER/EGFR tyrosine kinase inhibitor, Erlotinib, in combination with gemcitabine improved survival rates of pancreatic cancer patients; one year survival was increase to 23% in patients using combination treatment compared with 17% who used gemcitabine alone (Moore et al., 2007). A preliminary study in the Boyd laboratory (SIPBS) reported an additive effect using a combination of SU1261 and radition therapy, the results showed a siginificant difference in survival fraction (SF) during combined treatment compared with cells treated with irradiation alone, (Khalid personal communication). Therefore investigating the effect of combination therapy using selective IKKα inhibitors and irradiation could be an attractive approach in pancreatic cancer treatment.

This approach could be further developed when considering different sensitivies of pancreatic cancer cell to chemotherpay. Pan and co-worker have identified two groups of pancreatic cancer cell lines depending on their response to gemciatabine-induced apoptosis; sensitive (BxPC-3, l3.6pl and CFPAC-1) and resistance (Panc-1, MiaPaCa-2 and Mpanc96) (Pan et al., 2008b). In same study they examined the effect of p65 NFκB knockdown combined with gemciatabine treatment on both cells types, they reported enhanced gemciatabine-induced apoptosis following NFκB inhbition in the sensitive cell group compared with the resitant group (Pan et al., 2008b). In this thesis both Panc-1 and MiaPaCa-2 cells were used, both are in the resistance group, therefore using selective IKKα inhibitors combined with

gemciatabine treatment could be a good strategy to reduce the resistance of these cells to single drug challenge.

An important strategy to investigate the potential effect of IKKα inhibitors is the use of *in vivo* cancer models. Today there are 17 human pancreatic cancer cell lines; these model systems have many advantages, for example, easy manipulation of signalling pathways using pharmacological inhibitors and molecular tools such as siRNA. Despite this, there is a risk that some of these models lose features which mirror human disease as a result of long-term *in vitro* culture conditions (Lee et al., 2006). Therefore, using animal models would be a good strategy for confirming the potential clinical efficacy of IKKα inhibitors in particular, genetically engineered mouse models (GEMM) and primary xenografts.

For pancreatic cancer there are a number of GEMMs. For example, LSL-Keas^{G12D}, Pdx-1^{Cre}, Kras-p53^{Cre} and p48^{Cre}. These GEMMs have been used to evaluate the ability of new drugs to prevent the pancreatic cancer development, some of these models are applicable for testing NFκB inhibitors (Mohammed et al., 2015). These models could be used investigate the effect of IKKα inhibitors *in vivo* in further studies, either alone or combined with other pancreatic anti-cancer treatments. Primary xenografts or patients-derived xenografts have been examined in some instances utilising Panc-1 and MiaPaCa-2 tumours (Dey et al., 2016, Suenaga et al., 2016).

While the results in Chapter 5 link the IKKα pathway, for the first time, to the expression of a number of genes in Panc-1 cells, this work could be extended in a

number of ways. For example, one of the challenges is in identifying target engagement *in vivo* beyond just showing inhibition of tumour development *per se*. There have been few published studies, using tissues sections, which are able to examine the outcomes of IKKα inhibition *ex vivo*. One study used Western blotting following protein extraction of tissues samples (Jin et al., 2012). Direct inhibition of IKKα phoshorylation and p52 formation may not be easy to measure and quantify using such an approach. One strategy maybe to utilise surrogate markers particularly genes that have identified as being sensitive to pharmacological inhbition of IKKα for example, TNFAIP3, EZH2 and BBC3. In utilising the *in vivo* pancreatic cancer models, it would be important to ensure that the expression of these genes was abrogated in tissue sections from inhibitor treated mice. Preliminary results from the Boyd laboratory have shown regression of prostate cancer xenografts using SU1261, examining the effect of selective IKKα inhibitors in animal models of both prostate and pancreatic cancer must be accompanied by examination of surrogate IKKα-dependent gene marker.

In the last two years, novel biomarkers have been identified for early diagnosis of pancreatic cancer, such as Carbohydrate antigen 19-9 (CA19-9) and Carcinoembryonic antigen (CEA), CA19-9, and these biomarkers are not produced in all pancreatic tumours or tumour recurrence after surgery. To date there is no molecular targets which could be used in the clinic as a routine biomarker (Herreros-Villanueva and Bujanda, 2016), therefore assessing IKKα expression or IKKα dependent- target genes using siRNA or IKKα inhibitors in xenografts could be an initial step the generation of a panel of selective biomarkers for pancreatic cancer.

The four subtypes of pancreatic tumours encompass differences in the molecular changes during pancreatic cancer development and as such may enable selective therapies. For example, targeting KRAS signalling for the third tumour subtype may give rise to better clinical outcomes than observed previously (Collins and Pasca di Magliano, 2013). Recently, targeting the downstream-mediators of KRAS signalling has been considered as an alternative approach to the development of new pancreatic cancer treatments. One of these downstream-mediators is NFκB; therefore, a future strategy may be to investigate the role of the non-canonical NFκB pathway and the effect of IKKα inhibitors in the third tumour subtype. This might include again a combination/adjunct therapy approach depending of the sensitivity of the third tumour type to standard or targeted chemotherapy. As KRAS mutations increase non- canonical NFκB signalling the third tumour type might be initially tested.

In conclusion, this thesis has shown for the first time the development and utility of first-in-class selective IKKα inhibitors for use in pancreatic cancer cells, to prevent proliferation. However, considerably more studies include animal testing *in vivo* and clinical studies in humans are required to ensure a new medicine can be developed from this work.

Chapter Seven

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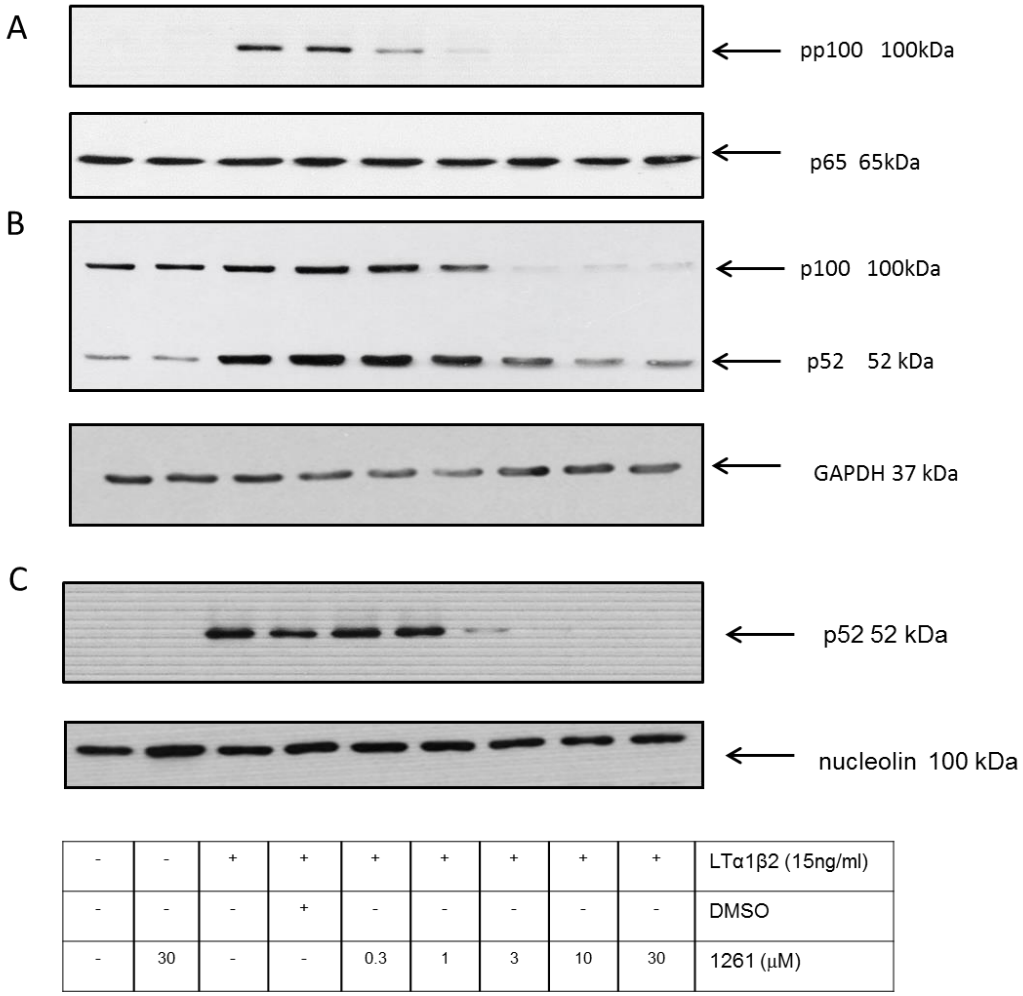
Appendixes



