Targeting inhibitory kappa B kinase alpha (IKKα) signalling in glioblastoma

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: *Haidar Al Moosawi*

Date: 01/03/2024

Dedication

This Thesis is dedicated to the memory of my father, who passed away during 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 him. I miss him and always I will.

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First and foremost I would like to thank Allah (God) for giving me the power to believe in myself and pursue my dreams. I could never have done this without the faith I have in you.

Happy, lost, tears, homesick, sad, stress, fun, crazy, excitement, overtime working, joys and why didn't work this time and what I did to myself, were the most words and phrases I had used during my PhD. There were many people who have given me endless advice and support to make my study easier. Firstly I would like to express my sincere and profound gratitude to Andrew Paul for giving me the opportunity to finish my study at his Lab. Thank you for spending a significant amount of time for guiding, discussing and providing feedback on my Lab techniques, writing, presentation and poster preparation which has really improved my skills. If there is a prize for the best supervisor, definitely you will get it.

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Abbreviations

ANOVA: Analysis of variance

APS: Ammonium Persulphate

BAFF: B cell activation factor

BLC: B lymphocyte chemoattractant

BSA: Bovine Serum Albumin

c-IAP: Cellular inhibitor of apoptosis protein

DMEM: Dulbecco's Modified Eagle Medium

DTT: Dithiothreitol

ECL: Enhanced chemiluminescence

EGFR: Epidermal growth factor receptor

EMSA: Electrophoretic Mobility Shift Assay

ERK: Extracellular Signal-regulated Kinase

HLH: Helix-loop-helix

IGF-1: Insulin–Like Growth Factor 1

IκB: Inhibitory kappa B

IKK: inhibitory kappa kinase B

IL6: Interleukin-6

IL-8: Interleukin-8

KRAS: Kirsten rat sarcoma viral oncogene homolog

LTβR: Lymphotoxin β receptor

MAP3K: Mitogen-activated protein/FRK kinase 3 family

MAP3K14: Mitogen activated protein kinase kinase kinsae 14

NFκB: Nuclear Factor Kappa B

NIK: NFκB-inducing Kinase

SDS: Sodium Dodecyl Sulphate

TEMED: N,N,N',N' tetramethendiaamine

TNFα: Tumour Necrosis Factor-alpha

TRAF2: TNFα receptor-associated factor 2

TRAF3: TNFα receptor-associated factor 3

TWEAK: TNFα-like Weak Inducer of Apoptosis

VEGF: Vascular endothelial growth factor

β-ME: β-mercaptoethanol

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Abstract

Introduction Cancer is a broad term, which covers a number of conditions characterised by uncontrolled cellular proliferation mainly due to genetic mutations. One of the most aggressive types of cancer is *Glioblastoma multiforme* (GBM). Presently, glioblastoma is considered an incurable type of cancer. Therefore, scientists are studying new patterns and mechanisms to supress GBM; one of which is through interrupting specific signalling pathways in tumour cells. One of the prominent pathways driving the tumour cell signalling responses and showing significant effects on cancer hallmarks, is the Nuclear Factor Kappa-B (NF-κB) pathway. ***Aim*** The aim of the study is to investigate the pharmacological effect of selective IKKα inhibitors through examining their selectivity within both the non-canonical and canonical NF-κB pathways and their effect on IKKα-dependent cellular processes that underpin the phenotypic outcomes that support GBM development. **Methods** Western Blotting was pursued with both whole cell extracts and crude nuclear extracts to determine the expression/phosphorylation/localisation of key signalling proteins. EMSA was used to determine the protein-DNA binding activity while MTT assay and clonogenic assay were used to identify cellular viability and clonogenic survival, respectively. All data shown were expressed as mean ± S.E.M. Statistical analysis was performed using GraphPad Prism version 10.1.0. 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). **Results** TWEAK and TNFα were used to stimulate T98G and/or UVW cells in increasing concentrations starting from 1 ng/ml up to 100 ng/ml or over time. Results showed that TWEAK was able to phosphorylate p100 in a concentration-dependent manner. 10 ng/ml of TWEAK were able stimulate p100 phosphorylation over time. It was also able to process p100 to p52/RelB and translocation to the nucleus in T98G GBM cells. TNFα was, to a lesser extent compared to TWEAK, able to phosphorylate p100 in a

concentration-dependent manner. 10 ng/ml of TNFα were able to stimulate p100 phosphorylation over time. It was also able to process p100 to p52/RelB and translocation to the nucleus of T98G GBM cells. SU1433 and SU1644, were both able to inhibit, in a concentration-dependent manner, TWEAK-stimulated phosphorylation of p100 as well as p52 processing and nuclear translocation. Furthermore, they affected the phenotypic characteristics of GBM cell lines as well as protein-DNA binding activity. **Conclusion** TWEAK can activate the non-canonical NF-κB pathway in glioblastoma cell lines in a concentration-dependent manner as well as over time. Moreover, SU1433 and SU1644 can inhibit TWEAK-stimulated non-canonical NF-κB pathway activation in Glioblastoma cell lines in a concentration-dependent manner. These compounds can, hence, affect the phenotypic characteristics of GBM cells and could be potentially developed as drugs for the future management of brain cancer.

Chapter 1

Introduction

Chapter 1 Introduction

1 Introduction

1.1 Cancer

Cancer is a broad term, which covers a number of conditions characterised by uncontrolled cellular proliferation mainly due to genetic mutations in certain genes according to the cancer type. These genes includ *KRAS* protein (which is accountable for the regulation and preservation of various cellular mechanisms, including proliferation, transformation, invasion, and survival), MutS protein homolog 1 and 6 **(***MSH1*, *MSH6*) (MutS protein is a constituent of a gene family that encodes proteins engaged in several cellular processes, including DNA mismatch repair, other forms of DNA repair, meiotic recombination, and other essential functions), mutL homolog 1 (*MLH1*), *PMS1* protein homolog 2 (*PMS2*) (both mutL and PMS proteins are involved in DNA mismatch repair), trypsin-1 (*PRSS1*) (encoding human cationic trypsinogen) and claudins (*CLDNs*) (which act as regulators of intercellular adhesion) and others (Kastrinos, et al., 2011; Howes et al., 2004; Whitcomb et al., 1996). Such mutations can transform almost any normal mammalian cell into an oncogenic (i.e. cancerous) cell. This transformation drives various regulatory downstream signalling pathways – the process through which cells respond to stimuli by extracellular signalling molecules through binding to specific receptors on the cell membrane or in the cellular cytoplasm which then transfers signals into the nucleus to induce corresponding gene expression, therefore producing biological effects and cellular responses (Xia et al., 2018). These signalling pathways are activated by different factors such as genetic predispositions, environmental influences, infectious agents and aging (Pulverer, 2001). This signalling process is not controlled in cancer cells leading to excessive cell proliferation, resistance to apoptosis, angiogenesis, invasion, and metastasis (Zhang et al., 2017; Sun et al., 2017). It is the complexity of the disease that challenged the development of effective and targeted therapies, which has led to cancer being one of the most frequent causes of death in the world (Xia et al., 2018) and according to the World Health Organisation (WHO), the incidence of common malignant tumours will increase from 14 million in 2012 to 19 million in 2025 and 24 million in 2035 (Torre et al., 2012; Cao et al., 2017). According to the Ministry of Health of the Sultanate of Oman, the total number of cancer cases reported in 2012 were

1212 cases and in 2019 the total number of cases increased to 2089 cases (MOH Oman, 2019). The total reported brain cancer cases were 28 in 2012 and 48 in 2019 (MOH Oman, 2019).

1.2 External causes of cancer

Approximately two hundred forms of cancer have been recognised, there are several organs that may be affected by cancer and it is difficult to identify the agent(s) that causes cancer (Sasco et al., 2004). However, several studies have discovered environmental factors, known as carcinogens that are linked with particular forms of cancer and these variables have been categorised into three primary groups: biological carcinogens, physical carcinogens, and chemical carcinogens (Sasco et al., 2004).

Biological carcinogens can be infectious agents, such as parasites, viruses, and bacteria; for instance, Kaposi's Sarcoma, which is a skin cancer linked with cutaneous lesions produced by Kaposi's Sarcoma Herpes Virus (KSHV), is a notable example (Schulz, 2000). Numerous studies have demonstrated a link between some bacterial species and the development of cancer, such as *Chlamydia pneumoniae* and lung tumours, *Bartonella* species and vascular tumours, and *Streptococcus bovis* and colon cancer (Ellmerich et al., 2000, Marshall and Windsor, 2005, Littman et al., 2005, Dehio, 2005).

Physical carcinogens are environmental elements that can contribute to the formation of tumours; for example, exposure of a foetus to high amounts of ionising radiation has been associated with an increased chance of developing juvenile leukaemia (Schmidt et al., 2021). Ultraviolet (UV) radiation is another example. UV is an electromagnetic radiation with wavelengths ranging from 10nm to 400nm and is a component of sunlight and electric goods that emit radiations such as Wi-Fi, 5G technology and others. 95% of skin malignancies (melanomas) are linked with long-term UV exposure, according to a number of studies (Andrade et al., 2012).

Lastly, chemical carcinogens include any chemical agent that causes cancer, such as water pollutants (arsenic) or components of cigarette smoke. There are around four thousand substances in tobacco smoke, and smoking has been linked to 93% of all lung cancers (Sasco et al., 2004, Villeneuve and Mao, 1994). A further illustration is the Aflatoxin B toxin, which is generated by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. This toxin is associated with hepatocellular cancers in Africa (Alpert et al., 1968). Bennett and Klich discovered that elevated amounts of this toxin caused the

growth of tumours in animal models (Bennett and Klich, 2003). Formaldehyde is another example of a chemical utilised in industry that is also found in some fruits and other foods. Formaldehyde is linked to a variety of human malignancies, including leukaemia and nasopharyngeal carcinoma, as well as haematological and lung tumours (Rager, et al 2011). As chemical carcinogenesis is predicted to have a growing impact on cancer formation, further research is required in this particular field.

1.3 Cellular features of cancer

As a disease, cancer is very diverse and there are more than two hundred types of cancer that have been identified affecting different body organs which makes it difficult to determine clearly the causative factor(s) (Sasco et al., 2004). However, studies have linked cancer to cellular and/or genetic factors which can be interconnected since cellular factors such as hormones, growth factors and intercellular pathways can result in direct or indirect genetic mutation within the cell which can lead to cancer (Todd and Wong, 1999).

Studies have suggested that growth factors and hormones play a key role in the development of cancer through regulating cell growth, cell proliferation and apoptosis (Lukanova and Kaaks, 2005) (Chuu et al., 2011). Oestrogen, for example, drives cancers such as uterine, cervical and breast cancer while prostate cancer is mainly caused by androgens (Lukanova and Kaaks, 2005). Chuu and co-workers suggested that about 80% of the prostate cancer cell proliferation is mainly due to high levels of androgens, which makes its suppression important in the treatment strategy of prostate cancer (Chuu et al., 2011). The effect of Insulin like Growth Factor 1 (IGF-1) in the tumourigenesis of prostate and breast cancers was also reported by Kaaks and Lukanova through its effect on reducing some pro-apoptotic proteins (Kaaks and Lukanova , 2001). Increasing insulin concentration enhances binding to IGF-1 receptors and activates signalling pathways that promote cancer growth (Moore et al., 1998). In addition, insulin has been linked to the metabolism and production of some pro-cancerous hormones, such as the androgens (Fairfield et al., 2002; Kaaks and Lukanova, 2001).

1.4 Genetic factors to cancer

There are two main types of genes, which are found in normal cells that can contribute to the development of cancer. The first type of genes are proto-oncogenes which are responsible for expressing

the proteins that control many critical functions (cell growth, proliferation, DNA damage repair and cell apoptosis) within the cells and these include *K-sam* and *RAS* (Doolittle et al., 1983). The other type of genes are the tumour suppressor genes which are able to control cell division and death; and these include *p53*, and breast cancer gene 1 & 2 encoding DNA repair associated proteins (*BRCA1* and *BRCA2*) (Todd and Wong, 1999). Other Key exemplar proto-oncogenes and tumour suppressor genes are summarised below in Table 1.1.

Studies have shown that a number of cancer types are related to genetic mutations that passed through generations. In colorectal cancer, for instance, scientists discovered several germline mismatch repair gene mutations, which are responsible to fix gene alterations or errors after cell mytosis, such as *MSH1, MSH6, MLH1* and *PMS2* (Kastrinos, et al., 2011). Another example is pancreatic cancer, which has been linked to a number of hereditary familial mutations such as *PRSS1* and *CLDN*, which cause premature activation of pancreatic enzymes that leads to inflammation within the the pancrease (Howes et al., 2004, Whitcomb et al., 1996). Moreover, Goldgar and co-workers have found that around 10% of pancreatic cancers were related to *BRCA2* germline mutations (Goldgar, et al, 1995). The *BRCA1* gene, on the other hand, is an example of a defective gene that can be both inherited and a consequence of somatic mutation owing to exposure to risk factors. Other examples include; mutations in *KRAS2* and *N-RAS* genes that are commonly mutated across most tumour types (Futreal et al., 2004). Overall, reduced exposure to both internal and external stimuli can lessen the development of tumours regardless of whether or not a person currently carries a mutation.

Table 1. 1 Common oncogenes and suppresser genes involved 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	Involved in lung cancer, This gene has intracellular function in ERK-MAP signalling. This gene codes for multiple nuclear phosphoproteins (De Greve, et al 1988).
Tumour suppressor genes	
BRCA1 and BRCA2	Involved in breast cancer, have a role in DNA repair damage.
P53	Tumour suppressor gene involved in many types of cancer which stops the formation of cancer.

RB1	Involved in bladder, bone, breast, retinoblastoma and small cell lung cancer.
APC	Involved in stomach and colon cancers, encodes APC protein, which plays a role in many cellular processes.
MTS1	Involved in many cancers, codes for p16 protein that has 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 NF-κB signalling pathway.
MYC *WNT9A*	Involved in pancreatic cancer, has a role in Wnt signalling pathway.

1.5 Carcinogenesis

Depending on the type of the mutation, there are many mechanisms that facilitate the change of a normal cell into a cancer cell; this process is known as tumourigenesis or carcinogenesis. Figure 1.1 depicts the three stages of tumourigenesis: initiation, promotion, and progression (Pitot, 1993, Barrett, 1993, Frank, 2007, Lapidot et al., 1994). These stages are supported by an irreversible genetic mutation in DNA (Barrett, 1993), which causes the DNA replication defect during the synthetic phase of the cell cycle or alterations in cellular metabolism, such as the generation of free radicals and reactive oxygen species (Chen et al., 2007, Pray, 2008). Some of the previously described environmental variables, such as UV radiation, X-rays, and smoking, definitely have the ability to cause DNA mutations. Exposure to these factors results in the formation of covalent connections inside DNA segments, known as DNA adducts; if not removed by the DNA repair mechanism prior to replication, this process can lead to mutation

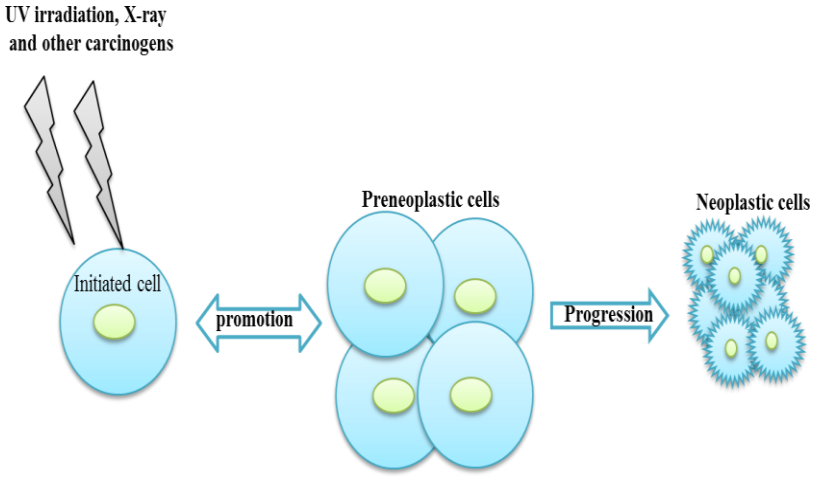


Figure 1. 1 Three major steps in the carcinogenesis process; initiation, promotion and progression (Barrett, 1993, Pray, 2008, Surh, 2003)

(Frank, 2007). According to the type of defect that occurs inside the DNA, direct DNA damage is divided into four distinct categories: substitution, loss or addition of bases, alteration of DNA chemical structure, and breaks in the DNA backbone (Pitot et al., 1993, Barrett, 1993, Frank, 2007).

During the promotion phase, cellular function may be disrupted, leading to a rise in the average rate of cell growth and division, which boosts the generation of daughter cells and errors at the cell cycle checkpoint. At this stage, cancer promoters no longer bind directly to DNA, as they did during the

initiation phase. Rather, activation of cell signalling pathways is associated to proliferation (Pitot et al., 1993). For instance, the increased synthesis of growth factors that bind to particular receptors on the membrane of the target cell might stimulate gene expression by activation of cell signalling pathways, or mutation of the intracellular pathways, inducing gene expression modifications without the necessity for receptor activation (Troll and Wiesner, 1985, Pitot et al., 1993).

The last stage is characterised by the ability of cells to form neoplastic cells or masses, which involves an increase in spontaneous DNA damage, mutation, or epigenetic alteration (i.e. changes that occur at the DNA). Through the mechanisms of migration and matrix de-regulation, cells at this stage proliferate at a rapid rate and have a larger potential for metastasis. These three stages can take a considerable amount of time, providing the potential to slow or stop cancer growth (Pitot, 1993, Barrett, 1993, Frank, 2007).

1.6 Cancer cell capabilities and enabling characteristics (Hallmarks)

According to Hanahan (Hanahan, 2022), there are several biological modifications in cancer that drive the transformation of normal cells to tumour cells (see Figure 1.2). These biological modifications are now recognised as cancer cell 'Hallmarks' and will be described below.

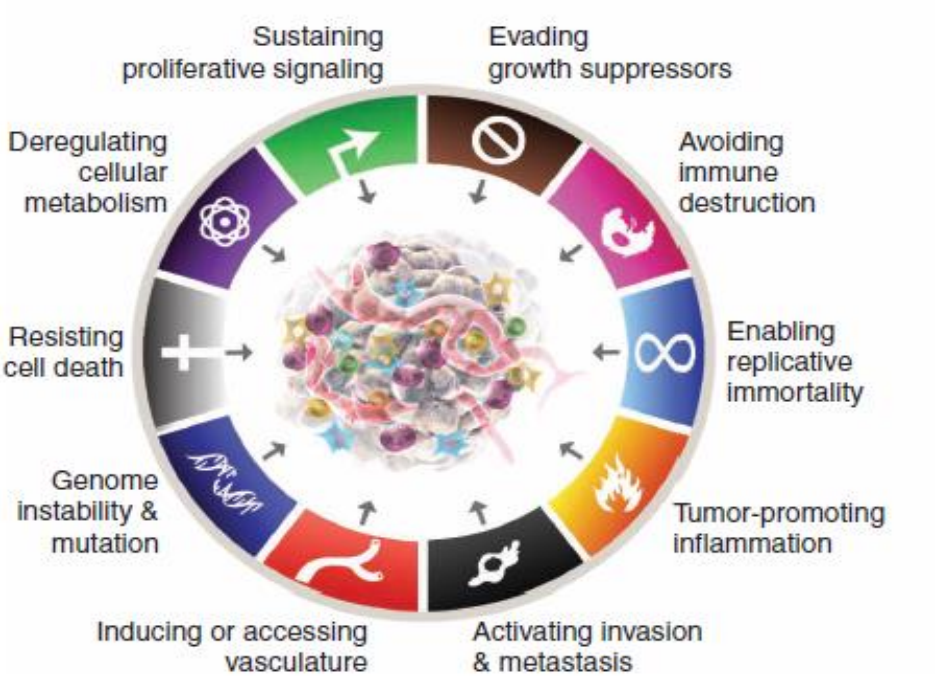


Figure 1. 2 Illustration of the ten cancer hallmarks (Sourced from Hanahan, 2022)

1.6.1 Sustaining proliferative signalling

Cancer cells have the ability to sustain cell proliferation using different mechanisms such as self-production of growth factors, or modifying the intracellular proteins, which independently mediate cell growth in the absence of an external signal (Hanahan, 2022). This is summarised in the second stage of the carcinogenesis process and is linked to oncogenic mutations that lead to increased growth factor production, increased growth factor receptor expression and overactive intracellular signalling pathways including Extracellular Signal-Regulated Kinase (ERK) and Phosphoinositide 3-kinase (PI3 Kinase) (Hanahan, 2022; Evan and Vousden, 2001).

1.6.2 Evading growth suppressors

There are a group of genes, called tumour-suppressor genes, which 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).

1.6.3 Activating invasion and metastasis

Metastasis is defined as the ability of the cancer cells to migrate and invade distant cells, which is a characteristic of a cancerous cell due to certain factors. One of the factors is the down regulation of E-cadherin, an adhesion molecule that helps normal cells to adhere to each other and reduce their migration (Berx and van Roy, 2009; Cavallaro and Christofori, 2004). Another component is the expression of certain transcriptional factors such as Snail, Slug, Twist and Zeb1/2, which have been found important in programming cell invasion and metastasis (Micalizzi et al., 2010; Taube et al., 2010; Schmalhofer et al., 2009; Yang and Weinberg, 2008).The process of cell metastasis is a multistep process often termed as the invasion-metastasis cascade which starts with local cells invasion, then intravasation of cancer cells through nearby blood or lymphatic vessels, followed by the escape of these cancer cells from such vessels into parenchyma of distant tissues (extravasation). Then the cells will form small nodules (micro-

metastases) which will eventually start to grow to form the macroscopic tumour (colonisation) (Hanahan, 2022).

1.6.4 Enabling replicative immortality

For cancer cells to make a macroscopic tumour, they require limitless replicative potential that leads to cell immortality (Hanahan, 2022). Multiple studies indicate that telomeres protecting the ends of chromosomes are centrally involved in the capability for unlimited proliferation (Blasco, 2005; Shay and Wright, 2000). Therefore, the length of telomeric DNA in a cell indicates how many successive cell generations its progeny can pass through before telomeres are largely eroded and have subsequently lost their protective functions (Hanahan, 2022). Telomerase, the specialised DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA, is almost absent in non-immortalised cells, but expressed at functionally significant levels in the majority (approximately 90%) of spontaneously immortalised cells, including human cancer cells. By extending telomeric DNA, telomerase is able to counter the progressive telomere erosion (Hanahan, 2022).

1.6.5 Inducing or accessing vasculature

Cancer cells continuous growth can lead to cell hypoxia, which can lead to cell death. Thus, for cancer cells to cope with hypoxic conditions, cells tend to form new capillaries to sustain the blood supply i.e. oxygen and nutrition; and this process is called angiogenesis (Bergers and Benjamin, 2003). For this process to happen, mediators such as vascular epithelial growth factor (VEGF), encoded by the VEGF gene, is released by the cancer cells that binds to specific receptors on the endothelial cell surface. VEGF expression is stimulated by conditions like hypoxia or oncogenic transformation (Ferrara, 2009, Mac Gabhann and Popel, 2008).

1.6.6 Resistance to cell death

Cells normally die due to factors such as damage, infection or inflammation in a programmed process, called ‘apoptosis’. This is not the case in cancer cells, since these cells are characterised by resisting apoptosis through direct alterations in signalling pathways or proteins that play a role in the apoptotic process such as the kinase p52 (Elmore, 2007). Another factor to resisting death is as described earlier,

the immortalisation of cancer cells, which is the ability to produce telomerase enzyme, which make telomeres' length longer.

1.6.7 Genome instability and mutation

Recent molecular genetic research of the genomes of the most common types of cancer cells have revealed a wide variety of mutations, demonstrating that genomic instability is heightened during tumour growth. Korkola and Gary utilised a genetic research technique known as comparative genomic hybridisation (CGH) in their investigation and discovered both increases and decreases in gene copy number in the cell genomes of a number of tumours. In the same study, the researchers determined that there is a breakdown in the control of genomic integrity in cancer cells; this disordered genomic maintenance reflects DNA repair system deficiencies and is believed to be a crucial factor in the progression of tumours (Korkola and Gray, 2010).

1.6.8 Tumour-promoting inflammation

Recent data indicates that inflammation has a role in the progression of cancer; it has been established that inflammation is active in the early stages of cancer development (Qian and Pollard, 2010). In addition, Grivennikov and colleagues discovered that during inflammation, a number of chemical mediators are released close to tumour cells, which promotes the proliferation of the tumour cells and the invasion of specific immune cells or progenitor cells that promote tumour growth; endothelial cell progenitor cells may also be involved (Grivennikov et al., 2010).

1.6.9 Deregulating cellular metabolism

There are two ways for normal cells to produce the energy required for cell function. Cells generate energy in the presence of oxygen by converting glucose to pyruvate in the cytosol and then, within the mitochondria, carbon dioxide is generated. However, in the absence of oxygen, cells release pyruvate via glycolysis. The capacity of cancer cells to utilise glycolysis even in the presence of oxygen, known as aerobic glycolysis (Warburg, 1956a, Warburg, 1956b), enables tumour cells to provide the energy necessary for their development.

The classification of cancer cells depends on the energy generation pathway; the first group uses lactose to generate energy, while the second group uses lactose produced by nearby cells (Kennedy and Dewhirst, 2010, Feron, 2009, Semenza, 2008).

1.6.10 Avoiding immune destruction

Normally, the immune system continuously monitors cells and tissues and routinely eliminates aberrant or tumour-forming cells. Tumour cells are increasingly able to evade identification by one arm of the immune system by altering the expression of cell surface proteins that the immune system would normally recognise. Immunocompromised persons can develop a variety of malignancies, demonstrating the crucial regulating function of the immune system (Teng et al., 2008, Kim et al., 2007)

1.7 Emerging hallmarks and enabling characteristics

There are, however, four more recently proposed hallmarks by Hanahan and co-workers (see Figure 1.3 below).

These potential novel hallmarks and enabling characteristics, may eventually become essential components of the idea of cancer hallmarks. These features are "unlocking phenotypic plasticity", "non-

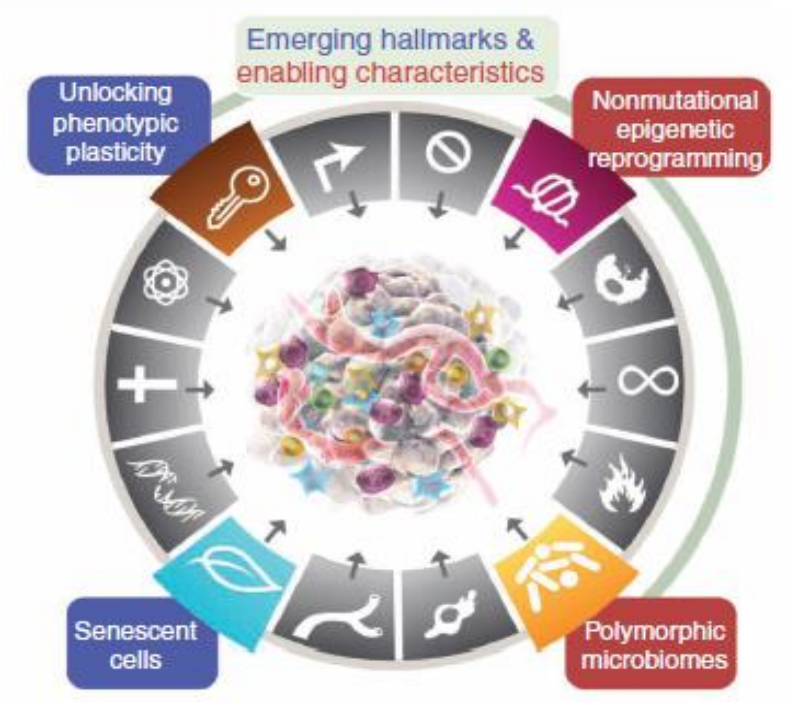


Figure 1. 3 Four newly proposed cancer hallmark capabilities by Hanahan and co-workers (Sourced from: Hanahan, 2022)

mutational epigenetic reprogramming", "polymorphic microbiomes", and "senescent cells" (see Figure 1.3 above).

1.7.1 Unlocking phenotypic plasticity

Unlocking the normally restricted capacity for phenotypic plasticity in order to evade or escape the condition of terminal differentiation is a crucial component of cancer aetiology, according to accumulating data (Yuan et al., 2019). This adaptability might emerge in numerous ways (see Figure 1.4). Thus, nascent cancer cells arising from a normal cell that has progressed along a pathway approaching or adopting a fully differentiated state can revert to progenitor-like cell states via de-differentiation (Hanahan, 2022). In contrast, neoplastic cells developing from a progenitor cell that is destined to follow a pathway leading to end-stage differentiation may circumvent the process, maintaining the spreading cancer cells in a progenitor-like, partially differentiated state (Hanahan, 2022). Alternatively, trans-differentiation may occur, whereby cells initially committed to one differentiation route convert to an altogether different developmental program, so acquiring tissue-specific characteristics that were not predetermined by their normal cell-of-origin (Hanahan, 2022).

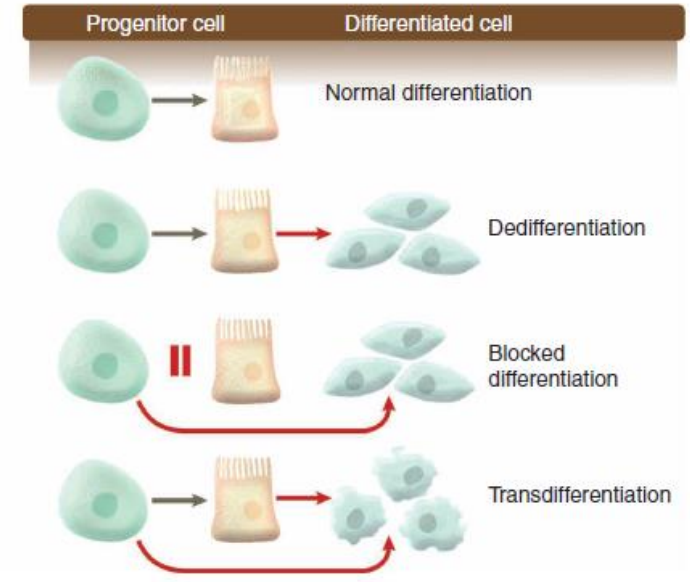


Figure 1. 4 Unlocking phenotypic plasticity. Three prominent modes of disrupted differentiation integral to cancer pathogenesis. (Hanahan, 2022)

1.7.2 Non-mutational epigenetic reprogramming

As the key mechanism governing embryonic development, differentiation, and organogenesis, the concept of non-mutational epigenetic regulation of gene expression is, of course, well-established (Zeng et al., 2019). Long-term memory in adults, for instance, requires changes in gene and histone

modification, chromatin structure, and the activation of gene expression switches that are maintained by positive and negative feedback loops throughout time (Kim et al., 2017). Similar epigenetic modifications may contribute to the acquisition of hallmark capacities throughout tumour formation and malignant progression, according to accumulating data (Hanahan, 2022).

1.7.3 Polymorphic microbiomes

There is a greater understanding that the ecosystems formed in the body by resident bacteria and fungi, the microbiomes, have a significant effect on health and disease (Thomas et al., 2017), a realisation powered by the ability to audit the populations of microbial species using next-generation sequencing and bioinformatics technologies (Hanahan, 2022). Polymorphic variation in the microbiomes across individuals in a population can have a substantial effect on cancer phenotypes, according to mounting data (Helmink et al., 2019). Human association studies and experimental manipulation in mouse cancer models are revealing specific microorganisms, primarily, but not solely bacteria that can have either protective or detrimental effects on cancer development, malignant progression, and therapeutic response. Likewise, the global complexity and composition of a tissue microbiome might vary significantly (Hanahan, 2022). Despite the fact that the gut microbiome has been the pioneer of this new frontier, various tissues and organs have associated microbiomes, each of which has unique characteristics in terms of population dynamics and diversity of microbial species and subspecies (Hanahan, 2022).

1.7.4 Senescent Cells

Cellular senescence is a generally irreversible feature of proliferative arrest that likely evolved as a protective mechanism for maintaining tissue homeostasis, ostensibly as a complement to programmed cell death that serves to inactivate and eventually eliminate diseased, dysfunctional, or otherwise unnecessary cells (Hanahan, 2022). Long considered a preventive mechanism against neoplasia, cellular senescence induces malignant cells to undergo senescence (Lee et al., 2019). There are a number of recognised initiators of senescence and key examples associated with malignancy include DNA damage as a result of aberrant hyperproliferation, so-called onco-gene-induced senescence due to hyperactive signalling, and therapy-induced senescence as a result of cellular and genomic damage caused by

chemotherapy and radiotherapy (Hanahan, 2022). Indeed, there are well-established examples of the preventive effects of senescence in preventing the progression of cancer (Kowlad et al., 2020). In fact, a growing body of evidence indicates the exact opposite: in certain situations, senescent cells drive tumour formation and malignant progression in numerous ways (Wang et al., 2016). In one illuminating case study, senescent cells were pharmacologically ablated by using a suicide gene $p16^{Ink4a}$ in aging mice, specifically senescent cells expressing the cell-cycle inhibitor p16INK4a: in addition to delaying multiple age-related symptoms, the depletion of senescent cells in aging mice resulted in decreased incidences of spontaneous tumourigenesis and cancer-related death (Baker et al., 2016).

Cancer hallmarks are relevant to all cancer types, each hallmark to varying degree of importance to each type of cancer and brain cancer is no exception as is one particularly aggressive form.

1.8 Brain cancer

There are numerous types of brain and central nervous system cancers, which accounted for 2.8% of all cancer diagnoses and 3.2% of all cancer deaths in the United Kingdom in 2011 (CRUK, 2015). The incidence of brain cancer increased steadily from 1979 to 2010 (CRUK, 2015), but this increase can be partially attributed to advancements in diagnostic imaging, which permited more accurate detection and diagnosis (McKinney, 2004). Comparing the incidence of all CNS tumours diagnosed between 1975-1979 and 1996-1999 reveals the same trend (Hoffman et al., 2006; Legler et al., 1999). The increased mortality and incidence of CNS cancers in both males and females in more developed regions relative to less developed regions (Torre et al., 2015) suggests that a westernised lifestyle may contribute to the development of these cancers. Alternatively, the fact that CNS cancers tend to be diagnosed in older patients may contribute to these statistics, given that these more developed regions have populations with a longer life expectancy. Additionally, it is possible that less developed regions lack the necessary equipment, resources, and clinical expertise to accurately diagnose these cancers, potentially skewing the data.

Statistics in the United Kingdom indicate that less than one percent of all diagnosed brain, CNS, and intracranial tumours are preventable by preventing the exposure to known causes (CRUK, 2015). High dose X-rays, such as those used in radiotherapy, and chemicals used in the petrochemical industry

are the only known causes, but they account for only a very small percentage of cases (Adamson et al., 2009). There is no evidence that mobile phones, smoking, alcohol, or specific pathogens cause brain or central nervous system cancers (McKinney, 2004).

A small number of genetic factors have been implicated as brain and CNS cancer causes. Neurofibromatosis (*NF1* gene), tuberous sclerosis (*TSC1/2* gene), Von-Hippel Landau disease (*VHL* gene), Li-Frauman syndrome (*p53* or *CHK2* gene), and Turcot syndrome, also known as mismatch repair cancer syndrome (*MLH1, MSH2, MSH6,* or *PMS2* genes), are examples of genetic conditions associated with an increased risk of brain and CNS tumours (Goodenberger and Jenkins, 2012). All of these conditions result in oncogenesis due to a loss of tumour suppressor gene function, an increase in oncogene activity, or the inability to repair DNA damage.

Despite significant research and clinical advancements, survival rates for brain and CNS tumours have remained low, with overall 5-year survival increasing from 7% to only 19% over the past 40 years (Cancer Research, 2014). This low survival rate is accompanied by an increasing incidence which as mentioned earlier is mainly attributed to the advancement in diagnostic imaging.

1.9 Glioma

Gliogenesis is the development process of glial cells within the peripheral and central nervous systems. Glial cells formation from neural stem cells occurs secondly to the formation of neurons. Glial cells have important roles in neural development and in the adult nervous system. There are different glial cells including radial glia, astroglia, oligodendroglia, microglia and schwann cells; each has its won stage for developmentt (Larjavaara et al., 2007). Any genetic mutation in glial cell development can lead to the formation of gliomas (Omuro and DeAngelis, 2013).

Glioma is the most prevalent brain or CNS cancer, accounting for 80% of all malignant brain tumours (Omuro and DeAngelis, 2013). Glioma can originate from various glial cells, including astrocytes, oligodendrocytes, and ependymal cells. Astrocytomas, which arise from astrocytes, account for 75% of all gliomas and 34% of all brain or CNS cancers (Ostrom et al., 2014). Gliomas are typically located in the frontal, temporal, parietal, and occipital lobes of the brain (Larjavaara et al., 2007).