-	-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	0.3	1	3	10	30	1261 (μM)

Panel A

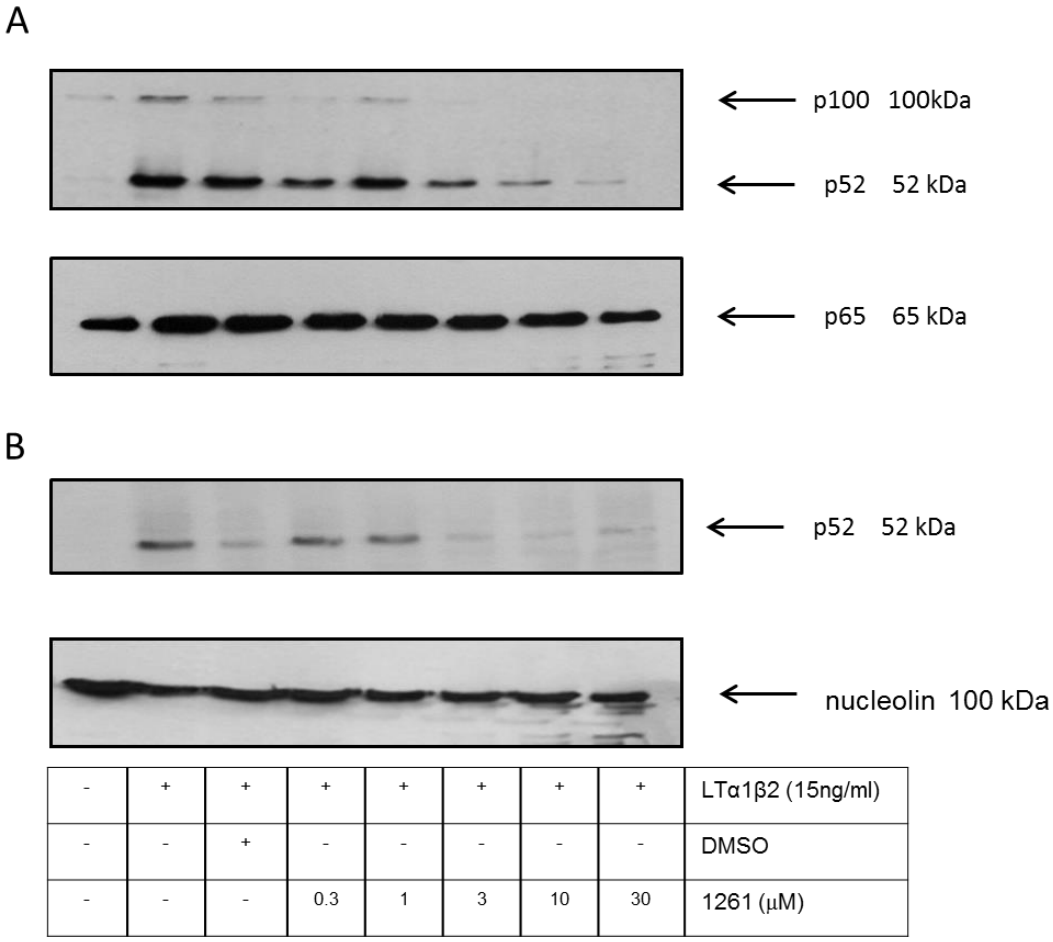
Panc-1 cells were pre-treated with 1261 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by W.B. assessed for p-p100 (100 kDa) and p65 (65 kDa) which was used as loading control

Panel B

Panc-1 cells were pre-treated with SU1261 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 24h. Whole cell extracts were preparedand proteins were separated by W.B. assessed for p52 (52 kDa) and p65 (65 kDa) which was used as loading control

Panel C

Panc-1 cells were pre-treated with SU1261 for 1h prior to stimulated with LTα1β2 (15ng/ml) for 24h. Nuclear extracts were prepared and then separated by SDS-PAGE and assessed for p52 (52 kDa) and nucleoin (100 kDa) which was used as loading. The results are representative two independent experiments



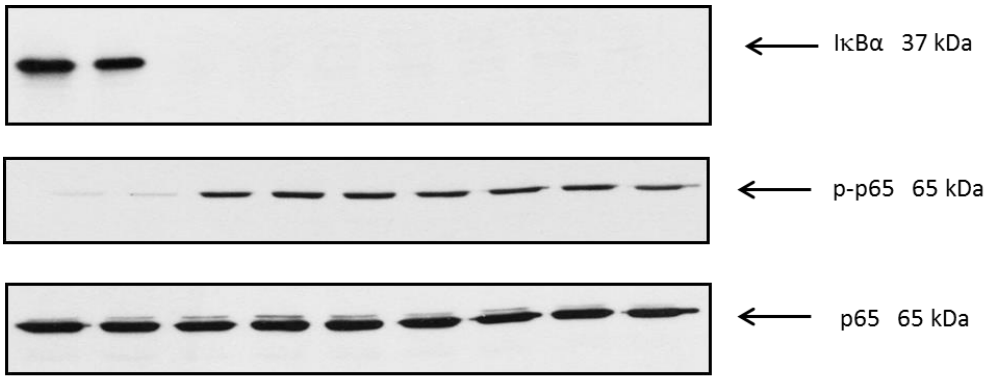
-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	+	-	-	-	-	-	DMSO
-	-	-	0.3	1	3	10	30	1261 (μM)

Panel A

MiaPaca-2 cells were pre-treated with SU1261 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 24h. Whole cell extracts were preparedand proteins were separated by W.B. assessed for p52 (52 kDa) and p65 (65 kDa) which was used as loading control