The World Health Organisation (WHO) classifies gliomas according to their aggressiveness and degree of cell differentiation. Grade I and II tumours are regarded as benign, but if left untreated, they will invariably transform into a malignant, aggressive growth (Omuro and DeAngelis, 2013). Gliomas of grades III and IV are highly differentiated, aggressive cancers with low survival rates (Ostrom et al., 2014).

Grade III gliomas are either anaplastic astrocytomas, oligodendrogliomas, or oligoastrocytomas, with astrocytomas having the lowest 5-year survival rate, ranging from 22 to 25.6% (Omuro and DeAngelis, 2013; Smoll and Hamilton, 2014). Glioma of grade IV is typically referred to as *Glioblastoma multiforme* or simply Glioblastoma. *Glioblastoma multiforme* arises and manifests via two distinct pathways, resulting in either a primary or secondary glioblastoma (Louis et al., 2016). Both primary and secondary glioblastoma, as the two distinct tumours are known, are WHO grade IV and are treated in the same manner. Secondary glioblastoma can arise directly from a WHO grade II astrocytoma or from a WHO grade II astrocytoma that has progressed to WHO grade III anaplastic astrocytoma prior to further progression to glioblastoma (Louis et al., 2016; Ohgaki and Kleihues, 2013).

Due to the aggressiveness of this cancer, glioblastoma is considered terminal, with 5-year survival rates in some patient groups as low as 1.9% historically (Perry et al., 2012). With the advent of more effective treatment options, however, 5-year survival has increased to between 8 and 10% (Perry et al., 2012; Preusser et al., 2011).

This pattern of increasing glioma incidence and continued low survival rates has necessitated the development of new and effective treatment options, and a deeper understanding of the molecular biology of glioma has led to the identification of a number of potentially exploitable targets.

1.10 *Glioblastoma multiforme* (GBM)

As mentioned above, GBM arises and manifests via two distinct pathways, resulting in either a primary or secondary glioblastoma. Both primary and secondary glioblastoma, as the two distinct tumours are known, are WHO grade IV and are treated identically, but their genetic, transcriptomic, and molecular patterns are distinct (Kim et al., 2015; Ohgaki and Kleihues, 2013; Tso et al., 2006).

Primary glioblastoma is a *de novo* tumour that typically manifests in the elderly without any prior clinical manifestation, whereas secondary glioblastoma is the progression of previously diagnosed glioma (Louis et al., 2016). Secondary glioblastoma can arise from a WHO grade II astrocytoma or a grade II astrocytoma that has progressed to grade III anaplastic astrocytoma prior to glioblastoma progression (Agnihotri et al., 2013; Louis et al., 2016). This process is illustrated in the figure below (Figure 1.5).

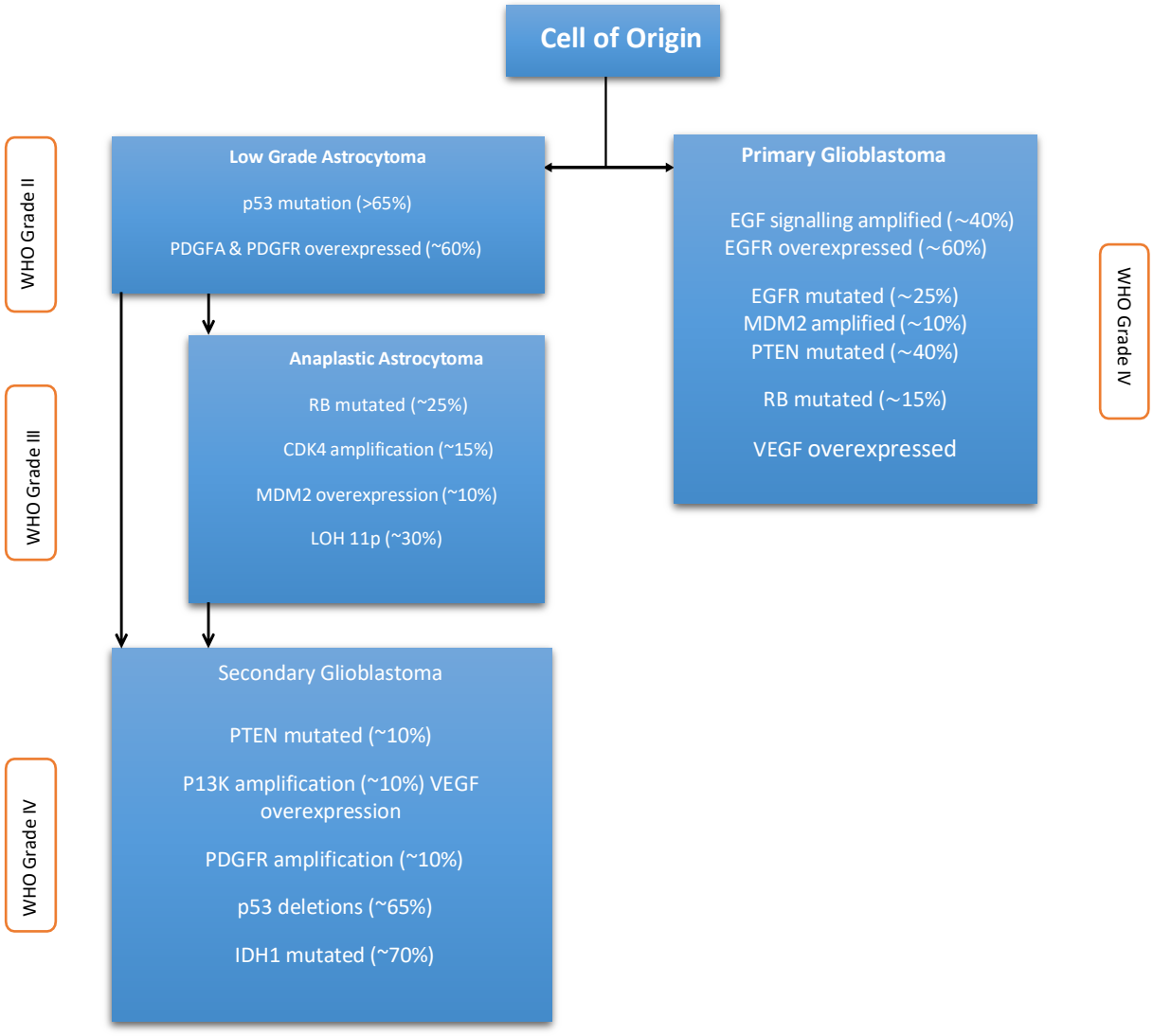


Figure 1. 5 Development of primary and secondary glioblastoma from an undefined cell of origin. Mutations associated with each cancer are also shown (adapted from Agnihotri et al., 2013; Louis et al., 2016).

There is no consensus regarding the cell type from which brain cancers such as glioma originate, so the origin cell in Figure 1.5 is left undefined. Additional independent research, on the other hand, has

identified Cancer Stem Cells (CSCs) as the likely cell of origin in glioma (Biserova et al., 2021). Although oligodendrocyte precursor cells appear to be the cell of origin for glial cancers, the mutation that induces these malignancies can occur in neural stem cells, the progenitor of oligodendrocyte precursor-cells (Liu et al., 2011). In addition, *p53, NF1*, and *TERT* mutations have been identified as drivers of this malignant divergence (Lee et al., 2018; Liu et al., 2011). Figure 1.6 illustrates this concept.

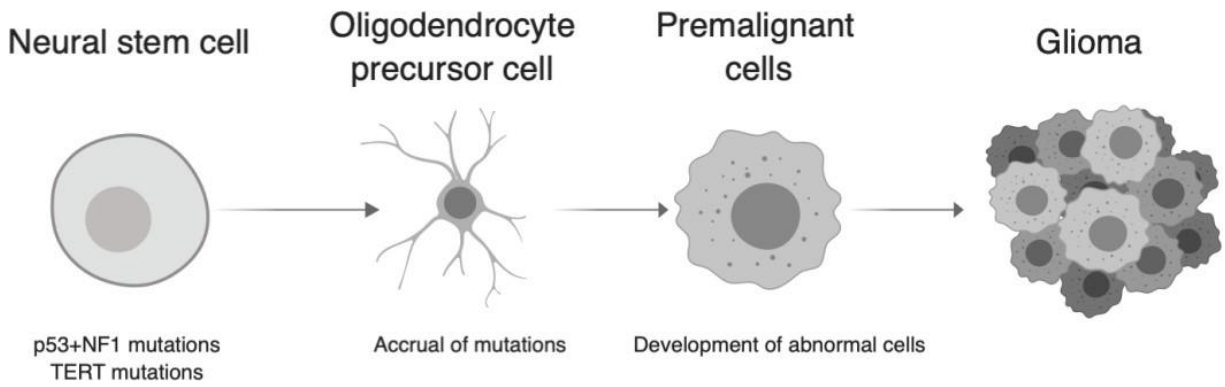


Figure 1. 6 Current paradigm for the origin of glioma as a divergence from typical neurodevelopment (adapted from Liu et al., 2011).

In general, the prognosis for secondary glioblastoma is better than that of primary glioblastoma (7.8 vs. 4.7 months) (Louis et al., 2016; Ohgaki and Kleihues, 2013). This is partially due to secondary glioblastoma presenting in younger patients who are more vulnerable to a more intensive treatment regimen than the generally older population in which primary glioblastoma presents (Ohgaki and Kleihues, 2013). Since secondary glioblastoma is the progression of a WHO grade II or III astrocytoma, there is a clinical history associated with every patient (Ohgaki and Kleihues, 2013). In secondary glioblastoma, a more detailed clinical history will enable clinicians to make better decisions regarding treatment deployment than in mutational primary glioblastoma (Ohgaki and Kleihues, 2013). Even though secondary glioblastoma has a considerably better prognosis, the majority of *Glioblastoma multiforme* diagnoses are for primary glioblastoma. Nearly 95% of cases of *Glioblastoma multiforme* meet the clinical criteria for a primary glioblastoma diagnosis (Ohgaki and Kleihues, 2013).

A distinct mutational, transcriptomic, and metabolomic profile is associated with primary and secondary glioblastoma, more so than clinical history and tumour grade at diagnosis (Tso et al., 2006). This is particularly evident at the transcriptomic level (Tso et al., 2006), where neural, classical,

mesenchymal, and proneural profiles are clearly defined and reported in the literature (Lottaz et al., 2010; Phillips et al., 2006; Verhaak et al., 2010). There is now evidence that the neural subtype is not a true glioma subtype, but rather contamination by healthy brain tissue (Li et al., 2017; Sidaway, 2017; Wang et al., 2017), as evidenced by the absence of genetic abnormalities in this grouping (Li et al., 2017; Sidaway, 2017; Wang et al., 2017). Now that these three profiles have been confirmed in a number of patient samples, additional analysis has revealed that the median survival for every subtype differs (Wang et al., 2017). The classical, mesenchymal, and proneural subtypes have respective median survival rates of 14.7, 11.5, and 17 months (Wang et al., 2017). Increased survivability in the proneural subtype commonly results from the almost exclusive presence of mutant *IDH,* the gene that encodes isocitrate dehydrogenase, in this population (Aldape et al., 2015).

Every one of these profiles has been linked to primary glioblastoma, with the exception of the proneural profile, which has been observed in both primary and secondary glioblastomas (Aldape et al., 2015). A DNA hypermethylation phenotype, known as the glioma-CpG island methylator phenotype (G-CIMP), has been identified as being exclusive to secondary glioblastoma and this subtype is more prevalent in proneural tumours (Aldape et al., 2015). This population was identified for the first time by Noushmehr, and it matches the secondary glioblastoma patient population (Noushmehr et al., 2010). It has been demonstrated that transition between these subtypes is a characteristic of glioma progression and development (Wang et al., 2017), and in some cases, progression would seem to be significantly affected by the tumour microenvironment, specifically the immune response and immune infiltration within the tumour (Wang et al., 2017).

1.10.1 Genetic basis to GBM

Each tumour has a combination of genetic alterations, which determine cancer prognosis and response to therapy. GBM is a very aggressive tumour that has high degree of proliferation, invasion, angiogenesis and resistance to treatment (Patil et al., 2015). Studies in the past 20 years showed different genetic alterations associated with GBM such as tumour protein p53 (*TP53*) mutation and loss, epidermal growth factor receptor (*EGFR*) amplification and mutation, cyclin dependent kinase 4 (*CDK*)

inhibitor p16/ADP-ribosylation factor (*INK4a/ARF*) mutation, phosphatase and tensin homolog (*PTEN*) mutation and loss of heterozygosity (LOH) in chromosome 10p and10q (Furnari et al., 2007).

In 2015, Patil and co-workers showed in their study (examining six different GBM cell lines; T98G, U87, U343, U373, LN 229 and LN18), that 55% of GBM tumour samples have telomerase reverse transcriptase (h*TERT*) promoter mutations and activation (in all cell lines). Its overexpression leads to cell immortalisation through the activation of the larger telomerase complex incorporating h*TERT* (Patil et al., 2015). Another mutation found was in *p53*, which is responsible for different cancer cell characteristics such as apoptosis, differentiation, proliferation and adhesion (Patil et al., 2015; Mollereau and Ma, 2014). Mutation of *NF1*, which is a negative regulator of *RAS* signalling pathway, was apparent mainly in U87 cells (Patil et al., 2015; Brennan et al., 2013). Moreover, they found that none of the six cell lines possessed mutations in mismatch repair (MMR) genes (*MSH2, MSH3, MSH6, MLH1, PMS2, MSH4, MSH5, MLH3, PMS1* and *PMS2L3*) (Patil et al., 2015). The table below shows more gene mutations and alterations related to GBM (Table 1.2).

Table 1. 2 Other gene mutations and alterations associated with GBM (Patil et al., 2015)

Genes	Function	Cell line
EGFR	Tumourigenesis	U373
PTEN	Tumour suppressor gene	T98G, LN18, LN229
ATRX	DNA break repair	U87
MLL3	Tumour suppressor gene	U87, T98G, LN18, U343, LN229
BRCA2	DNA break repair	LN18

Glioma, like many other malignancies, contains a number of frequently mutated or overexpressed genes. The DNA damage repair protein *MGMT*, the pro-inflammatory transcription factor NF-κB, the isocitrate dehydrogenase (*IDH*) gene family, and the h*TERT* are examples.

1.10.2 *MGMT*

Glioma cells include a multitude of DNA repair mechanisms aimed at repairing accumulated DNA damage. *MGMT* is one of these DNA damage repair proteins (O^6-methylguanine DNA methyltransferase). Under normal conditions, *MGMT* largely prevents the epigenetical silencing of

tumour suppressor genes by methylation (Christmann et al., 2011; Nakamura et al., 2001). *MGMT* specifically reverses methylation at the O^6 location of the guanine base by transferring the methyl group to a neighbouring cytosine base (Christmann et al., 2011). The removal of the methyl group from the O^6 position of guanine by *MGMT* is an irreversible process, which poisons the enzyme's active site irreversibly (Christmann et al., 2011).

The methylation of guanine to O^6-methylguanine is uncommon, comprising fewer than 8% of all DNA alkylation events (Fan et al., 2013; Kaina et al., 2007). O^6 methylation of guanine is extremely hazardous because the cell is unable to effectively link the methylated guanine with a complementary base pair. Induction of the mismatch repair pathway ensues (Fan et al., 2013). A cell's persistent failure to effectively couple the methylated guanine base results in failed mismatch repair cycles, which creates DNA double strand breaks (Fan et al., 2013).

Methylation of the O^6 position of guanine is the primary mechanism by which temozolomide acts as an alkylating agent (Nakamura et al., 2001). Expression of the DNA damage repair protein O^6-methylguanine DNA methyltransferase (*MGMT*) is therefore utilised therapeutically to predict response to temozolomide, the primary chemotherapeutic agent for glioblastoma (Nakamura et al., 2001).

However, in high grade glioma the *MGMT* promoter is epigenetically methylated (Nakamura et al., 2001). Methylation of the promoter inhibits the expression of the *MGMT* protein, hence enhancing the therapeutic efficacy of temozolomide (Nakamura et al., 2001). Promoter methylation and hence null expression are more prevalent in low-grade astrocytoma (48%) than in secondary glioblastomas (WHO grade IV) that have progressed from low-grade astrocytoma (75%) (Ohgaki and Kleihues, 2013). In primary glioblastomas, the frequency of *MGMT* promoter methylation was shown to be much lower (36%) (Nakamura et al., 2001). This suggests that methylation of the *MGMT* promoter may be an early event in the formation of these malignancies, and the lack of *MGMT* activity in secondary glioblastoma may explain why these tumours respond considerably better to temozolomide treatment (Ohgaki and Kleihues, 2013).

1.10.3 Isocitrate dehydrogenase (*IDH*)

IDH-1 and *IDH-2* are metabolic enzymes that generate the Kreb's Cycle intermediate α-ketoglutarate (Reitman et al., 2011). Secondary glioblastoma contains *IDH1* mutations more frequently than main glioblastoma, and these mutations are considered to emerge early in the development of these malignancies (Frezza et al., 2010). As previously stated, secondary glioblastoma is a progression from a lower grade glioma, most commonly anaplastic astrocytoma (Louis et al., 2016; Ohgaki and Kleihues, 2013). *IDH* mutation at codon 132 has been identified in 60% of cases of anaplastic astrocytoma but in only 7.2% of cases of primary glioblastoma (Hartmann et al., 2010; Sanson et al., 2009).

Following mutation, *IDH* will exist as a heterodimer consisting of one wild-type and one mutant subunit (Zhao et al., 2009). Unusually for a tumour suppressor gene, only a single allele mutation is required for function loss to occur. This mutation decreases intracellular α-ketoglutarate levels, which increases intracellular levels of the hypoxia marker, hypoxia inducible factor 1- (HIF1-), as α-ketoglutarate is implicated in the oxygen-dependent degradation of HIF (Fu et al., 2010). Despite mutation in a tumour suppressor gene, patients with an *IDH* mutation had much greater survival rates, and it is hypothesised that this is because mutant *IDH* consumes NADPH instead of creating it (Christensen et al., 2010). This will prevent the depletion of the free-radical scavenger glutathione, causing chemo- and radiosensitisation (Christensen et al., 2010; Fu et al., 2010; Houillier et al., 2010).

SongTao and co-workers, discovered in 2012 that secondary glioblastoma patients with *IDH1/2* mutations lived 4.3 months longer than those with *IDH1/2* wild type (8.4 months vs 12.7 months) (SongTao et al., 2012) and 2.7 years longer (1.1 years vs 3.8 years) than those with *IDH1* mutations (Parsons et al., 2008). Overall, patients with both *IDH* mutation and *MGMT* promoter methylation have a better prognosis than patients with either *MGMT* promoter methylation or *IDH* mutation (1,311 days vs. 331 days mean survival) (Hartmann et al., 2010; Molenaar et al., 2014).