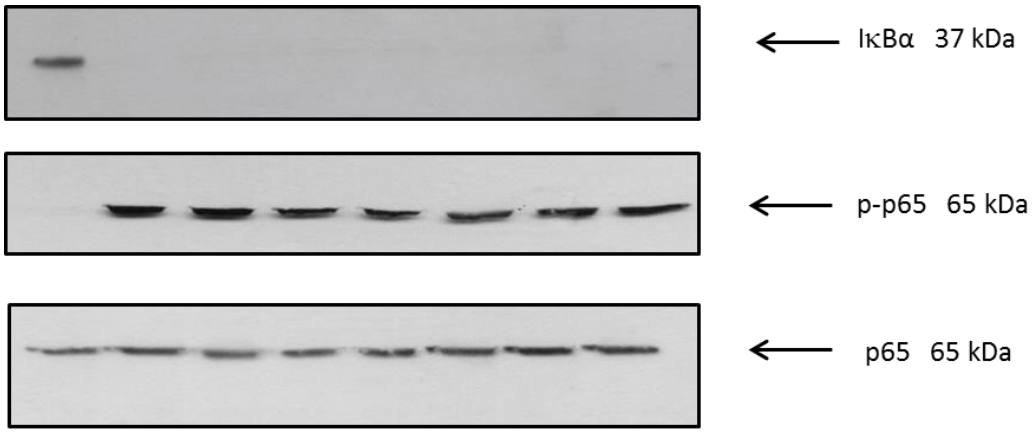
Panel B

MiaPaca-2 cells were pre-treated with SU1261 for 1h prior to stimulated with LTα1β2 (15ng/ml) for 24h. Nuclear extracts were prepared and then separated by SDS-PAGE and assessed for p52 (52 kDa) and nucleoin (100 kDa) which was used as loading. The results are representative two independent experiments



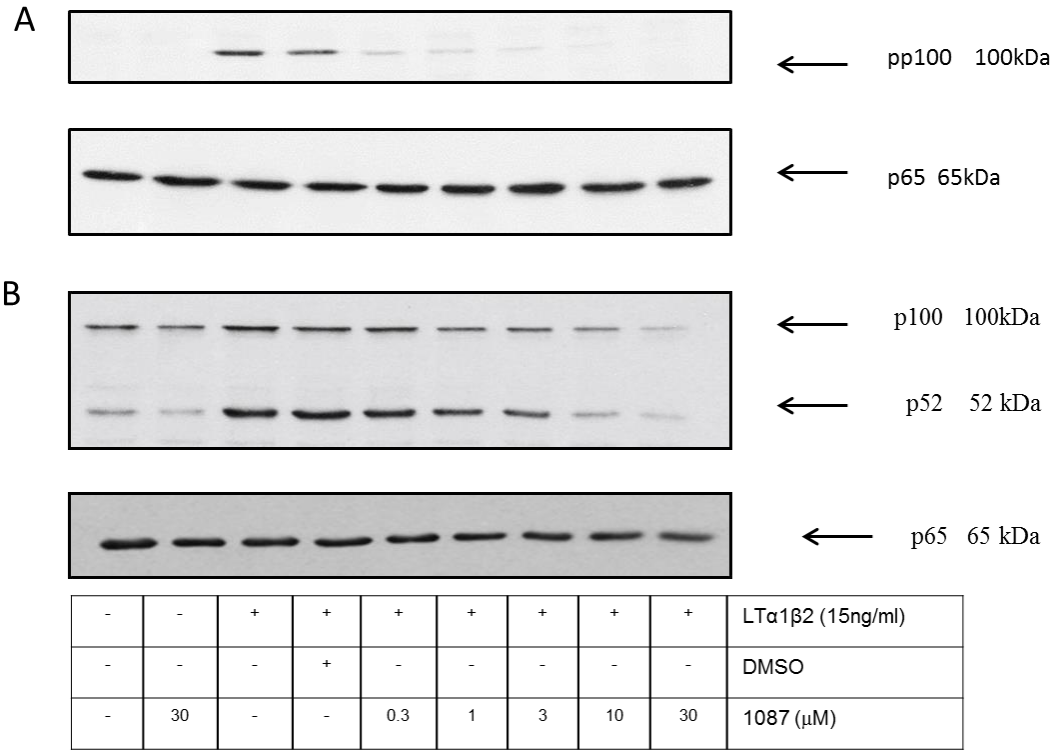
-	-	+	+	+	+	+	+	+	TNFα (10ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	.3	1	3	10	30	1261 (μM)

Panc-1 cells were pre-treated with SU1261 for 1h before stimulation with TNFα for 15 min. Whole cells extracts were prepared, and then blotted for IkBα (37 kDa), p-p65 (65 kDa) and p65 (65 kDa) which was used as loading control



-	+	+	+	+	+	+	+	TNFα (10ng/ml)
-	-	+	-	-	-	-	-	DMSO (30 μM)
-	-	-	.3	1	3	10	30	1261 (μM)

MiaPaca-2 cells were pre-treated with SU1261 for 1h before stimulation with TNFα for 15 min. Whole cells extracts were prepared, and then blotted for IkBα (37 kDa), p-p65 (65 kDa) and p65 (65 kDa) which was used as loading control.



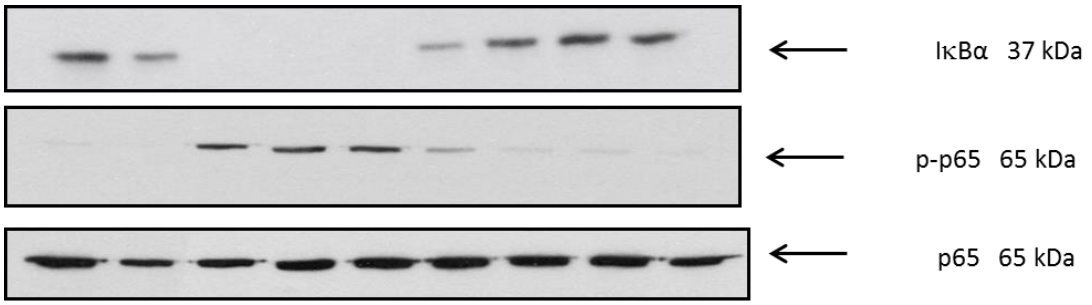
-	-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	0.3	1	3	10	30	1087 (μM)

Panel A

Panc-1 cells were pre-treated with 1087 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by W.B. assessed for p-p100 (100 kDa) and p65 (65 kDa) which was used as loading control

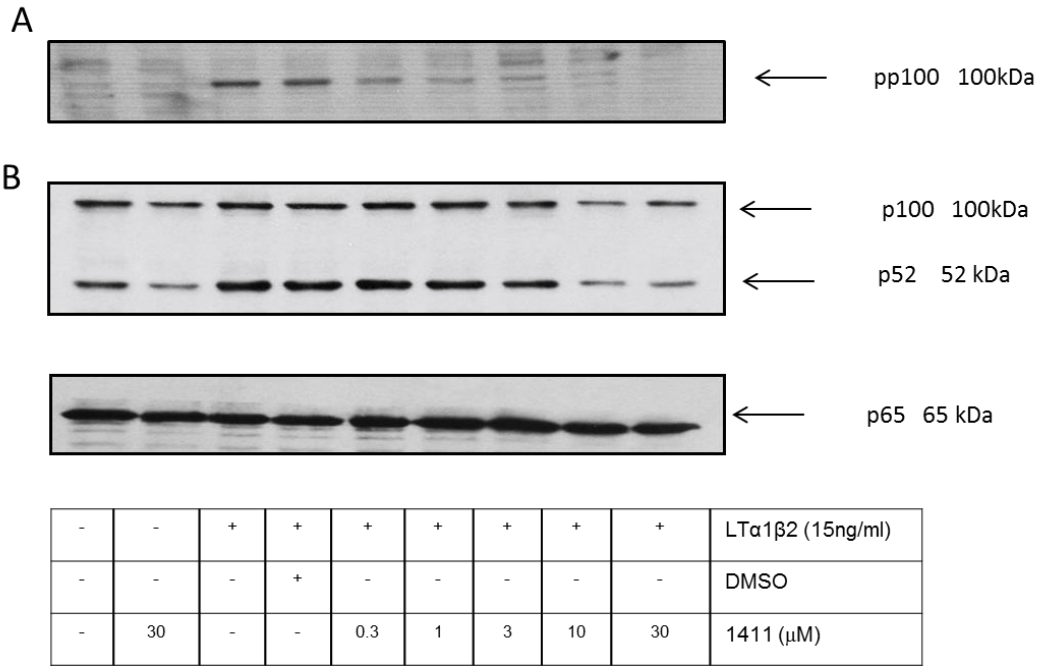
Panel B

Panc-1 cells were pre-treated with SU1087 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 24h. Whole cell extracts were preparedand proteins were separated by W.B. assessed for p52 (52 kDa) and p65 (65 kDa) which was used as loading control



-	-	+	+	+	+	+	+	+	TNFα (10ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	.3	1	3	10	30	1087 (μM)

Panc-1 cells were pre-treated with SU1087 for 1h before stimulation with TNFα for 15 min. Whole cells extracts were prepared, and then blotted for IkBα (37 kDa), p-p65 (65 kDa) and p65 (65 kDa) which was used as loading control.



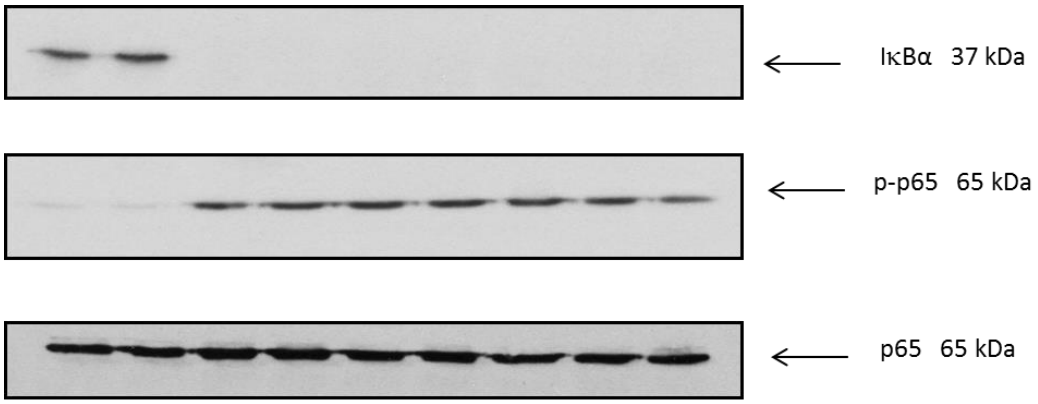
-	-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	0.3	1	3	10	30	1411 (μM)

Panel A

Panc-1 cells were pre-treated with 1411 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by W.B. assessed for p-p100 (100 kDa)

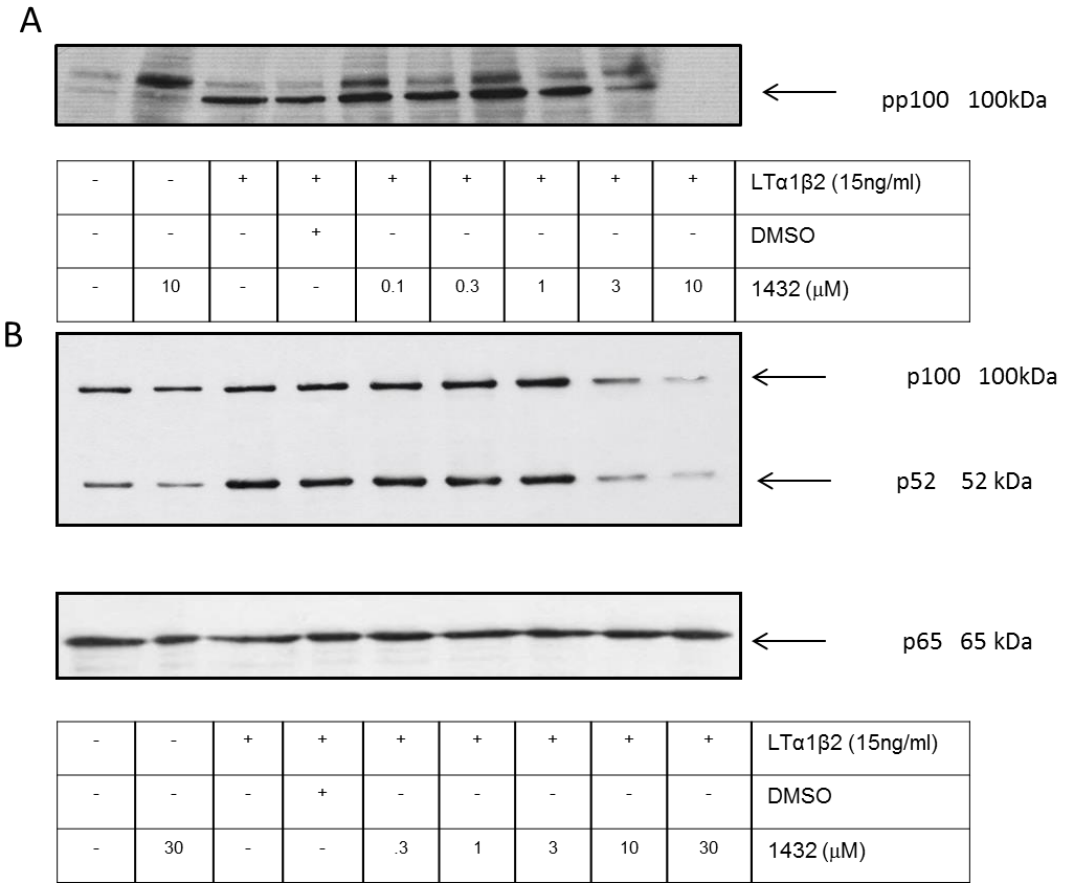
Panel B

Panc-1 cells were pre-treated with SU1411for 1h prior to stimulation with LTα1β2 (15ng/ml) for 24h. Whole cell extracts were preparedand proteins were separated by W.B. assessed for p52 (52 kDa) and p65 (65 kDa) which was used as loading control



-	-	+	+	+	+	+	+	+	TNFα (10ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	.3	1	3	10	30	1411 (μM)

Panc-1 cells were pre-treated with SU1411 for 1h before stimulation with TNFα for 15 min. Whole cells extracts were prepared, and then blotted for IkBα (37 kDa), p-p65 (65 kDa) and p65 (65 kDa) which was used as loading control.