1.10.4 Telomerase reverse transcriptase (*TERT*)

Telomeres are region of repetitive nucleotide sequences at the end of the chromosomes and they protect the chromosomal DNA from progressive degradation after cell mitosis. The telomerase reverse transcriptase (*TERT*) gene encodes the catalytic subunit of the telomerase complex, which is required to

counteract the negative impacts of continuous telomere shortening (Huang et al., 2013). In contrast to stem cells, telomerase function is often minimal or absent in the majority of somatic cells due to transcriptional suppression of *TERT*'s promoter (Günes, & Rudolph, 2013). Therefore, reactivation of the *TERT* gene via transcriptional de-repression of its promoter is the rate-limiting stage in restoring telomerase activity, a necessity for the development of the majority of human cancers (Low et al., 2013). In addition to its role in telomere lengthening, other extra-telomeric activities of *TERT* that are essential for cancer development (Ghosh et al., 2012) have been identified. Obviously, understanding how the human *TERT* promoter is triggered in cancers is essential for elucidating a fundamental mechanism of tumorigenesis. Two mutually exclusive and substantially recurrent mutations in the main *TERT* promoter, C250T or C228T, were identified, giving a suitable vantage point for investigating the process of *TERT* reactivation (Li et al., 2014). Many transcription factors, particularly Myc, β-catenin, and NF-κB, have been reported as drivers of the *TERT* promoter, based on the existence of binding sites for these factors on the *TERT* promoter (Greider et al., 2012). NF-κB is a well-known transcription factor that regulates numerous biological processes, including tumorigenesis (Shin et al., 2014). NF-κB signalling can influence *TERT* expression *in vitro* (Ghosh et al., 2012), and the *TERT* promoter contains two putative NF-κB-binding motifs (Yin et al., 2000).

1.10.5 The tumour microenvironment

In the tumour setting, it is also recognised that a number of cytokines and chemokines are overexpressed in GBM such as TWEAK, TNFα, RANKL, IL-8, IL-11 and IL-6, which can contribute in the activation of different signalling pathways (Nogueira et. al., 2011). The activation of these pathways is responsible for driving the inflammatory mechanisms in both the stromal and parenchymal cells of the tumour microenvironment. This process is crucial in initiating and developing the fundamental characteristics of cancer, as previously discussed.

1.10.6 Symptoms associated with GBM

Initial symptoms of glioblastoma may include headaches (30%), weakness and hemiplegia (Paralysis of one side of the body) (20.3%), fits (17%), memory loss and confusion (15%), visual disturbance (10%),

speech deficits (8%) and unconsciousness (5%) (Yuile et al., 2006). Overtime these symptoms individually and collectively worsen and so indicate disease progression.

1.10.7 Treatment of GBM

Presently, glioblastoma is considered an incurable type of cancer and the current management scheme is multimodal which includes surgery, radiotherapy and chemotherapy (e.g. temozolamide, carmustine), as described below. However, as mentioned earlier, glioblastoma reoccurs and overall 5-year survival increased slowly from 7% to only 19% over the past 40 years (Cancer Research, 2014). Therefore, scientists are studying new patterns and mechanisms to suppress cancer in general and *Glioblastoma multiforme* specifically; one strategy is using targeted approaches to interrupt the signalling pathways in tumour cells, which may antagonise the development of certain cancer cell Hallmark(s). One example is, immune checkpoint inhibition using antibody-based therapies (Ming et al., 2014) as well as other examples, also described below.

1.10.7.1 Surgery

Surgery is only performed when it is safe and appropriate to perform so, but it remains the primary treatment option for all glioma patients who qualify. Radiotherapy and chemotherapy can be used to eliminate any remaining cancer cells that have the potential to reform into a tumour. On the other hand, chemotherapy and radiotherapy can be utilised prior to surgery to reduce tumour size (Reed, 2009). This is not always a viable treatment option, such as in the case of brain stem gliomas located deep within the brain or diffuse tumours like diffuse intrinsic pontine glioma (DIPG) (Balogun and Rutka, 2018).

The location of the tumour mass is one of the most restricting aspects of surgery. Glioma is primarily found in the eloquent cortex of the brain, specific areas that control function directly, and so surgeons will err on the side of caution when debulking the tumour mass, as damaging these areas could significantly impair the patient's motor control and speech (Vives and Piepmeier, 1999). However, resection is frequently necessary in these areas because the tumour exerts a mass effect, which can affect a patient's quality of life (Mikuni and Miyamoto, 2010).

The sensitive areas in which glioma grows and the aggressive nature of glioma make it difficult to determine and completely resect the tumour margins. Glioma has a strong affinity for myelinated

tracts and can penetrate the contralateral hemisphere using these tracts to cross the corpus callosum (Giese et al., 2003). Glioma is so invasive that even extreme surgical procedures, such as radical hemispherectomy, have not been successful in curing it (Dandy, 1928). The use of 5-aminolevulinic acid (5-ALA), a fluorescent dye that stains cancerous cells preferentially, has improved surgery. The use of 5-ALA has significantly increased the extent of surgical resection and the time to tumour progression compared to non-fluorescent guided surgery (Eljamel, 2015; Ishizuka et al., 2011).

Peripheral, established 'invader' glioma cells have the capacity to regrow and reform the tumour mass; hence, this is always a fatal, treatment-resistant cancer (Sneed et al., 1994). This recurrence normally occurs within 7-9 months, and in over 90% of cases, it is within 2 cm of the tumour margins (De Bonis et al., 2013). This study also revealed that recurrence occurred in over 65% of instances following prolonged resection of the tumour mass, and in over 85% of cases following a basic resection of high grade glioma and *Glioblastoma multiforme* (De Bonis et al., 2013). Intriguingly, distant recurrences from the primary tumour location had considerable genetic differences from the primary tumour. This genetic difference is not as pronounced in local recurrences (Kim et al., 2015).

In low-grade glioma, in which patients have a much improved prognosis, patients whose cancer is less than 70% excised have a significantly worse 5-year survival rate (41%) than patients who had greater-than 70% tumour resection (84%) (Ius et al., 2012). However, this operation can postpone the growth of the malignancy and lower the likelihood of anaplastic transformation (Keles et al., 2006). This emphasises the need for maximum glioma excision. Regarding the effectiveness of surgery as a therapy option for high-grade glioma, however, there is considerable debate. It is acknowledged that tumour debulking can boost survival rates (Pang et al., 2007), but whether extensive debulking is an appropriate treatment choice is contested (Mitchell et al., 2005). Nevertheless, eliminating the tumour bulk can improve the efficacy of chemotherapy (Ng et al., 2007), which may be a valid justification for surgery.

1.10.7.2 Radiation

Radiotherapy is used in nearly all glioma patients when it is safe to do so, and radiotherapy after surgery has been the standard of care since the 1970s. This is nearly always external beam radiation from an X-ray source, supplied in increments of 1.8-2Gy up to 60Gy (Weller, 2011). However, there is a growing

interest in stereotactic radiotherapy, which delivers very high doses of radiation to a very specific region of the brain in fewer exposures (Baskar et al., 2012; Fogh et al., 2010).

Since the late 1990s, intensity-modulated radiation treatment (IMRT) has been offered. IMRT is a relatively recent method of external beam radiotherapy that employs exceedingly precise X-rays to deliver radiation to the tumour. This has been found to protect vital areas, such as the brain stem, from excessive exposure to ionising radiation without reducing the dose reaching the target (Hermanto et al., 2007). Studies indicate that there is no substantial gain in survival when IMRT is used to treat high-grade glioma, and therefore that the high expense of IMRT may not justify its use in all instances (Fuller et al., 2007).

Proton beam therapy is an emerging treatment option for glioma, but it is more frequently used to treat brain cancers in children. Proton beam therapy deposits highly localised protons with high energy (Loeffler and Durante, 2013). If this region encompasses the area where glioma is anticipated to spread, high-grade glioma can be confined by ionising radiation and subsequent proton beam therapy (Mizumoto et al., 2015). Proton beam therapy is restricted for tiny tumours with well-defined borders, such as ependymoma, chondrosarcoma of the base of the skull, and base of the skull and spinal chordoma (Brada et al., 2007; Crellin, 2018). It has also been demonstrated that proton beam therapy targets glioma stem cells *in vitro* by generating high levels of oxidative stress, which causes irreversible DNA damage (Mitteer et al., 2015). However, proton beam therapy has also been demonstrated to promote glioma cell invasion *in vitro*, likely via SOX-2-mediated matrix metalloproteinase (MMP-9) overexpression (Park et al., 2006; Zaboronok et al., 2014). External beam X-irradiation has also been demonstrated to enhance glioma invasion via MMP-9, unlike proton beam therapy (Park et al., 2006).

External beam radiotherapy is a treatment that utilises ionising radiation, such as X-rays, to harm cells. X-rays are bundles of photons with a wavelength between 0.1 and 10 nanometres that are absorbed by water within tissue, and it is through this absorption that X-rays cause damage. The majority of this damage is indirect DNA damage caused by the interaction of radiation with water molecules absorbed by tissue. The Compton Effect causes the ionisation of water molecules in tissues where radiation is absorbed. This is the interaction of photons with a relatively low-energy or ‘free’ electron. The photon

is then deflected and loses a substantial amount of energy, and the free electron becomes a fast-moving electron that is capable of generating free radicals by ionising other molecules, which is a form of indirect damage, and cleaving bonds in vital molecules, which is a form of direct damage (Dunne-Daly, 1999; Hall, 2000). Figure 1.7 illustrates this procedure.

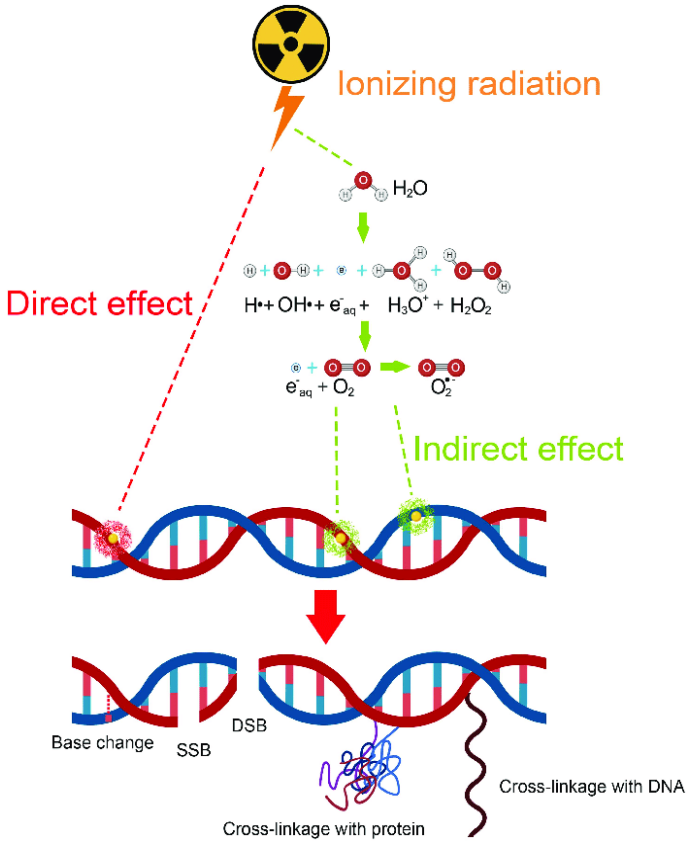


Figure 1. 7 A simplified schematic of how radiation exerts damage on a strand of DNA (Adapted from Liuyun et al., 2021).

The Compton Effect tends to generate electrons with either high or low energy, with varying energy transfer values. Approximately one-third of these electrons are high energy and, as a result, have a high linear energy transfer (LET) value, which means they go further through the cell (Hall and Giaccia, 2012). As a result of the extremely localised ionisation that these electrons generate, it can be difficult for the cell to repair this type of damage (Hall and Giaccia, 2012). The remaining two-thirds of the electrons produced by irradiation are composed of electrons with lower Compton energy (Hall and Giaccia, 2012). Due to the fact that these electrons have a low LET and ionise sparingly along their journey, they cause a high level of cellular ionisation. The vast majority of free radicals are created in this manner (Hall and Giaccia, 2012; Niemantsverdriet et al., 2012).

As it scavenges hydrogen from the base thymine and combines with the double bonds of the bases and the deoxyribose that forms the backbone of the DNA helix, the extremely reactive hydroxyl (OH-) radical is the most damaging to DNA (Cooke et al., 2003). This can lead to both single- and double-stranded DNA breaks. Following a conventional single dose of 1-2Gy of radiation, a single cell's DNA may include up to 1,000 single strand breaks, 40 double strand breaks, and numerous locally damaged sites (McMillan et al., 2001; Vignard et al., 2013). These DNA breaks trigger a DNA damage response that precedes DNA replication in the cell cycle and involves DNA repair proteins like ATM, *MGMT*, and BRCA (Jackson and Bartek, 2009). In response to irradiation, ATM-mediated phosphorylation of p53 arrests the cell cycle in the G_1, S, and G_2 stages, preceding the mitotic phase (Banin, 1998; Saito et al., 2002). At these checkpoints, the cell seeks to repair damage caused by radiation. Due to the lack of an intact template strand, DNA double-stranded breaks are more difficult to repair, causing the cell to induce p53-mediated apoptosis more frequently (Nelson and Kastan, 1994).

In cancer cells with faulty cell cycle checkpoints, abnormal cell cycle arrest after irradiation results in mitotic catastrophe, a kind of cell death. These cells undergo a brief G_2 arrest and initiate mitosis prematurely despite considerable unrepaired DNA damage (Vakifahmetoglu et al., 2008). After mitosis, the cells fail to divide and they re-enter the G_1 phase with a tetraploid DNA level (Weaver and Cleveland, 2005). This results in the formation of enormous, multinucleated cells that live for several days before undergoing delayed apoptosis, necrosis, or senescence and dying (Eriksson and Stigbrand, 2010).

1.10.7.3 Chemotherapy

There are numerous chemical compounds developed to selectively target and eliminate cancer cells. These chemotherapeutic medications target fast dividing cells, which provides the therapeutic advantage for the bulk of anti-cancer cytotoxic agents because cancer cells divide more rapidly than normal cells.

There are numerous kinds of anti-cancer chemicals, each with a unique mode of action, such as DNA-damaging alkylating agents, spindle-poisoning taxols, and growth-inhibiting anti-metabolites (McKnight, 2003). These medications are nearly always used in combination to maximise their efficacy,

which permits a lower dose of each drug to be prescribed, hence minimising the total side effect profile and the risk of drug resistance.

There is no defined chemotherapy regimen for glioma; however, a regimen consisting of procarbazine, lomustine, and vincristine (PCV) has been developed, however it was never universally adopted as the gold standard treatment (Kappelle et al., 2001). Prior to the widespread use of temozolomide, PCV therapy was the first-line treatment choice, and temozolomide was given only when PCV therapy failed (Brandes et al., 2001). This PCV therapy has been demonstrated to be superior to carmustine therapy (Newton et al., 1993). Carmustine has been reintroduced to the clinic as a result of the establishment of local delivery networks (Perry et al., 2007). These chemotherapeutics are considered below.

1.10.7.3.1 Temozolomide

Temozolomide is the first-line therapy for glioma (Beier et al., 2008), and it has dramatically increased survival rates since its debut (Perry et al., 2012). Temozolomide is an alkylating compound that alkylates preferentially the N^7 or O^6 position of the base guanine and the O^3 position of adenine (Friedman et al., 1998). Methylation of these bases causes DNA aggregation, which may contribute to the cytotoxicity of temozolomide, as it does with other alkylating agents, but the main cytotoxic effects of temozolomide are believed to result from a failure of the DNA mismatch repair mechanism to detect appropriate complementary base for the methylated guanine. During these mismatch repair attempts, improper pairing of methylation guanine and thymine might occur, forcing the cell to re-enter the mismatch repair pathway. This results in double-strand breakage (Sarkaria et al., 2008). These DNA nicks cause G_2/M cell cycle arrest (Barciszewska et al., 2015; D'Atri et al., 1998; Friedman et al., 1998; Li, 2008). The cell

will trigger p53-mediated apoptosis following a protracted cell cycle halt and inability to repair damaged DNA (Hirose et al., 2001; Roos et al., 2007). This process is illustrated in Figure 1.8. Deficiency in mismatch repair has been demonstrated to contribute to temozolomide resistance because a competent mismatch repair pathway is required (von Bueren et al., 2012; Cahill et al., 2007; Hunter et al., 2006).

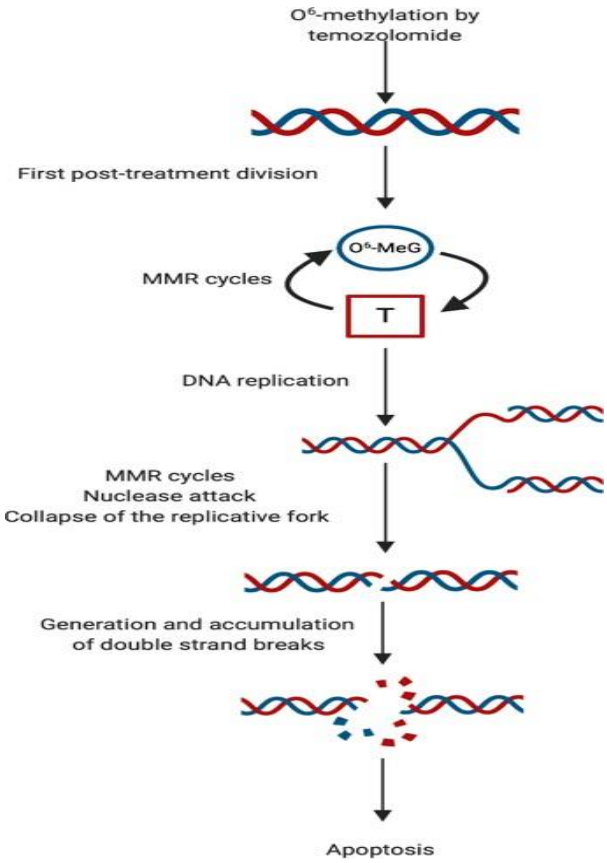


Figure 1. 8 The suggested mechanism of action of temozolomide

(Adapted from Hirose et al., 2001; Roos et al., 2007).