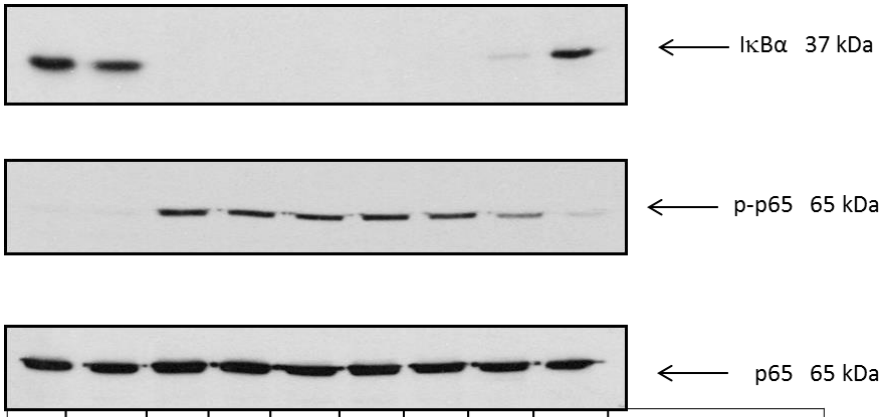


Panel A

Panc-1 cells were pre-treated with 1432 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by W.B. assessed for p-p100 (100 kDa)

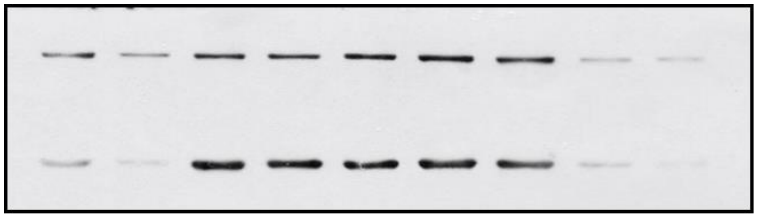
Panel B

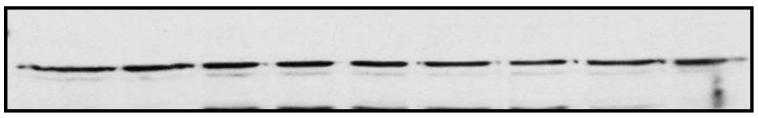
Panc-1 cells were pre-treated with SU1432for 1h prior to stimulation with LTα1β2 (15ng/ml) for 24h. Whole cell extracts were preparedand proteins were separated by W.B. assessed for p52 (52 kDa) and p65 (65 kDa) which was used as loading control



-	-	+	+	+	+	+	+	+	TNFα (10ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	.3	1	3	10	30	1432 (μM)

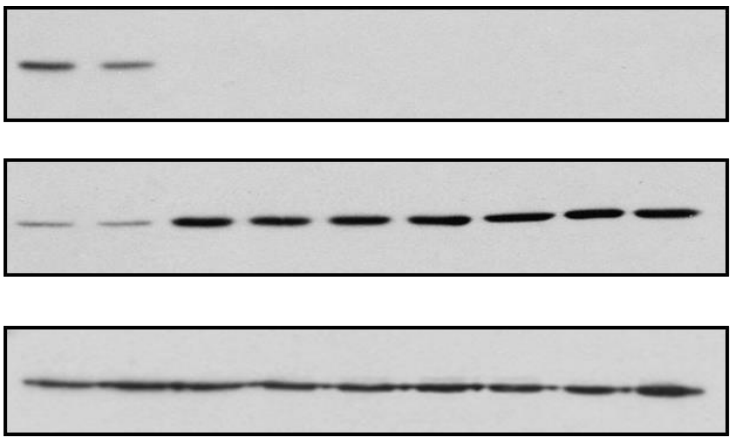
Panc-1 cells were pre-treated with SU1432 for 1h before stimulation with TNFα for 15 min. Whole cells extracts were prepared, and then blotted for IkBα (37 kDa), p-p65 (65 kDa) and p65 (65 kDa) which was used as loading control.





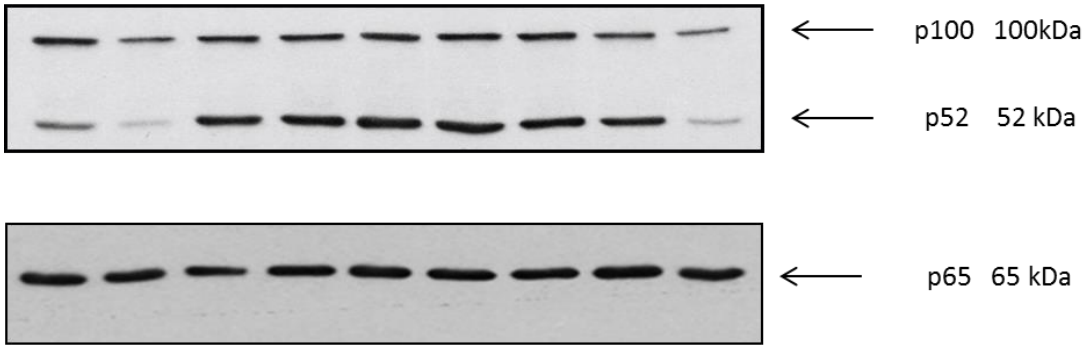
-	-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	0.3	1	3	10	30	1434 (μM)

Panc-1 cells were pre-treated with SU141 1for 1h prior to stimulation with LTα1β2 (15ng/ml) for 24h. Whole cell extracts were preparedand proteins were separated by W.B. assessed for p52 (52 kDa) and p65 (65 kDa) which was used as loading control



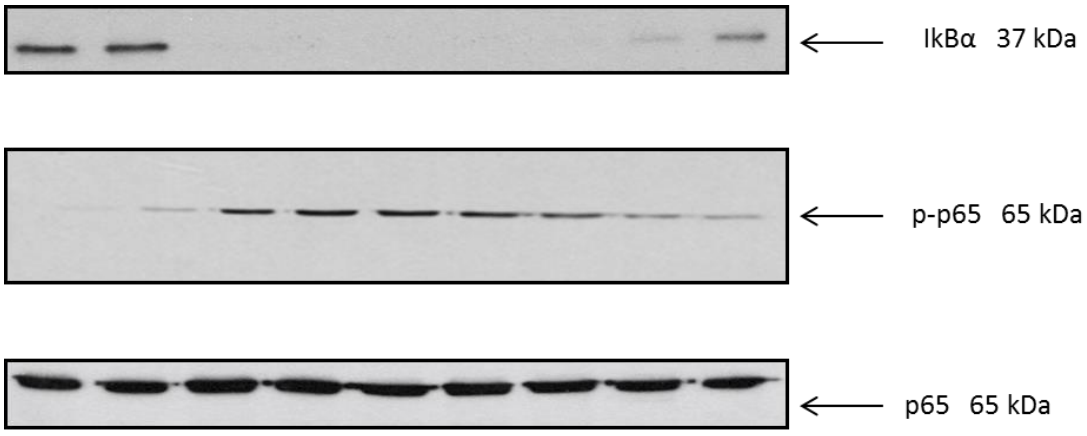
-	-	+	+	+	+	+	+	+	TNFα (10ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	0.3	1	3	10	30	1434 (μM)

Panc-1 cells were pre-treated with SU1434 for 1h before stimulation with TNFα for 15 min. Whole cells extracts were prepared, and then blotted for IkBα (37 kDa), p-p65 (65 kDa) and p65 (65 kDa) which was used as loading control



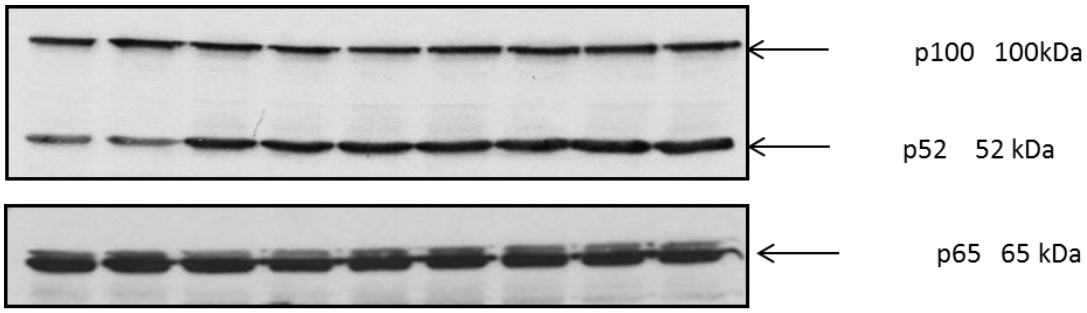
-	-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	0.3	1	3	10	30	1436 (μM)

Panc-1 cells were pre-treated with SU1436 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 24h. Whole cell extracts were prepared and proteins were separated by W.B. assessed for p52 (52 kDa) and p65 (65 kDa) which was used as loading control.



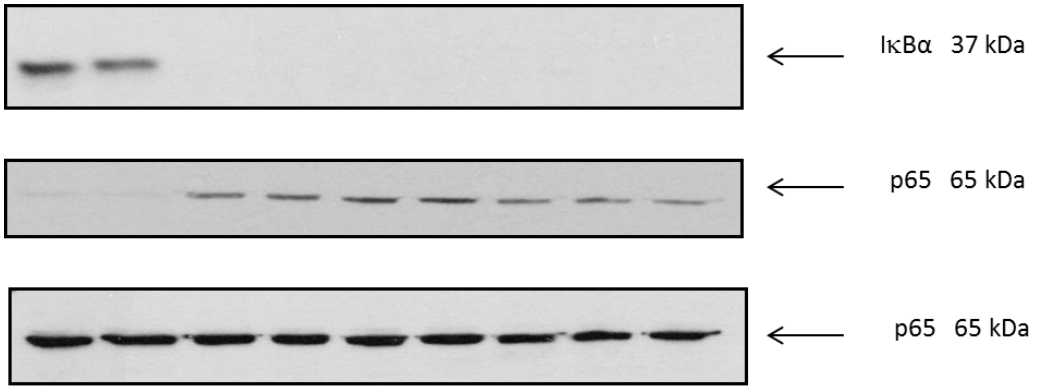
-	-	+	+	+	+	+	+	+	TNFα (10ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	0.3	1	3	10	30	1436 (μM)

Panc-1 cells were pre-treated with SU1436 for 1h before stimulation with TNFα for 15 min. Whole cells extracts were prepared, and then blotted for IkBα (37 kDa), p-p65 (65 kDa) and p65 (65 kDa) which was used as loading control



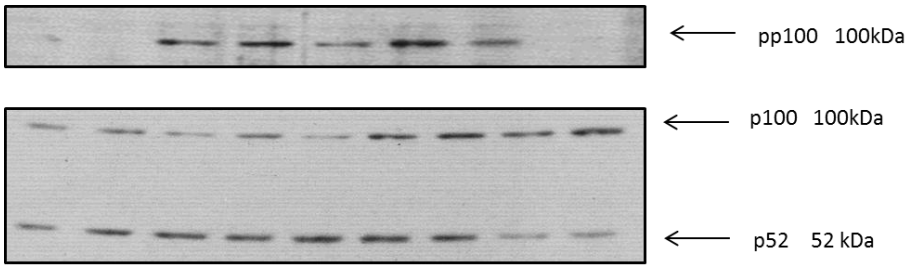
-	-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	0.3	1	3	10	30	1392 (μM)

Panc-1 cells were pre-treated with SU1392 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 24h. Whole cell extracts were preparedand proteins were separated by W.B. assessed for p52 (52 kDa) and p65 (65 kDa) which was used as loading control



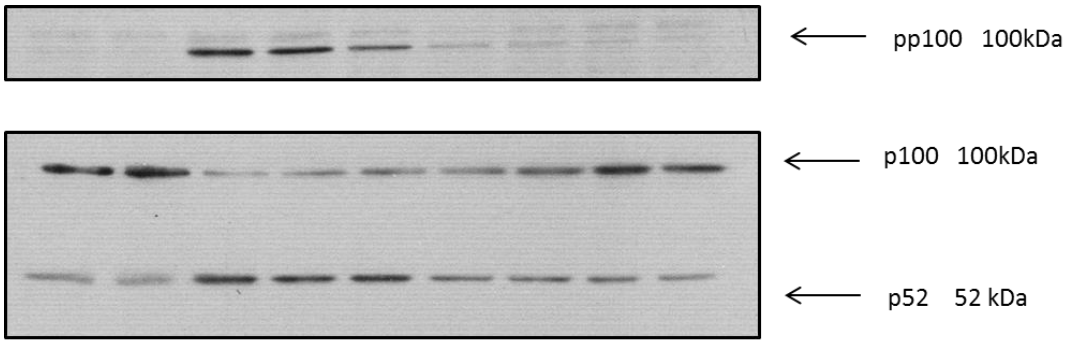
-	-	+	+	+	+	+	+	+	TNFα (10ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	0.3	1	3	10	30	1392 (μM)

Panc-1 cells were pre-treated with SU1392 for 1h before stimulation with TNFα for 15 min. Whole cells extracts were prepared, and then blotted for IkBα (37 kDa), p-p65 (65 kDa) and p65 (65 kDa) which was used as loading control



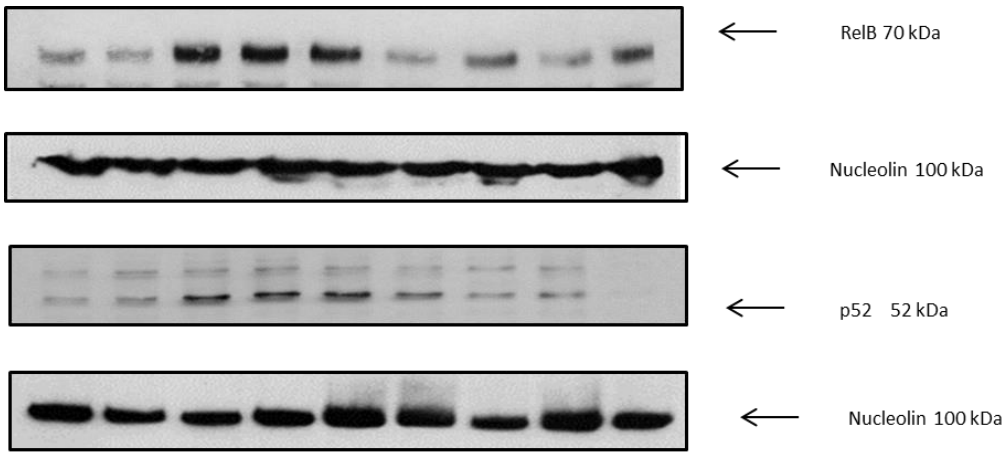
-	-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	10	-	-	0.1	0.3	1	3	10	1399 (μM)

Panc-1 cells were pre-treated with 1399 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by W.B. assessed for p-p100 (100 kDa) and p52/p100 (52/100 kDa) which was used as loading control



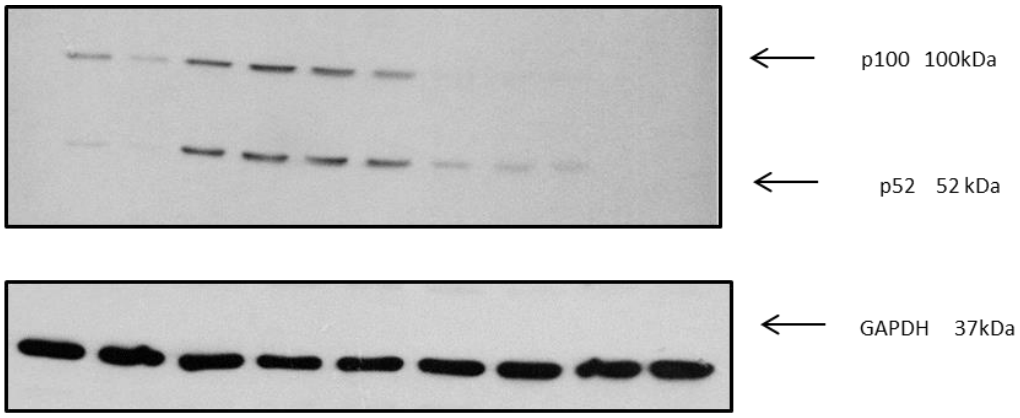
-	-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	-	+	-	-	-	-	-	DMSO (30 μM)
-	10	-	-	0.1	0.3	1	3	10	1349 (μM)