Mutations or hypermethylation of the MSH6 gene, which encodes the guanine/thymine binding protein and is one of the mismatch repair mediators, have been demonstrated to cause therapeutic resistance to temozolomide (Hunter et al., 2006; Xie et al., 2016). There is some evidence that the recurrence of treatment-resistant glioblastoma is caused by the clonal growth of temozolomide-treated cells, specifically mismatch repair-deficient cells (Hunter et al., 2006). It has been demonstrated that in recurrent glioblastoma there is a considerable loss of mismatch repair proficient cells; this shows that first line treatment with temozolomide is positively selecting sensitive cells, which may result in the recurrent tumour becoming treatment resistant (Felsberg et al., 2011).

A DNA hypermethylation phenotype is induced by alkylating chemotherapeutic drugs such as temozolomide, which is one of their disadvantages (Hunter et al., 2006; Kim et al., 2015). Bodell et al., 2003 have shown enhanced mutagenicity following temozolomide treatment, as well as increased

silencing of MSH6, conferring increased chemoresistance. Approximately 25% of temozolomide-treated patients exhibit this symptom (Hunter et al., 2006; Johannessen and Bjerkvig, 2012).

In addition to mismatch repair deficit, *MGMT* expression imparts temozolomide resistance in glioblastoma; *MGMT* status is utilised therapeutically to determine a patient's responsiveness to temozolomide therapy (Hermisson et al., 2006; Paz and Yaya-Tur, 2004). As described in Section 1.2.2.1, the *MGMT* promoter can be epigenetically repressed in glioma, preventing the production of the protein and resulting in a temozolomide-sensitive tumour (Cabrini et al., 2015; Hegi et al., 2004). This is supported by clinical trials, in which *MGMT* promoter methylation has been demonstrated to correlate with therapy success (Hegi et al., 2005).

O^6-benzylguanine has been used clinically to improve the efficacy of temozolomide in response to *MGMT*-mediated temozolomide resistance. In clinical trials, however, O^6-benzylguanine was found to reduce systemic levels of *MGMT*, necessitating a reduction in temozolomide dosage (Koch et al., 2007), which decreased temozolomide's therapeutic efficacy. The injection of O^6-benzylguanine did not increase the sensitivity of temozolomide-resistant gliomas to the drug, according to a separate clinical investigation (Quinn et al., 2009). Prior to the development of temozolomide, O^6-benzylguanine was combined with the alkylating agent carmustine. This increased the cytotoxicity of carmustine (Dolan et al., 1991).

Despite the frequency of *MGMT*-mediated chemoresistance, one of the key advantages of temozolomide is that it can also work as a radiosensitiser; considerably enhancing the efficacy of radiation, an effect that has been demonstrated in both clinical and *in vitro* investigations (Grossman et al., 2010). This enhanced cytotoxicity is considered to be produced by an increase in double-strand breaks or a failure in effective double-strand break repair, which in turn increases the number of cells experiencing mitotic catastrophe (Kil et al., 2008). Temozolomide is therefore administered concurrently and as an adjuvant to radiation. This regimen has dramatically increased glioblastoma patients' overall survival (Stupp et al., 2009, 2010).

Different organisations have published inconsistent results from experiments utilising temozolomide as a radiosensitiser considering whether *MGMT* status influences radiosensitisation. Only

in an orthotopic mouse tumour model in which the *MGMT* gene was epigenetically silenced was radiosensitisation demonstrated in one study (Carlson et al., 2009). Others maintain that *MGMT* is not a determinant of radiosensitisation (Bobola et al., 2010; KA Van Nifterik et al., 2007).

1.10.7.3.2 Carmustine

Following surgical resection, the intracranial administration of carmustine wafers is one of the therapy choices for glioblastoma (Perry et al., 2007). Similar to temozolomide, the alkylating drug carmustine has been utilised as an initial treatment for glioma (Engelhard, 2000). Carmustine, commonly known as bis-chloroethylnitrosourea (BCNU), is an alkylating chemical that inhibits DNA synthesis and repair by generating inter-strand linkages in the DNA chain. The formation of chloroethyl adducts at the O^6 site of guanine is analogous to the mechanism of action of temozolomide. The combination of chloroethylation and chlorine displacement results in the development of an ethyl bridge across the opposing DNA strands (Bota et al., 2007). This will prevent DNA from unravelling, hence inhibiting DNA replication and triggering cell death (Dronkert and Kanaar, 2001).

Carmustine-mediated apoptosis is caused by the collapse of the replicative fork, which results in double-stranded DNA breaks and a G_2/M cell cycle arrest (Roos and Kaina, 2006). Chloroethylating chemicals such as carmustine are substantially more hazardous to p53-mutated cells with weak cell cycle arrest capability, suggesting that double-stranded breaks induced by interstrand linkages cannot be repaired (Batista et al., 2007).

Carmustine resistance is mediated by *MGMT* (Friedman et al., 1998) and functions in the same manner as temozolomide resistance, with *MGMT* eliminating chloroethyl adducts from the O^6-position of guanine (Esteller et al., 2000). Therefore, silencing the *MGMT* gene increases the effectiveness of carmustine, and inhibitors such as O^6-benzylguanine can be employed to artificially reduce *MGMT* levels (Wedge and Newlands, 1996).

Combining temozolomide and carmustine is an appealing therapy option. This combination has been reported to be safe and effective (Barrié et al., 2005), and with the rising use of carmustine implants, it has been claimed that this combination will become the new first-line therapy for glioma (McGirt and

Brem, 2010). Due to the impact of genes such as *TP53* and *MGMT* on the treatment outcome, the use of carmustine is contingent on the careful selection of patient groups (Batista et al., 2007; Hegi et al., 2005; Hirose et al., 2001).

1.10.7.4 Targeted approaches – use of Bevacizumab

Glioma is distinguished by its extensive vascularisation and continuous angiogenesis. Overexpression of vascular endothelial growth factor (VEGF) is associated with increased angiogenesis and blood brain barrier vascular permeability. Regarding angiogenesis, glioma exhibits a certain degree of cause and effect. In response to hypoxia and the synthesis of HIF-1, substantial amounts of VEGF are produced; this results in an increase in tumour vascularisation and growth (Jensen et al., 2006). Nonetheless, when the tumour expands, the hypoxic component of the tumour will also expand, resulting in increased VEGF synthesis and activity. This enhanced vascularisation and vascular permeability is related with metastasis in conventional malignancies; however, the synthesis of VEGF in glioma is associated with growth and invasion via CXCR4 (C-X-C chemokine receptor type 4) signalling (Hong et al., 2006; Zagzag et al., 2006).

Vascular endothelial growth factor (VEGF) is regarded as the most effective mediator of angiogenesis in glioma; hence, numerous techniques have been implemented to target and suppress VEGF. VEGF-receptor inhibitors, VEGF trapping, suppression of VEGF downstream signalling using multiple tyrosine kinase inhibitors (mTKIs), and direct inhibition of VEGF are examples (Weathers and De Groot, 2015). Bevacizumab is a humanised monoclonal antibody that targets vascular endothelial growth factor (VEGF). In 2005, the European Medical Association (EMA) authorised it as the first anti-VEGF therapeutic for the treatment of breast, colorectal, and lung malignancies. In 2009, the FDA approved bevacizumab for the treatment of *Glioblastoma multiforme* in the United States (Cohen et al., 2009), although approval has not yet been granted in Europe for the treatment of GBM (11/2023).

However, it has not been documented that bevacizumab improves overall survival when taken with temozolomide and radiation. Nevertheless, it has been demonstrated to increase progression-free survival in high-grade gliomas, but with a higher frequency of side events (Chinot et al., 2014; Gilbert et al., 2014). Bevacizumab has a threshold effect on glioblastoma, and increasing concentration provide

no meaningful survival benefit, according to a meta-analysis of 15 clinical studies (Wong et al., 2011). It has also been demonstrated that following bevacizumab treatment, it is common to observe a non-enhancing pattern on contrast-weighted MRI, which is usually indicative of a lack of tumour invasion, a low-grade cancer, or, most likely, a decrease in tumour vascularisation (Iwamoto et al., 2009; Norden et al., 2008; White et al., 2007). This decrease or lack of tumour enhancement has been observed with various anti-VEGF therapies (Batchelor et al., 2007), suggesting that a drop in enhancement may be attributable to a reduction in angiogenesis and vascularisation.

1.11 Signalling pathways that contribute to GBM

There are a number of signalling pathways that support accelerated cell growth in cancer cells such as extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK) which is one of the mitogen-activated protein kinase (MAPK) signalling pathway major cassettes; microtubule-associated protein 1S (MAP1S) and others (Tong et al., 2015; Ming et al., 2014). However, one of the prominent pathways driving the tumour cell signalling responses, which is showing significant effect on cancer Hallmarks, is the Nuclear Factor kappa-B (NF-κB) pathway, which involves a number of proteins controlling various biological responses (Paul et al., 2018).

1.11.1 The Nuclear Factor kappa-B (NF-κB) pathways

Nuclear Factor kappa-B (NF-κB) proteins are five transcription factor subunits: Rel (cRel), p65 (RelA, NFκB3), RelB, p105/p50 (NF-κB1), and p100/p52 (NF-κB2) (Kim et al., 2017; Xing, 2016). These proteins play a role in different biological responses such as inflammation, immune response, cell growth, proliferation, apoptosis and cell differentiation and development (Paul et al., 2018).

All NF-κB subunits possess an N-terminal Rel homology domain named ‘RHD’, which is responsible for DNA binding, nuclear translocation, protein dimerisation and interaction with specific IκB proteins (Hayden and Ghosh, 2004). Moreover, NF-κB is further classified according to the presence of C-terminal transactivation regions as either class 1 or 2 (Brown et al., 1994). Class 1 NF-κB isoforms do not possess a C-terminal transactivation region; these are p50/p105 and p100/p52, which 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). Class 2 NF-κB subunits, on the other hand, have C-

terminal transactivation regions and these subunits consist of RelA, c-Rel and RelB (Dobrzanski et al., 1993).

NF-κB signalling is driven by either the canonical or non-canonical pathway, which remain inactive by the association with inhibitory kappa B (I-κB) proteins α, β and ε isoforms (Paul et al., 2018). According to studies, each I-κB protein is specific for each Rel/NF-κB protein. For instance, RelB only binds to p100/p105 (Dobrzanski et al., 1995), whilst I-κBα and I-κBβ bind to and strongly inhibit RelA/p52 dimer (Baeuerle and Baltimore, 1989, Beg et al., 1992, Thompson et al., 1995).

Both the non-canonical and the canonical NF-κB signalling pathways can be activated by a number of extracellular ligands such as lymphotoxin-β (LTβ), Receptor-activator of NF-κB ligand (RANKL), B-cell activating factor (BAFF) or CD40; and cytokines, pathogen-activated molecular patterns (PAMPs), Growth Factors (GFs) or androgens, respectively (see Figure 1.9) (Paul et al., 2018).

Following cellular activation the NF-κB/I-κB complex dissociates due to the activity of the Inhibitory kappa B kinases (IKKs), which allows the transcription factor to translocate into the nucleus,

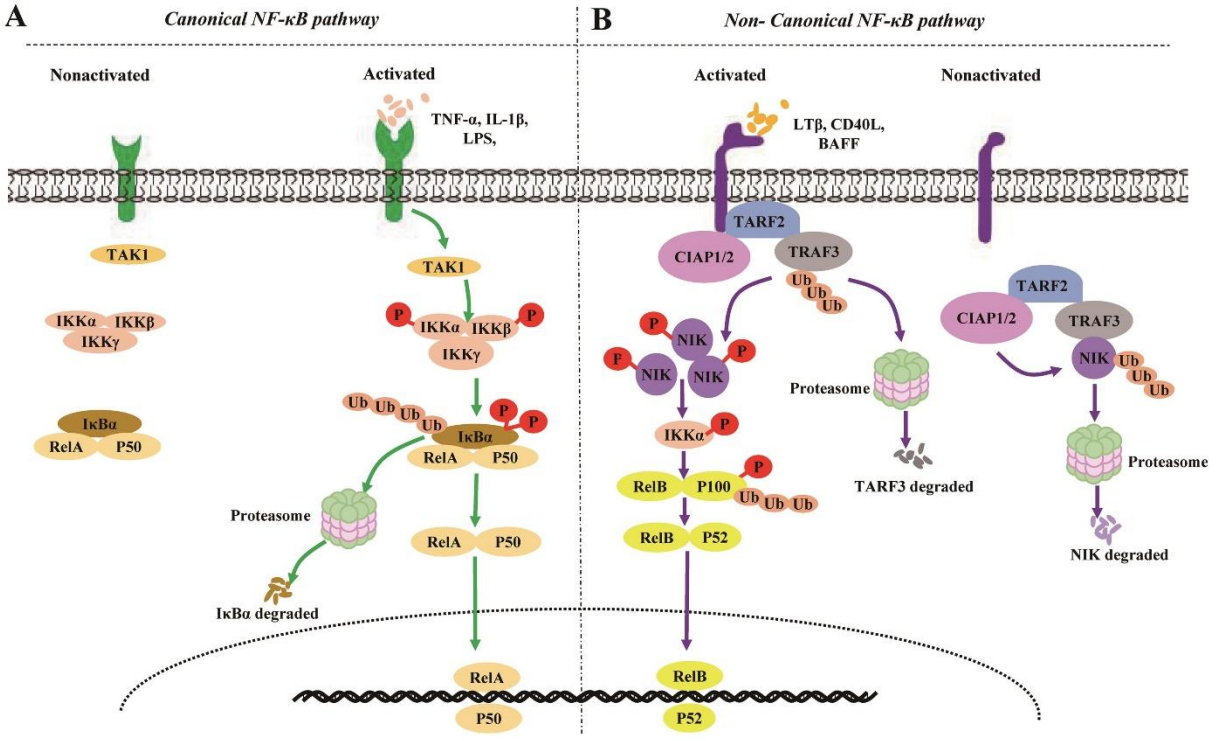


Figure 1. 9 The canonical (classical) and non-canonical (alternative) NF-κB signalling pathways (Sourced from Chen Q. et al., 2021).

where it binds to promotor regions of different genes (Gamble et al., 2012a). Therefore, IKKs seem to be potential targets for cancer therapy.

1.11.2 The role of Inhibitory kappa B Kinases (IKKs) in regulating NF-κB pathways

For NF-κB to be activated, I-κB is phosphorylated by IKKs, which exhibit as a complex of α, β and γ isoforms (α, β which are catalytically active; and γ which is a scaffolding protein). Both IKKα and IKKβ have a similar structure with C-terminal helix – loop – helix (HLH) domains, leucine–zipper motifs and N-terminal kinase domains (see Figure 1.10) (Woronicz et al., 1997). However, based on knockout (KO) studies using IKKα and IKKβ KO mice, it was found that IKKα predominantly regulates the non-canonical NF-κB pathway while IKKβ regulates the canonical NF-κB pathway (Gamble et al., 2012).

1.12 The regulation of the canonical NF-κB pathway

In relation to physiological responses, the canonical NF-κB pathway is activated primarily by cytokines such as TNFα and IL-1β. However, for this pathway particularly, the array of stimuli is very diverse and includes multiple activators of Toll-like Receptors (TLRs), UV light, reactive oxygen species and neurotransmitters (Hayden and Ghosh, 2008).

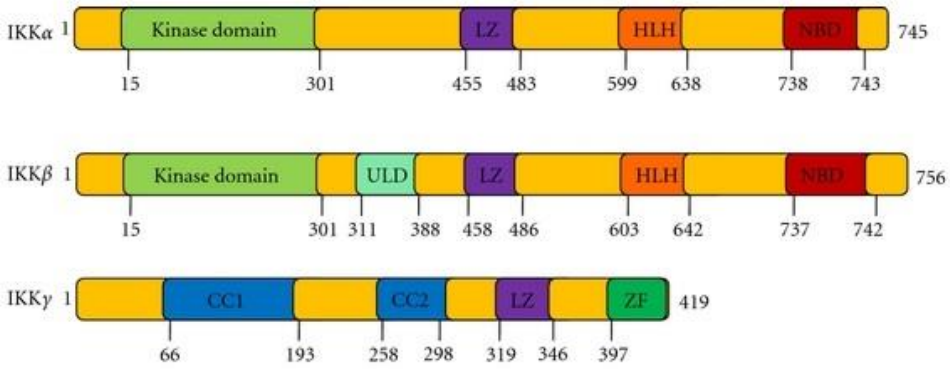


Figure 1. 10 Structures of different IKKs. Abbreviations: LZ=leucine zipper, HLH=helix-loop-helix, NBD=NEMO binding domain, CC= coiled coil region and ZF=zinc finger (Sourced from: Hindawi, 2012).

Regardless of stimuli, a crucial integration point in this pathway is the activation of IKKβ, which mediates the phosphorylation of IκBα facilitating its ubiquitination and degradation. However, there are a number of key intermediate proteins that link the IKK complex to the related receptor, for example TNFα stimulates the 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 NF-κB binding to DNA following the over-expression of TRAF2 (Devin et al., 2001).

While TRAF2 engagement is linked to the activation of TNFR1 by TNFα, activation of the classical NF-κB pathway by IL-1 occurs after TRAF6 engagement. Following the stimulation by IL-1, recruitment of TAB2 (an adaptor protein) occurs and translocates from the plasma membrane into the cytosol, associating with TAK-1 and TRAF6 (Takaesu et al., 2000). In addition, Kishida and co-workers found that TAK-1 facilitated the ubiquitination of TRAF6 during stimulation of cells with IL-1 to transduce the signal from TRAF6 to the IKK complex (Kishida et al., 2005).

1.13 The regulation of the non-canonical NFκB pathway

A number of studies have confirmed that the activation of the non-canonical NFκB pathway is dependent on IKKα as a key factor (Liang et al., 2006, Senftleben et al., 2001; reviewed in Paul et al., 2018). Such activation requires phosphorylation and processing of the p100 subunit to p52 which 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).

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 TNFα like weak inducer of apoptosis (TWEAK) (Cherry et al., 2015), have all been shown to couple to the non-canonical NF-κB pathway.

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 stabilisation and elevated expression and activation of NF-κB-inducing Kinase (NIK). NIK in turn activates IKKα that regulates the processing of p100 to p52 (Xiao et al., 2001a).

In addition, a study showed that NIK has the ability to regulate processing of p100 through a region named the NIK responsive domain (NRD), a mutation in NIK led to decreased processing of p52

in lymphoplasia mice (Xiao et al., 2001b). These results gave the impression that IKKα and NIK both have a vital role in the activation of the non-canonical NF-κB pathway (Razani et al., 2010). Thus, both of these kinases within the non-canonical NF-κB cascade could be potential targets for inhibition of this pathway.

1.14 Nuclear Factor Kappa B Pathways in Cancer

A number of studies suggest that NF-κB signalling pathway could contribute to a number of cellular processes related to cancer development such as proliferation and cell survival (Valentine et al., 2010). In 2010, Tysnes emphasised two cellular processes that are linked to NF-κB activity and tumour development. These were, firstly, increased expression of survival genes which made cells resistant to apoptosis and secondly, increased expression of cell cycle genes such as cyclin D1 and cyclin D2 (Tysnes, 2010) that determine cellular proliferation.

Such involvement of NF-κB in cancer has been demonstrated to associate with clinical outcomes. For instance, NF-κB has been shown to be active in around 95% of cancers such as pancreatic, colorectal and lung cancer (Lu et al., 2004, Lu and Stark, 2004, Senegas et al., 2015). Moreover, high levels of NF-κB were measured in primary breast cancer tissue and mammary carcinoma cell lines of both humans and rodents (Cogswell et al., 2000). Karin and co-workers found a strong link between NF-κB activity and the development of melanoma metastasis, as well as leukaemia and lymphoma (Karin, 2006). NF-κB signalling was also confirmed to be active in different brain cancer cell lines including *Glioblastoma multiforme* cells (Cherry et al., 2015).

Studies have also examined the role of the IKKs in cancer development which showed that constitutive activation of the IKK complex has been measured in a number of cancer cell lines such as those derived from the breast (Romieu-Mourez, 2001), the prostate (Gasparian et al., 2002) and in the colorectal setting (Charalambous et al., 2003). 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). Moreover, a decrease in the activity of IKKβ has been associated with reduced development of myeloma (Yang, 2010). Also, pre-treatment with PS1145, an IKKβ inhibitor which blocks classical NF-κB activity, resulted in the death of myeloma cancer cells (Castro et al., 2003).

Many studies have observed a potential role for IKKα in a number of cancers such as breast, prostate, lung, colorectal, pancreatic and brain cancer through the effect on cell cycle progression and apoptosis (Fernandez-Majada et al., 2007b; Park et al., 2005; Hirata et al., 2006; Luo et al., 2007; Cherry et al., 2015). Studies demonstrate that IKKα can regulate cyclin D1 independently of NF-κB 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). In addition, 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 kinase A (Prajapati et al., 2006). Several other studies have involved IKKα in the regulation and phosphorylation of a number of proteins that increase cell proliferation in diverse cancer models including cyclin D1, Amplified in breast cancer 1 (AIB1), β-catenin and others (Pui, 2009; Zardawi et al., 2010; Cantarini et al., 2006). These and additional studies collectively suggest an important role for NF-κB signalling and the increase in the non-canonical NF-κB cascade in the regulation of multiple processes that lead to cancer development (Lee and Hung, 2008; Luo et al., 2007).

1.15 IKK-NF-κB signalling in GBM

As in other types of cancer, elevated activation of NF-κB has also been observed in glioblastoma (GBM) tumours, wherein the expression of NF-κB was significantly greater in GBM tissue as compared to non-GBM tissue (Avci et al., 2020). The NF-κB transcription factor is known to play a crucial role in regulating several cellular processes such as tumour cell proliferation, migration, immune response, and apoptosis as well as the development of chemoresistance to temozolomide (TMZ) and the regulation of *MGMT* activity in GBM (Avci et al., 2020). This is achieved by NF-κB binding to certain locations within the *MGMT* promoter, hence increasing the expression of the *MGMT* gene (Avci et al., 2020). Moreover, recent studies have established a correlation between recurrence and the reactivation of the h*TERT* promoter, a promoter that is known to be mutated in approximately one-third of GBM cancer patients (Li et al., 2015).

The h*TERT*, which is responsible for encoding the catalytic subunit of the telomerase complex (Li et al., 2015), plays a crucial role in counteracting the negative consequences of gradual telomere

shortening (Li et al., 2015). Two distinct and frequently occurring mutations in the core *TERT* promoter, specifically the C250T and C228T alterations, have been documented in previous studies. These mutations offer a promising avenue for investigating the reactivation mechanism of *TERT* (Horn et al., 2013). Although the two hotspot mutations generate a comparable binding motif for E-twenty-six (ETS) transcription factors, they are distinctive in that the C250T *TERT* promoter, in contrast to the C228T *TERT* promoter, is activated by non-canonical NF-κB signalling (Li et al., 2015). The binding of ETS alone to the C250T mutant *TERT* promoter does not provide sufficient activation for transcription, and the reactivation of *TERT* at this mutant promoter is dependent on the collaboration with p52, which occurs downstream of non-canonical NF-κB signalling (Li et al., 2015). Therefore, inhibiting the non-canonical NF-κB pathway through interfering with IKKα is considered a potential route for novel anti-cancer drug therapy. Targeting IKKα in GBM tumours therefore may beneficially block a variety of cancer hallmarks and therefore tumour development in approximately 30% of all GBM cases that carry the C250T mutation in the h*TERT* promoter, by specific targeting of p52-driven transcription.

1.16 Potential anti-cancer effect of IKK inhibitors

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 chemotherapy (Park et al., 2005; Hirata et al., 2006; Fernandez-Majada et al., 2007a; Doppler et al., 2013; Paul et al., 2018). Today there are a number of IKKα/IKKβ or selective IKKβ inhibitors, however, these inhibitors have issues, which prevent 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 results in inhibition of the non-canonical NF-κB pathway which is IKKα dependent; activation of this pathway leads to a slow and maintained NF-κB signal in comparison with the IKKβ-dependent pathway (the canonical NF-κB pathway), which is a rapidly generated response. This difference may allow for the use of selective IKKα inhibitors in disease targeting (reviewed in Sun, 2012; Paul et al., 2018).

1.17 Aims and objectives

Glioblastoma has a very poor prognosis, with surgery currently being the only solution for prolonged survival (approximately 15 months) because most patients are either resistant to chemotherapy or become so over time. Many studies have demonstrated that the NF-κB pathway plays a role in the resistance to chemotherapy, cancer cell invasiveness and growth, in addition to recurrence, which studies linked to reactivation of the h*TERT* promoter, a promoter that is found mutated in 1 of 3 patients with cancer (Li et al., 2015).

Given recent studies have investigated NF-κB pathways as a new strategy for developing new anti-cancer drugs. Such studies have suggested that IKKα has a key role in the activation of the non-canonical NF-κB pathway and other related transcriptional cascades, it is implicated in the regulation of growth, invasion and metastasis. Thus, targeting IKKα may be a promising strategy to the development of new glioblastoma medications**.**

Study Hypothesis

Null Hypothesis (H_0): Inhibiting IKKalpha will have no significant effect on NFkB activation and nuclear translocation in GBM cells.

Alternative Hypothesis (H_1): Inhibiting IKKalpha will result in decreased NFkB activation and impaired nuclear translocation in GBM cells, leading to reduced expression of NFkB target genes associated with tumor aggressiveness.

Therefore, the aims of this study are to:

1. Characterise the activation of the non-canonical and canonical NF-κB pathways in glioblastoma cell lines that represent different forms of GBM with varying genetic backgrounds (T98G and UVW) using inflammatory and growth supporting stimulants that are typically overexpressed in GBM.
2. Investigate the pharmacological effect of selective IKKα inhibitors through examining their selectivity within both the non-canonical and canonical NF-κB pathways and their effect on IKKα-dependent cellular processes that underpin the phenotypic outcomes that support GBM development.

The objectives of the study are to:

1. Investigate the impact of inhibiting IKKα on the activation and nuclear translocation of NF-kB biomarkers in T98G cells.
2. Assess the alterations in GBM cell proliferation and viability upon inhibition of IKKα-mediated NF-kB signalling pathway.
3. Examine the influence of IKKα inhibition on the expression levels of NF-kB target genes associated with GBM aggressiveness, such as those involved in cell survival, invasion, and immortality.
4. Evaluate the efficacy of IKKα inhibitors as potential therapeutic agents for GBM treatment through in vitro studies, including cell culture assays.

Chapter Two

Materials and Methods

Chapter 2 Materials and Methods

2 Materials and Methods

2.1 General reagents

All reagents and materials used were supplied by Sigma-Aldrich Chemical Company Ltd. (Pool, Dorest UK) or other companies, as described below.

Thermo Fisher Scientific UK Ltd (Leicestershire, UK)

Bovine Serum Albumin (BSA)

L – Glutamine Gibco™

Penicillin-Streptomycin (Antibiotics)

Trypsin

Gibco™ Fetal Bovine Serum

Modified Eagle Medium (MEM)

Bamford Laboratories

Ethanol

Boehringer Mannheim Ltd (East Sussex, UK)

DTT

Bio-Rad Laboratories (Hertfordshire, UK)

Bio-Rad DC™ Protein Assay Dye Reagent Concentrate

Pre-stained SDS-PAGE molecular weight markers

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 (Buckinghamshire, UK)

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

Sarstedt AG & Co LTD (Leicester, UK)

Serological pipettes 5, 10, 25 ml

2.2 Anti-bodies

Abcam (Cambridge, UK)

Rabbit monoclonal anti-IKKβ (Y466)

Cell Signaling Technology Inc. (MA, USA)

Rabbit monoclonal anti-p-NF-kappa B2 p100 (Ser866/870) (4810L)

Rabbit monoclonal IgG anti-GAPDH (14C10)

Rabbit polyclonal anti-p-NF-kappa B2 p65 (Ser536) (3031L)

Rabbit monoclonal anti-IκBα (92424L)

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

Mouse monoclonal anti-IKKα (14A231)

Anti-NFκB p52 (32534)

Santa Cruz Biotechnology Inc (California, USA)

Rabbit polyclonal anti-NFκB p65 (C-20)

LI-COR Biosciences UK ltd. (Cambridge, UK)

EMSA oligo pan-NFκB IRDye700 (D20209-03)

Odessey EMSA Buffer Kit (D20124-02)

Integrated DNA Technologies (IDT) (Iowa, USA)

C250T TERT promoter (Ref. no. 233059171)

WT TERT promoter (Ref. no. 233059168)

2.3 Pharmacological agonists

Insight Biotechnology Limited (Wembley, UK)

Human TNF-related weak inducer of apoptosis (TWEAK/TNFSF12)

Recombinant Human Tumour Necrosis Factor-α (TNFα)

2.4 Pharmacological inhibitors of the IKKs

Selective small molecule IKKα kinase inhibitors (SU compounds, University of Strathclyde):

IKKα-selective kinase inhibitors, SU1433 (K_i IKKα vs IKKβ: is 11 vs. 2295 nM) and SU1644 (K_i IKKα vs. IKKβ: is 20 nM vs. > 5000 nM), were kindly provided by Professor S. Mackay, SIPBS.

Selective small molecule IKKβ kinase inhibitor: MLN120B (ML120B) as a potent, ATP competitive, and orally active inhibitor of IKKβ with an *in vitro* IC_{50} against IKKβ of 60 nM (K_i IKKβ is 30nM) (no data on IKKα found) was obtained from MedChemExpress (MCE), USA. (Catalgue no.783348-36-7)

2.5 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.

T98G cell line

Human Caucasian glioblastoma (T98G) cells, modal no. 92090213, were derived from glioblastoma multiform tumour from a 61-year-old Caucasian male. The cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC).

UVW cell line

Human brain astrocytoma (MOG-G-UVW) cells, modal no. 86022703, were established from an anaplastic astrocytoma of normal adult brain. The cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC).

2.5.1 Culture of T98G and UVW GBM Cells

The normal medium for these cell lines was MEM medium supplemented with 10% (v/v) foetal bovine serum, 0.5% (v/v) penicillin/streptomycin and 1% (v/v) pyruvate and 1% (v/v) non-essential amino acids. Cells were incubated at 37°C in presence of a mixture of 95% air, 5% CO_2.

2.5.2 Trypsinisation and subculture

Once the cells reached approximately 80-90% confluency, they were sub-cultured. Firstly, the media was aspirated and the cells washed twice with a sterile solution of 5% (w/v) trypsin. After trypsin was removed, the flask was placed in the incubator at 37°C, at 5% CO_2 for 2-4 min. Once the cells displayed a round morphology, flasks were given a gentle tap to ensure cells were fully detached. The flask was then washed with 10ml MEM to re-suspend the recovered cells, which were used for seeding into fresh flasks (75 cm^2) or multi-well culture plates as required.

2.5.3 Cell freezing and resuscitation

Once the cells reached approximately 80-90% confluency, they were sub-cultured. Firstly, the media was aspirated and the cells washed twice with a sterile solution of 5% (w/v) trypsin. After trypsin was removed, the flask was placed in the incubator at 37°C, at 5% CO_2 for 2-4 min. Once the cells displayed a round morphology, flasks were given a gentle tap to ensure cells were fully detached. The flask was then washed with 10ml of (1ml 0.1% SDS, 4ml MEM and 5ml foetal bovine serum) to re-suspend the recovered cells, which were transferred into 2 ml cryotubes ready for freezing in liquid nitrogen. To resuscitate, cells were defrosted at room temperature and used for seeding into fresh flasks (75 cm^2).

2.6 Western Blotting

2.6.1 Maintaining cells in culture and rendering quiescent ahead of experiments

Cells were grown in a cell incubator at 37°C, and the media containing 10% (v/v) FBS was changed every two days until the growth reached 90%. To render cells quiescent prior to signalling experiments, cells were starved of serum for 24 hours before any stimulation using FBS free media or containing a significantly lower concentration of FBS e.g. 1-2% (v/v) FBS/media.

2.6.2 Preparation of whole cell extracts

Cells grown on 6- or 12-well plates were exposed to suitable concentrations of agonist (TWEAK) for specific periods of time or varying concentrations, plates placed on ice to terminate the reaction, and then the media aspirated. Cells were washed once with 500 µl cold PBS, and 250 µl of Laemmli sample buffer containing DTT (63 mM Tris-HCl, (pH 6.8), 2 mM $Na_4P_2O_7$, 5 mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50 mM DTT, 0.007% (w/v) bromophenol blue) added. The cells were scraped and the

chromosomal DNA sheared by repeatedly passing through a 21 gauge needle. Cell extracts were transferred into 1 mltubes with a hole pierced in the lid, then boiled for five minutes and stored at -20°C until use.

2.6.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated by SDS-PAGE. 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 and flush. The resolving gels were prepared by mixing a suitable volume (7.5% (v/v)) of acrylamide (acrylamide 30%: N, N'- methylenebis-acrylamide 0.8% (37.5: 1)), 0.375 M 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 manufacturer's instruction (Bio-Rad) and 200 µl of 0.1% (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) of acrylamide (acrylamide 30%: : N, N'- methylenebis-acrylamide 0.8% (37.5:1)), 0.125 M 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 well combs inserted immediately into the stacking gel solution. After the polymerisation of the gel, the combs were removed and the gel assembled in a Bio-Rad Mini-PROTEAN II^{TM} electrophoresis tank. Aliquots of samples (10-20 µl) were loaded into the wells using a micro-syringe. A pre-stained SDS-PAGE molecular weight marker of known molecular weights was run concurrently in order to identify the molecular weights of polypeptides of interest. Samples were electrophoresed at a constant voltage of 130V, until the bromophenol dye had reached the bottom of the gel.

2.6.4 Electrophoretic transfer of proteins onto nitrocellulose membrane

Separated proteins present in acrylamide gels were transferred to a 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, 0.25M Tris (pH 8.3)

and 20% (v/v) methanol) in a Bio-Rad mini Trans-Blot^{TM} tank and a constant current of 300mA was applied for 150 minutes. During this time the tank was cooled by inclusion of an ice reservoir.

2.6.5 Immunological detection of proteins

Following the transfer of proteins to nitrocellulose membranes, the membranes were removed and the non-specific binding of proteins was blocked by incubation in a solution of 3% (w/v) BSA in NATT buffer (20 mM Tris, 150 mM NaCl (pH 7.4), 0.03% (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 0.5% (w/v) BSA in NATT overnight. The following day, the membranes were washed in NATT every 15 minutes, 5 times, with gentle shaking. Following this the membranes were incubated for one and a half hours with secondary horseradish peroxidase-conjugated IgG directed against the first immunoglobulin diluted to a suitable concentration in NATT buffer containing 0.2% (w/v) BSA. The membrane was then washed in NATT every 15 minutes, 5 times, and the bands of immune-reactive protein were detected by incubation in enhanced chemiluminescence (ECL) reagents (reagent 1 contains 1 M Tris pH8.5, 250 mM luminol, 250 mM p-cymuric acid and distilled H_2O,; reagent 2 contains 1 M Tris pH8.5, 0.19% H_2O_2 and distilled H_2O) for 2 min with gentle shaking. The membranes were then placed in a photographic cassette and covered with cling film, then exposed to X-ray film for the required time under darkroom conditions and developed using a JPI AUTOMATIC X-RAY FLIMPROCESSOR MODEL JP-33.

2.6.6 Re-probing and stripping of nitrocellulose membrane

To re-probe the nitrocellulose membrane for additional proteins, the membranes were stripped of antibodies. The nitrocellulose was incubated in 15 ml of stripping buffer (0.05 M Tris-HCl, 2% (v/v) SDS, and 0.1 M 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 5 min in NATT to remove the residual stripping buffer. After the last wash the membrane was incubated with primary antibody overnight with 0.5% (w/v) BSA in NATT. The blots at this stage were then ready for the immunological detection protocol as explained previously.

2.6.7 Preparation of nuclear extracts

To prepare crude nuclear extracts, cells were grown on 6-well plates (in duplicates or triplicates). Once cells were 90% confluent they were rendered quiescent by incubation in serum free media (or media containing 1-2% (v/v) FBS) for 24 hours and cells were then exposed to appropriate agonists over a specific time course. The media then was aspirated and the cell monolayers scraped into 0.5 ml PBS and transferred into 1.5 mL tubes. The tubes were centrifuged at 4°C for 13,000 rpm for 1 minute and the supernatants removed. 400 µl of buffer 1 (10 mM Hepes pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml pepstatin) was added to each pellet, and following resuspension incubated for 15 min. Following the addition of 25 µl of 10% (w/v) NP-40 the samples were vortexed for 10 seconds 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 re-suspended in 50 µl of buffer 2 (20 mM Hepes, (pH 7.9), 25% (v/v) glycerol, 0.4 M NaCl, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/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 rpm for 15 min. Supernatants (the soluble nuclear extracts), were stored at -80 °C until further use.

2.6.8 Determination of protein concentration in the nuclear extracts

The concentration of protein in nuclear extracts was determined using the Bio-Rad DC™ protein assay dye reagent by the Bradford assay method. A range of standards were prepared using different concentrations (2-20µg) of BSA. The dilutions of the standards and the nuclear extract samples were made up in sterile dH_2O (790-795 µl) and mixed with 200 µl of dye agent. The samples 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.