Panc-1 cells were pre-treated with 1349 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by W.B. assessed for p-p100 (100 kDa) and p52/p100 (52/100 kDa) which was used as loading control

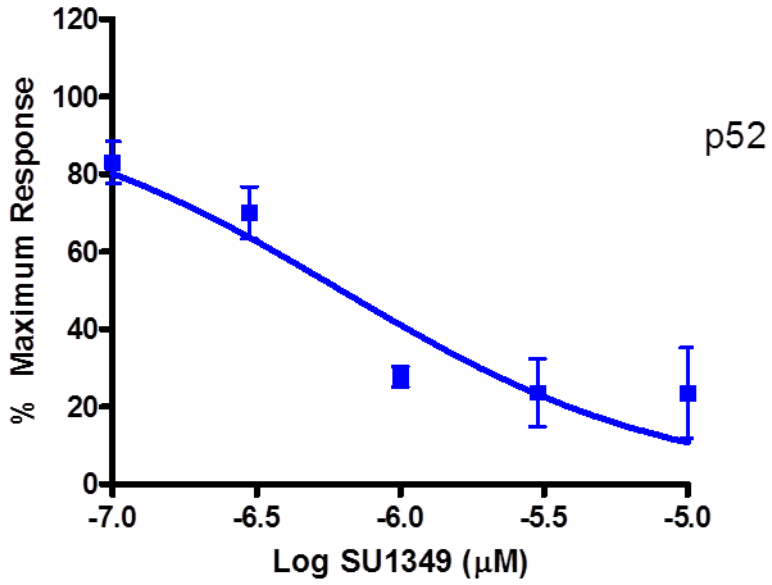


-	-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	-	+	-	-	-	-	-	DMSO (30 μM)
-	10	-	-	0.1	0.3	1	3	10	1349(μM)

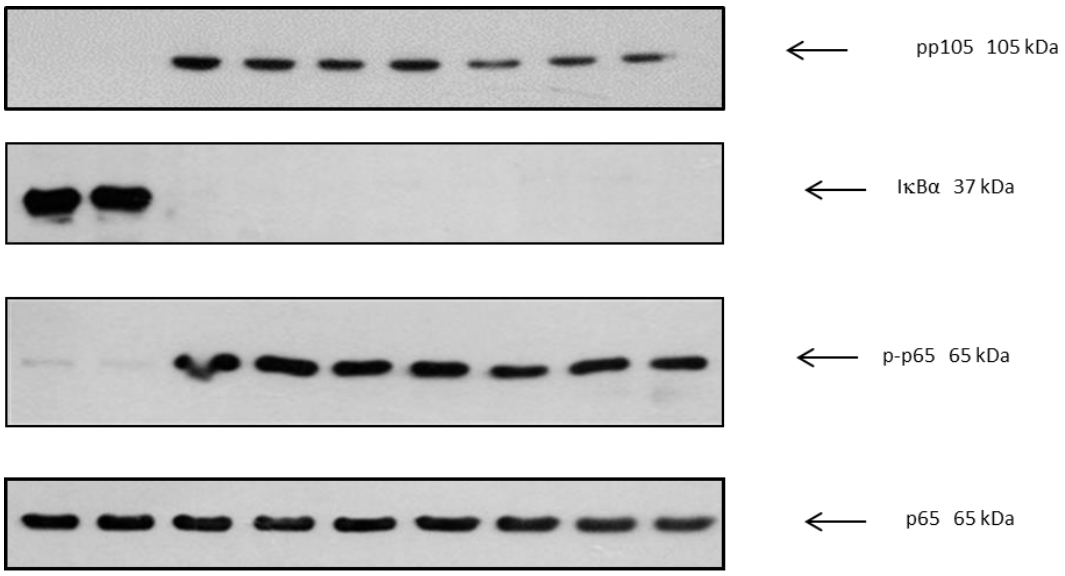
Panc-1 cells were pre-treated with SU1349 for 1h prior to stimulated with LTα1β2 (15ng/ml) for 4h. Nuclear extracts were prepared and then separated by SDS-PAGE and assessed for, RelB (70kDa) p52 (52 kDa) and nucleoin (100 kDa) which was used as loading. The results are representative two independent experiments



-	-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	10	-	-	0.1	0.3	1	3	10	1349(μM)

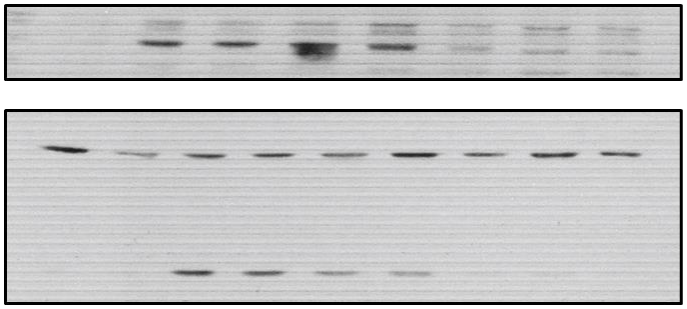


Panc-1 cells were pre-treated with SU1349 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 24h. Whole cell extracts were preparedand proteins were separated by W.B. assessed for p52 (52 kDa) and p65 (65 kDa) which was used as loading control



-	-	+	+	+	+	+	+	+	TNFα (10ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	10	-	-	0.1	0.3	1	3	10	1349 (μM)

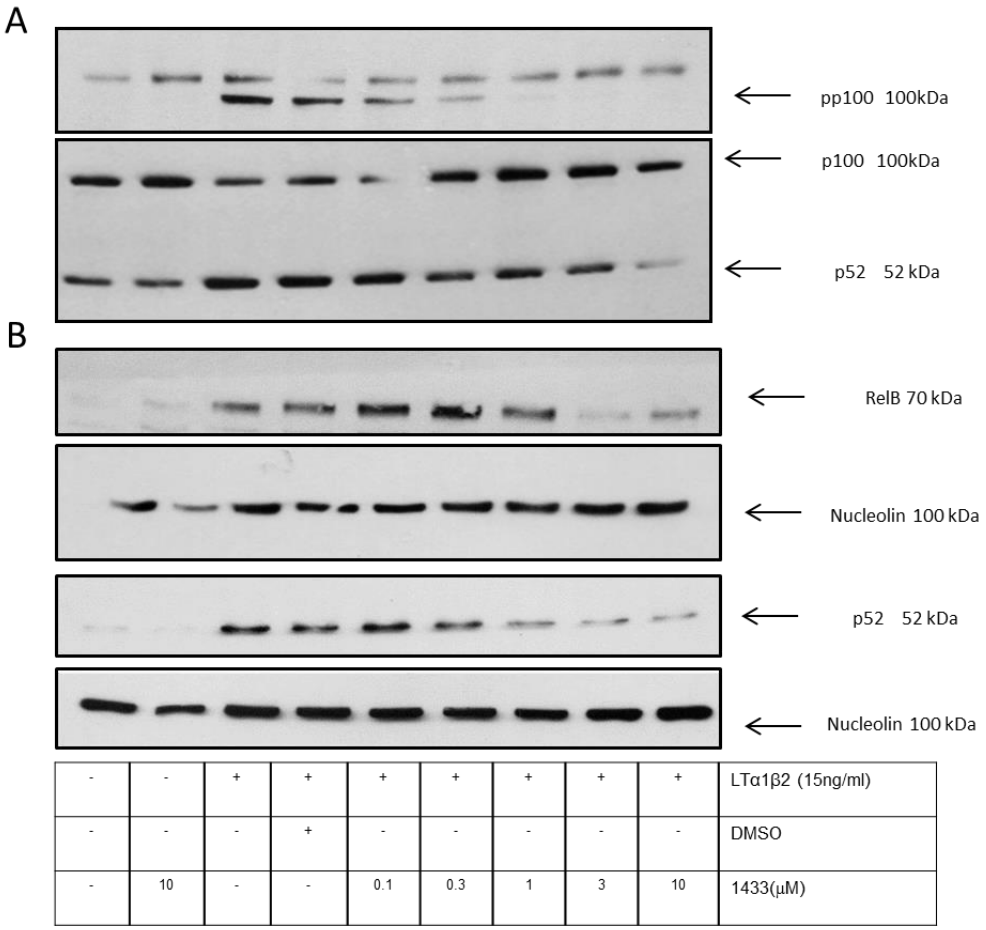
Panc-1 cells were pre-treated with SU1349 for 1h before stimulation with TNFα for 15 min. Whole cells extracts were prepared, and then blotted for IkBα (37 kDa), p-p65 (65 kDa) and p65 (65 kDa) which was used as loading control.