2.7 EMSA assay

Electrophoretic Mobility Shift Assay (EMSA) is a commonly used method to detect protein-DNA interactions *in vitro*. The following was the methodology for performing EMSA:

Preparation of probes: Single-stranded oligonucleotides containing the DNA sequences of interest were synthesised and labeled with a fluorescent tag (IRDye 700). Preparation of nuclear extract: Cells were harvested and lysed to release their nuclear contents as mentioned above. The nuclear extract was prepared by centrifugation and quantified using a Bradford assay. A binding reaction was set up by incubating the labelled DNA probe with the nuclear extract in the presence of a buffer containing a carrier DNA (salmon sperm DNA) and a non-specific competitor used to minimize the binding of nonspecific proteins to the labeled target DNA, such as poly(dl-dC). The binding reaction mixture was then loaded onto a native polyacrylamide gel and subjected to electrophoresis. The gel was run at a low voltage (100V) to prevent denaturation of protein-DNA complexes. The gel was scanned to detect the labeled DNA probe using a LI-COR Odyssey Infrared Imager. Protein-DNA complexes migrate slower than unbound probes, resulting in a shift or retardation in the mobility of the labeled probe.

The short fluorescently-labeled DNA sequences derived from the h*TERT* promoter sequences that were used in these experiment were as follows:

TERT C250T duplex

5′- GTCCCGACCCCTTCCGGGTCCCCGGC -3′

3′- CAGGGCTGGGGAAGGCCCAGGGGCCG -5′

TERT WT duplex

5′- GTCCCGACCCCTCCCGGGTCCCCGGC -3′

3′- CAGGGCTGGGGAGGGCCCAGGGGCCG -5′

2.8 MTT assay

The MTT assay is a colorimetric assay that is commonly used to measure cell viability and proliferation in vitro through measuring cell metabolic actvity. The following was the methodology for performing the MTT assay:

T98G and UVW cells were seeded into a 96-well plate at a density that allows for exponential growth (5 x 10^4/well). The cells were then cultured under optimal conditions (37°C in presence of a mixture of 95% air, 5% CO_2), for a predetermined length of time to allow for cell attachment and growth. The cells were then treated with the compound of interest, typically small molecule IKK kinase inhibitors (SU1433, SU1644 and ML120B). The MTT reagent was added to each well, and the plate was

incubated at optimal conditions for a predetermined length of time, usually 2-4 hours. The medium was aspirated, and the formazan crystals were solubilised with DMSO. The absorbance of the formazan product was measured spectrophotometrically at a wavelength of 570 nm using a microplate reader. The absorbance values correlate with the number of viable cells, and therefore cell viability or proliferation can be quantified.

2.9 Clonogenic assay

To assess the replicative potential of cells, under varying conditions, a colorimetric clonogenic assay was utilised. Cells were grown to around 70% confluence, the media was removed and the cells were washed with 2 ml of 5% (w/v) trypsin. Cells were detached using 1 ml of trypsin (0.05% (w/v)) then the cells were collected in a 15 ml tube and 5-10 ml of media was added. The cell number was determined using a haemocytometer and the appropriate number of cells (150 cells) seeded onto 60 mm petri dishes in 3 ml of media in triplicate for each treatment. After 24 h, cells were treated with different concentrations of compound for 24 h and the media removed and 5 ml of growing media added. Following 7-14 days of incubation, the media was removed and the colonies washed with 3 ml of cold PBS and the cells fixed with 100% methanol for 15 min. The cells were stained with 3 ml of Giemsa for 15 min then washed with tap water and dried. The number of colonies were counted manually. Plating efficiency (PE) and survival fraction (SF) were calculated from the following equations:

$$\underline{\mathbf{PE}} = \frac{\textbf{average no. of control colonies formed}}{\textbf{no. of seeded cells}}$$

$$\frac{\textbf{average no. of colonies formed after treatment}}{\textbf{no. of seeded cells (untreated cells)} \times \textbf{PE}}$$

2.10 Statistical analysis

All data shown were expressed as mean ± S.E.M and were representative of at least three separate experiments unless stated otherwise. Optical density of bands was analysed using Scion image software. Statistical analysis was performed using GraphPad Prism version 10 (Version 10.1.0, GraphPad Software Inc, USA). 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 EC_{50} for TWEAK and the IC_{50} for the SU compounds, GraphPad Prism version 10 (Version 10.1.0, GraphPad Software Inc, USA) was used. X values were the logarithms of either the agonist or the inhibitor concentrations and Y values were the reading of optical density using Scion image software. Non-linear regression analysis was used to determine the concentration response curve and calculation of the EC_{50} and IC_{50}. Graphs were plotted in GraphPad Prism version 10 (Version 10.1.0, GraphPad Software Inc, USA).

Chapter 3

Characterisation of the NF-κB signalling pathways in the T98G glioblastoma cell line

Chapter 3 Characterisation of the NF-κB signalling pathways in the T98G glioblastoma cell line

3.1 Introduction

According to Oeckinghaus and Ghosh (2009), NF-κB plays a significant role in several cellular functions, including growth and apoptosis. In addition, NF-κB plays a crucial role in numerous processes that lead to tumour progression, including proliferation, anti-apoptotic responses, cellular motility, angiogenesis, metastasis, and drug resistance (Hoesel and Schmid, 2013). Despite the fact that the overwhelming majority of these studies are linked to the canonical NF-κB pathway, activation of the non-canonical NF-κB pathway is gaining prominence. Different stimuli, such as TWEAK, stimulate the non-canonical NF-κB pathway, resulting in the phosphorylation of IKKα and subsequent phosphorylation of p100, its subsequent ubiquitination and processing to p52, and the formation/liberation of p52-RelB heterodimers (Paul et al., 2018, Oeckinghaus and Ghosh, 2009). These dimers translocate into the nucleus and bind to specific DNA sites to activate the transcription of a number of cancer-related genes (Paul et al., 2018). Birbrair et al. (2015) found that IKKα mediated regulation of gene products included MMP-9, IL-8, and VEGF. In addition, it was demonstrated that IKKα activation increased the expression of a number of genes, including Chemokine (C-C motif) ligand 19 (CCL19), CCL21 and CCL22, by more than threefold (Birbrair et al., 2015) and collectively these induction events support phenotypic outcomes such as inflammation and the development of cancer 'hallmarks, as detailed previously (section 1.6).

To date, however, relatively little is known about the status of the non-canonical NF-κB pathway in the context of brain cancer or its potential contribution to disease progression. Consequently, the purpose and initial aims of this chapter were to investigate the characteristics of the non-canonical NF-κB pathway activation relative to that of the canonical NF-κB pathway, assessing the components within each of these related, yet distinct signalling cascades in a glioblastoma cell line. These experiments were designed in the T98G glioblastoma cells line with specific end-points of assessment;

1) TWEAK and TNFα cytokines abilities to activate the non-canonical NF-κB cascade, as measured by p-100 phosphorylation, p100/52 processing and nuclear translocation of p52/RelB complexes.
2) In parallel, the ability of TWEAK and TNFα cytokines to activate the canonical NF-κB cascade by measuring the degradation of IκB-α, the phosphorylation and nuclear translocation of p65.

3.2 Materials and Methods

3.2.1 Cell lines and routine cell maintenance

All routine maintenance of cell lines was performed as described in Section 2.5.

Cell stimulations were performed using TNFα and TWEAK as described in section 2.6.

3.2.2 Western Blotting

Western Blotting was performed using different antibodies (details in table below) as described in methods section 2.6.

Table 3. 1 Antibodies used throughout chapter 3

Antibody	Type	Dilution	Remarks
Anti-p-NF-kappa B2 p100	Rabbit	1:1000	Cell signalling Technology Inc. (USA)
Anti-NFκB p52	Mouse	1:20,000	Millipore (U.K.) Limited (UK)
Anti-RelB	Rabbit	1:3000	Cell signalling Technology Inc. (USA)
Anti-IκB-α	Rabbit	1:2000	Cell signalling Technology Inc. (USA)
Anti-p-NF-kappa B2 p65	Rabbit	1:2000	Cell signalling Technology Inc. (USA)
Anti-NFκB p65 (C-20)	Rabbit	1:2000	Santa Cruz Biotechnology Inc (USA)

3.3 Characterisation of TWEAK-mediated non-canonical of NF-κB pathway activation in T98G glioblastoma cells

Initial experiments sought to examine the kinetics of activation of the non-canonical and canonical NF-κB pathways. This was investigated utilising two agonists: TWEAK, and additionally TNFα, which have been reported to activate both the canonical and non-canonical NF-κB pathways in a variety of cell types including a number of cell lines derived from tumours (reviewed in Paul et al., 2018).

3.3.1 The effect of TWEAK on p100 (Ser866/870) phosphorylation in T98G glioblastoma cells

The key marker for activation of the non-canonical NF-κB pathway is phosphorylation of p100 NF-κB2 (Ser866/870). In order to assess the effect of TWEAK on the phosphorylation of p100 in T98G cells, first, the cells were treated with increasing concentrations of TWEAK (0-100 ng/ml) at 4 hours based on previous studies that described TWEAK to stimulate the non-canonical NF-κB pathway in glioblastoma cell lines; two studies used 100 ng/ml to activate the pathway while one study showed that TWEAK activated the pathway with a concentration as low as 10 ng/ml (Tran et al., 2005; Fortin et al., 2009; Cherry et al., 2015). Taking these reports into account, experiments were constructed examining the impact of TWEAK at increasing concentrations, from 0 to 100 ng/ml.

Following treatment, cells were washed by the procedure described in the methods section and WCEs prepared at 4 hours' time. In Figure 3.1, p100 phosphorylation was analysed by Western Blotting following TWEAK stimulation. Phosphorylation of p100 was variable and low at the first two concentrations (1 and 3 ng/ml) with a significant increase in the effect seen at 20, 30 and 50 ng/ml (the highest effect measured at 20 ng/ml). Phosphorylation of p100 was maintained thereafter with a sharp decrease at 100 ng/ml (see Figure 3.1). This indicated that TWEAK activated the non-canonical NF-κB pathway in the T98G cell line with 10 ng/ml being the best concentration to use in further examinations, as it achieved EC_{75}=12.95 ng/ml, being comparable to the work of Cherry et al. (2015).

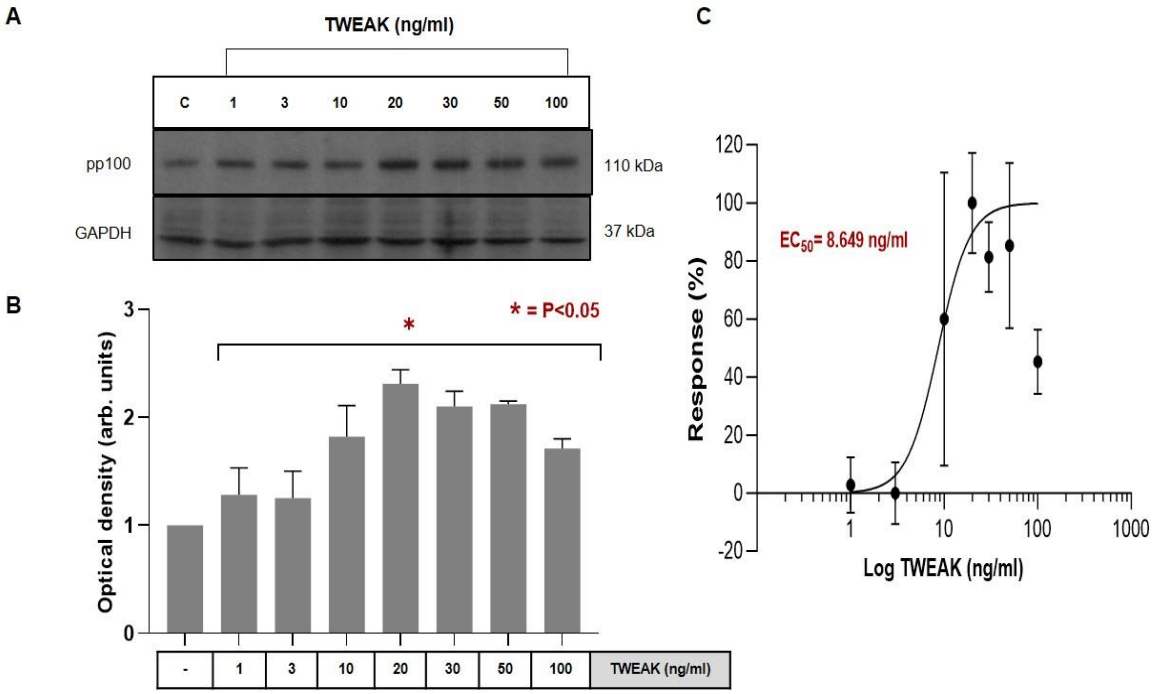


Figure 3. 1 The effect of TWEAK on p100 phosphorylation in T98G glioblastoma cells.

T98G cells were grown in a 12 well plate to near confluence and rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cells were then treated with different concentrations of TWEAK (1-100 ng/ml) for 4 hours. Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies (name the ones relevant for this experiment). Detection of GAPDH was used as a loading control. **(A)** Western Blot bands representing the phosphorylation of p100 (pp100) following the exposure to different concentrations of TWEAK (1-100 ng/ml) for 4 hours. **(B)** Bar chart showing a semi-quantitative densitometry of the blot. Results expressed as fold stimulation relative to control. **(C)** A concentration curve that represents p100 phosphorylation following increasing concentrations of TWEAK (EC_{50} = 8.65 (see graph) ng/ml). Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

To examine the effect of TWEAK on p100 phosphorylation over time, T98G cells were exposed to TWEAK over a 24 hours' time course, as shown in Figure 3.2. Phosphorylation of p100 was noticeable from 30 minutes (0.5 h) followed by an increasing pattern. After the first two hours, a significant elevation was seen at 4 hours with the highest level at 8 hours, and a sharp reduction at 24 hours. This indicates that TWEAK can activate the non-canonical NF-κB pathway in the T98G glioblastoma cell line.

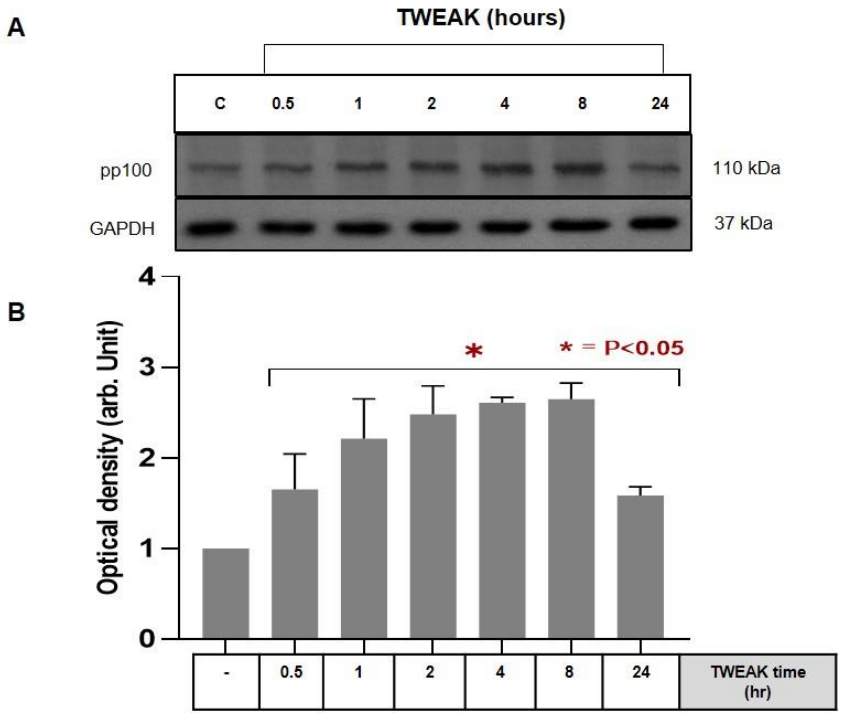


Figure 3. 2 The effect of TWEAK on p100 phosphorylation in T98G glioblastoma cells.

T98G cells were grown in 12 well plates to near confluence and rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cells were then treated with 10 ng/ml of TWEAK over time (0-24 hours). Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. Detection of GAPDH was used as a loading control. **(A)** Western Blot bands representing the phosphorylation of p100 over time following the exposure to 10ng/ml TWEAK. **(B)** Bar chart showing the blots assessment using semi-quantitative densitometry. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

3.3.2 The effect of TWEAK on p100/p52 processing in T98G glioblastoma cells

In order to examine the effect of TWEAK on the processing of p100 to mature p52, whole cell extracts samples of T98G cells were exposed to TWEAK over a 24 hours' time course, as shown in Figure 3.3. The results showed that the processing of p100 to p52 was noticeable at one hour the levels of p100 started to decrease and as a result, in parallel, the levels of p52 increased until 8 hours (significant increase compared to control was seen at 4-8 hours). Thereafter, p52 production/expression decreased. This indicated that TWEAK can activate cytoplasmic processing of p100 to p52 in the non-canonical NF-κB pathway in the T98G glioblastoma cell line.

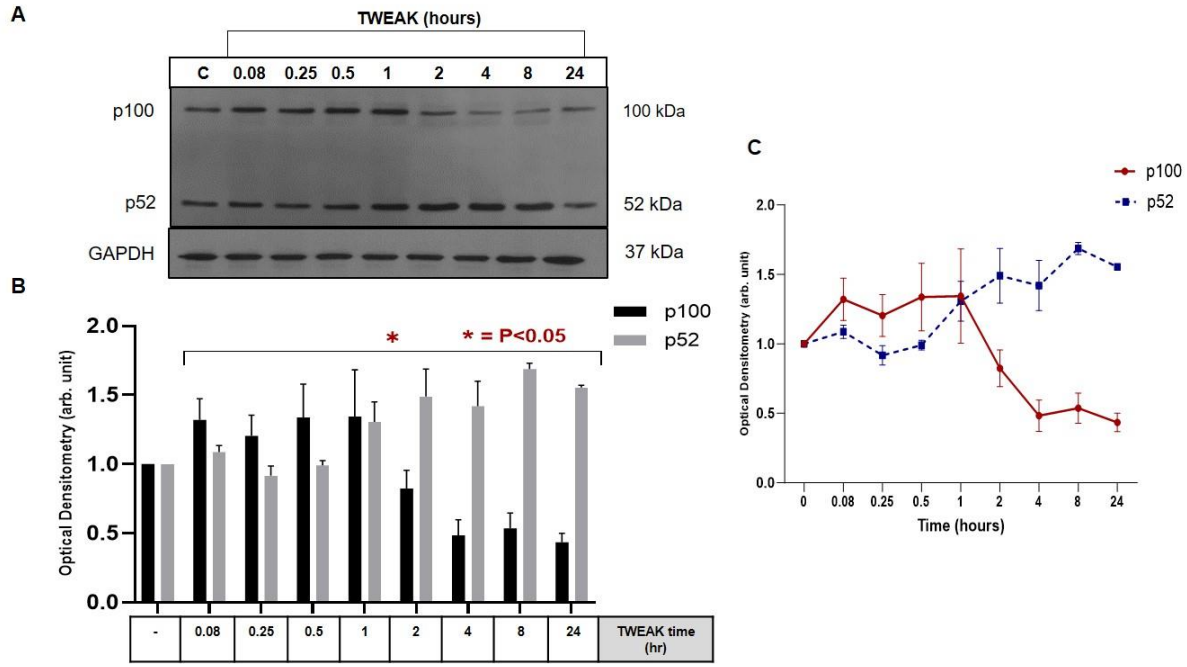


Figure 3. 3 The effect of TWEAK on the expression of p100/p52 in T98G glioblastoma cells.