-	-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	10	-	-	0.1	0.3	1	3	10	1499 (μM)

n=1

Panc-1 cells were pre-treated with SU1499 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by W.B. assessed for p-p100 (100 kDa) and p52/p100 (52/100 kDa) which was used as loading control

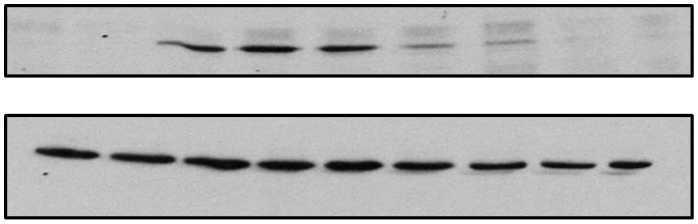


Panel A

Panc-1 cells were pre-treated with SU1433 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by W.B. assessed for p-p100 (100 kDa) and p65 (65 kDa) which was used as loading control

Panel B

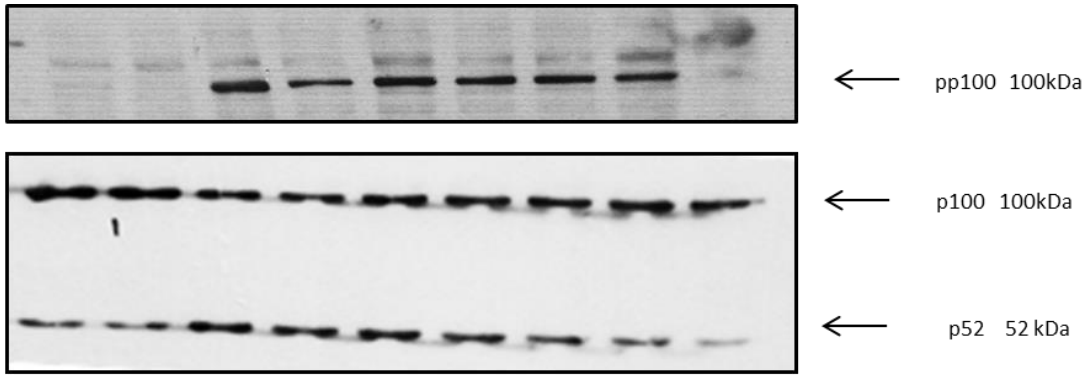
Panc-1 cells were pre-treated with SU1433 for 1h prior to stimulated with LTα1β2 (15ng/ml) for 4h. Nuclear extracts were prepared and then separated by SDS-PAGE and assessed for RelB (70kDa), p52 (52 kDa) and nucleoin (100 kDa) which was used as loading. The results are representative two independent experiment



-	-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	.3	1	3	10	30	1438 (μM)

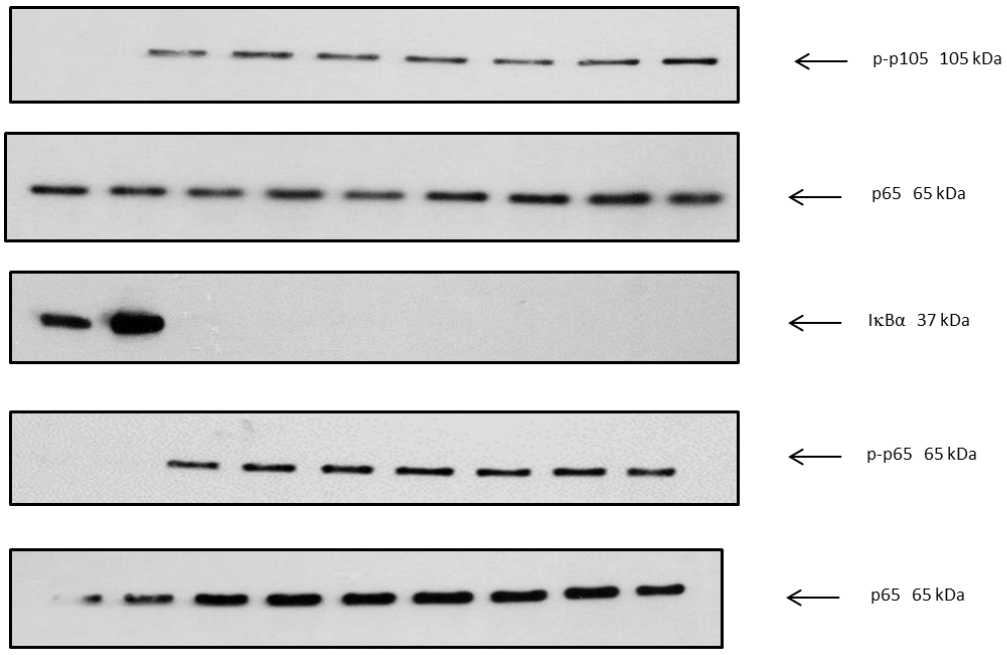
n=3

Panc-1 cells were pre-treated with 1438 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by W.B. assessed for p-p100 (100 kDa) and p65 (65kDa) which was used as loading control



-	-	+	+	+	+	+	+	+	LTα1β2 (15ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	30	-	-	.3	1	3	10	30	1497 (μM)

Panc-1 cells were pre-treated with 1497 for 1h prior to stimulation with LTα1β2 (15ng/ml) for 4h. Whole cell extracts were prepared and proteins were separated by W.B. assessed for p-p100 (100 kDa) and p65 (65 kDa) which was used as loading control



-	-	+	+	+	+	+	+	+	TNFα (10ng/ml)
-	-	-	+	-	-	-	-	-	DMSO
-	10	-	-	0.1	0.3	1	3	10	15407(μM)

Panc-1 cells were pre-treated with CW15407 for 1h before stimulation with TNFα for 15 min. Whole cells extracts were prepared, and then blotted for p-p105 (105 kDa), IkBα (37 kDa), p-p65 (65 kDa) and p65 (65 kDa) which was used as loading control.