T98G cells were grown in 12 well plates to near confluence and rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cells were then treated with 10 ng/ml of TWEAK over time (0-24 hours). Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western Blot bands representing the processing of p100 to p52 over time following the exposure to 10ng/ml of TWEAK. Detection of GAPDH was used as a loading control. **(B and C)** A bar chart and a line chart that show the blots' assessment using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

3.3.3 The effect of TWEAK on nuclear translocation of p52/RelB in the T98G glioblastoma cell line

The results obtained in previous figures demonstrate that exposure to TWEAK induced the formation of p52 in whole cell extracts of T98G cells. In order to investigate the ability of TWEAK to induce p52/RelB translocation to the nucleus of T98G cells, nuclear extracts from cells treated with vehicle (DMSO) or 10 ng/ml of TWEAK were prepared as outlined in methods section 2.6.7. The results in Figure 3.4 showed that 2 hours after TWEAK stimulation, a continuous increase in the level of nuclear p52 as well as RelB was observed. A significant nuclear accumulation of p52 was noticed at 8 hours post-stimulation with no significant accumulation observed for RelB. These results suggested that TWEAK can mediate the processing of p100 to p52/RelB and translocation to the nucleus.

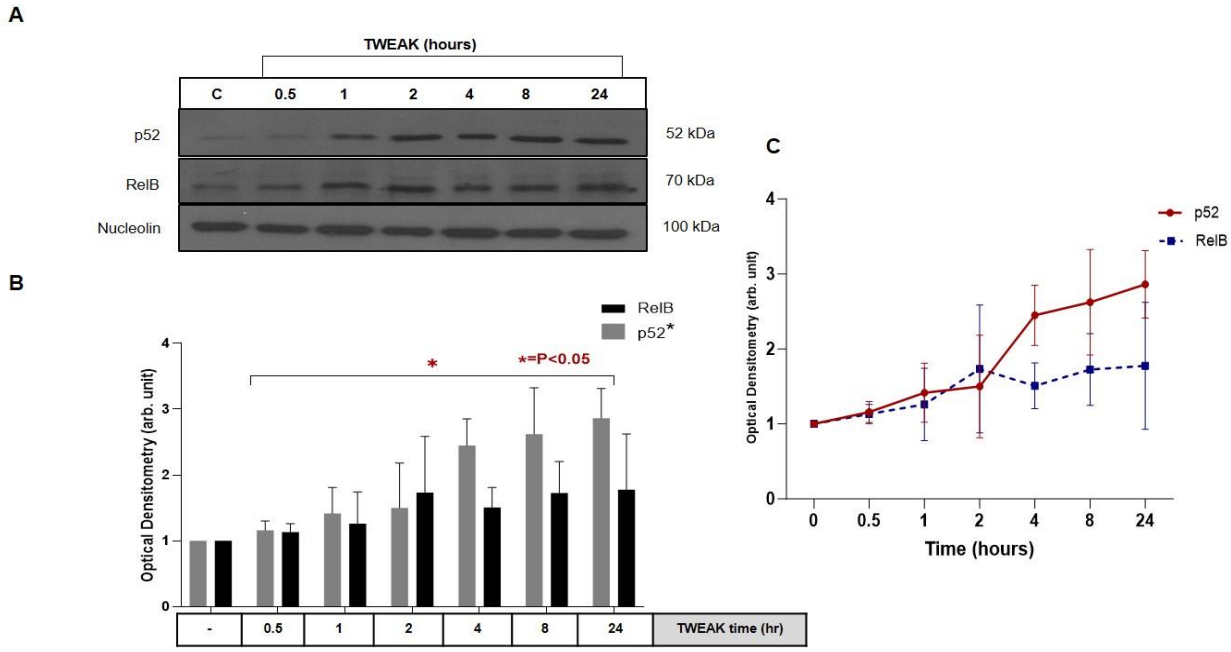


Figure 3. 4 The effect of TWEAK on nuclear translocation of p52/RelB in T98G cells.

T98G cells were grown to near confluency in a 6 well plate (in triplicates) rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cells were then treated with 10 ng/ml of TWEAK over time (0-24 hours). Nuclear Extracts were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western Blot bands representing the nuclear translocation of p52 and RelB over time following the exposure to 10ng/ml of TWEAK. Detestion of nucleolin was used as a loading control. **(B and C)** A bar chart and a line chart that show the blots' assessment using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

3.3.4 The effect of TWEAK on IKKα and IKKβ expression in T98G glioblastoma cells

As levels of p100 increased in response to TWEAK stimulation, the levels of cellular IKKα and IKKβ in T98G cells were investigated. Cells were exposed to TWEAK for the same time points used in previous experiments. IKKα and IKKβ levels were assessed by Western blotting as described in the methods section. The results showed minimalvariation in the levels of both IKKα and IKKβ protein expression across all time points (Figure 3.5).

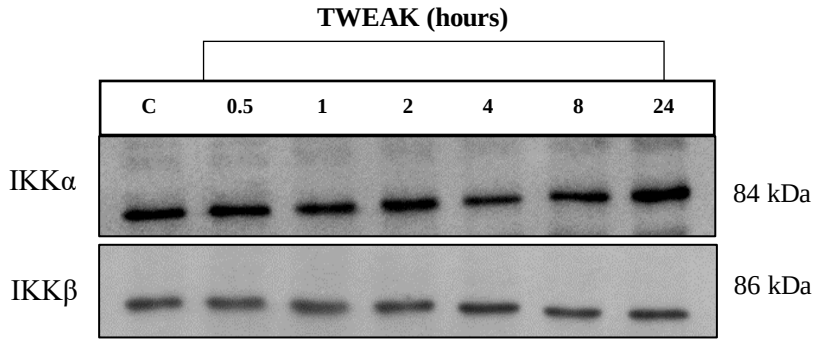


Figure 3. 5 Effect of TWEAK on expression of IKKα and IKKβ in T98G cells.

T98G cells were grown in 12 well plates to near confluence and rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cells were then exposed to TWEAK (10ng/ml) for the times indicated, whole cell extracts were prepared and analysed by Western blotting for expression of IKKα and IKKβ. The blot is representative of two independent experiments.

3.4 Characterisation of TWEAK-mediated canonical NF-κB pathway activation in T98G glioblastoma cells

3.4.1 The effect of TWEAK on the degradation of IκB-α in T98G glioblastoma cells

It is well recognised that a number of cytokines and/or members of the TNF superfamily (e.g. TWEAK, TNFα etc.), in a variety of cellular settings, can activate the classical NF-κB pathway (Paul et al., 2018). To assess whether in glioblastoma cells TWEAK was able to activate the classical NF-κB pathway by affecting the degradation of IκB-α over time, T98G cells were exposed to TWEAK over a 24 hours' time course, as shown in Figure 3.6. No noticeable degradation of IκB-α was seen from 0 minutes until 24 hours. This indicated that TWEAK did not activate the canonical NF-κB pathway in the T98G glioblastoma cell line.

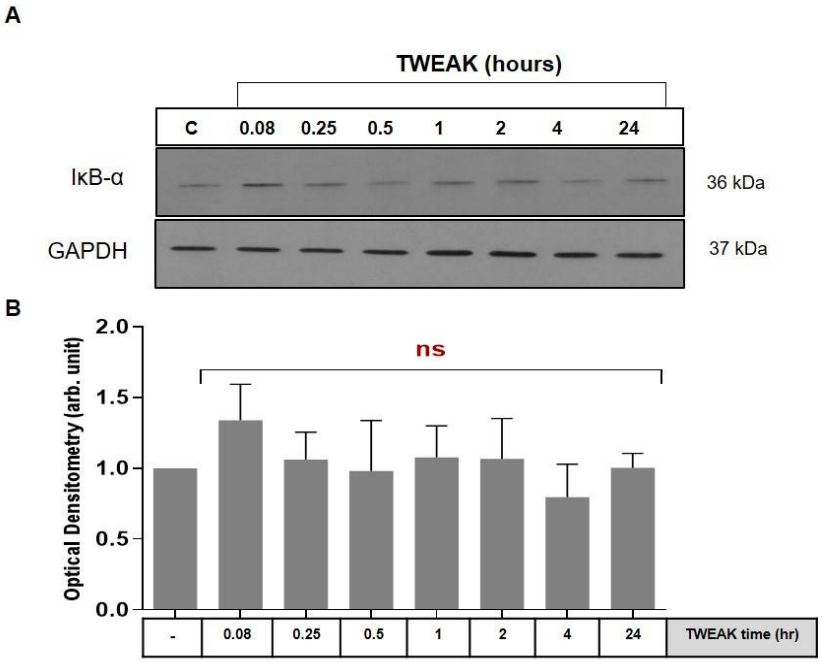
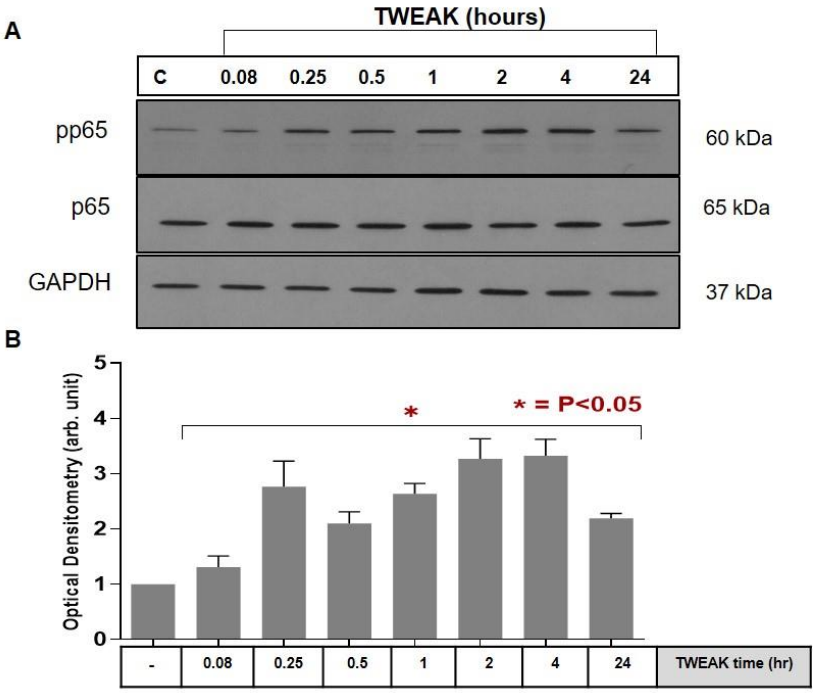


Figure 3. 6 The effect of TWEAK on the degradation of IκB-α in T98G glioblastoma cells.

T98G cells were grown in 12 well plates to near confluence and rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cells were then treated with 10 ng/ml of TWEAK over time (0-24 hours as indicated). Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots representing the phosphorylation of IκB-α over time following the exposure to 10ng/ml of TWEAK. Detection of GAPDH was used as a loading control. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

3.4.2 The effect of TWEAK on p65 (Ser536) phosphorylation in T98G glioblastoma cells

To examine the effect of TWEAK on the p65 phosphorylation (Ser536) over time, T98G cells were exposed to TNFα over a 24 hours' time course, as shown in Figure 3.7. Significant phosphorylation of p65 was noticed from 5-15 minutes (0.08-0.25 h) with the maximum at 4 hours followed by a gradual decrease. This indicated that the TWEAK may have some ability to part-modulate components of the canonical NF-κB pathway in the T98G glioblastoma cell line and may indicate an ability to impact p65 transactivation and associated transcriptional activation. However, further examinations are required in this regard.



3.4.3 The effect of TWEAK on nuclear translocation of p65 in T98G glioblastoma cells

The results obtained in previous experiments demonstrate that exposure of T98G glioblastoma cells to TWEAK produced no effect on IκB-α expression and minimal effect on p65 (Ser536) phosphorylation in whole cell extracts. In order to investigate the potential ability of TWEAK to induce p65 translocation to the nucleus of T98G cells, nuclear extracts from cells treated with 10 ng/ml of TWEAK were prepared as outlined in the methods section. The results in Figure 3.8 show that TWEAK stimulation produced minimal variation in the level of nuclear p65 over 24 hours. This result suggests that TWEAK cannot fully mediate the processing and translocation of p65 to the nucleus.

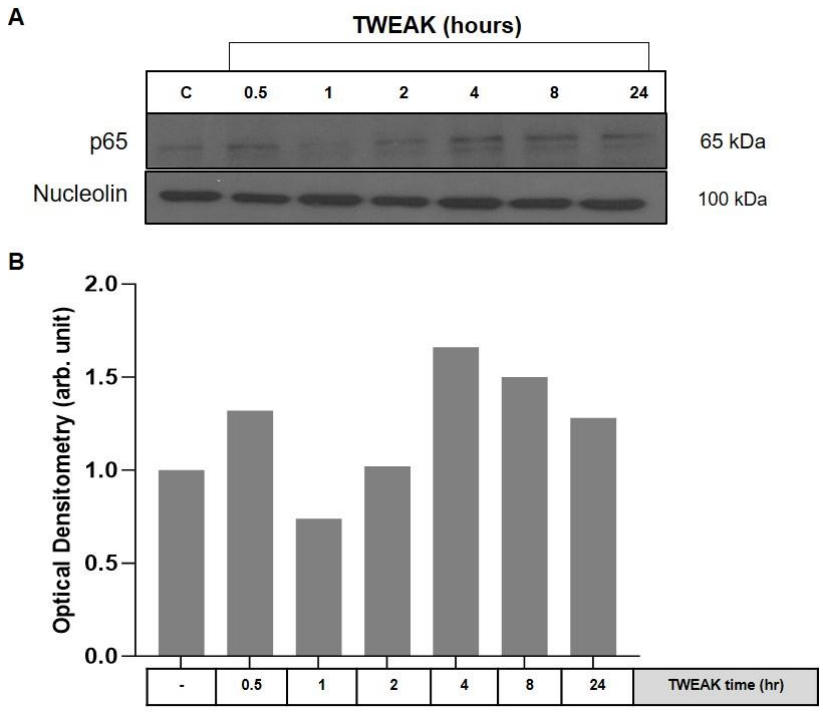


Figure 3. 7 The effect of TWEAK on nuclear translocation of p65 in T98G glioblastoma cells.

T98G cells were grown to near confluency in 6 well plates (in triplicates) rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cells were then treated with 10 ng/ml of TWEAK over time (0-24 hours). Nuclear extracts were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots representing the nuclear translocation of p65 over time following the exposure to 10 ng/ml of TWEAK. Nucleolin was used as a loading control. **(B)** Bar chart showing the blots' assessment using semi-quantitative analysis. Results expressed as fold stimulation relative to control. (n=1)

3.5 The characterisation of TNFα-mediated non-canonical NF-κB pathway activation in T98G glioblastoma cells

3.5.1 The effect of TNFα on p100 (Ser866/870) phosphorylation in T98G glioblastoma cells

TNFα has been documented to induce activation of both the canonical and non-canonical NF-κB pathways in various cell types, including several tumour-derived cell lines (Paul et al., 2018).To examine the effect of TNFα on p100 (Ser866/870) phosphorylation over time in the T98G GBM cell line, T98G cells were exposed to TNFα over a 24 hours time course, as shown in Figure 3.9. Phosphorylation of p100 was noticeable from 2 hours post-stimulation without statistical significance compared to the control, followed by a decreasing trend until 24 hours. This indicated that TNFα can minimally activate the non-canonical NF-κB pathway in T98G glioblastoma cells.

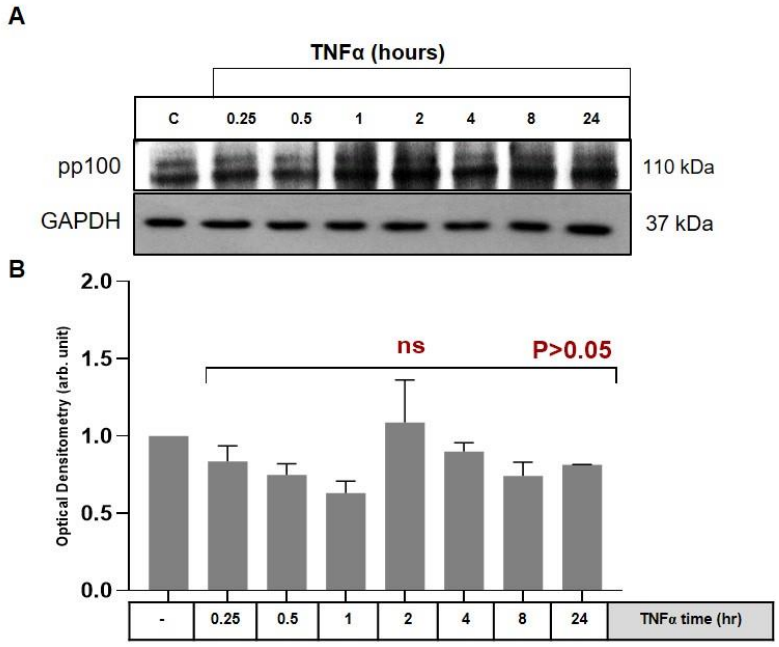


Figure 3. 8 The effect of TNFα on p100 phosphorylation in T98G glioblastoma cells.

T98G cells were grown in 12 well plates to near confluence and rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cells were then treated with 10 ng/ml of TNFα over time (0-24 hours). Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western Blots representing the phosphorylation of p100 over time following the exposure to 10ng/ml of TNFα. GAPDH was used as a loading control. **(B)** A bar chart the shows the blots assessment using semi-quantitative densitometry. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

3.5.2 The effect of TNFα on p100/p52 processing in T98G glioblastoma cells

Next, in order to examine the effect of TNFα on the processing of p100 to mature p52, T98G cells were exposed to TNFα over a 24 hours time course, as shown in Figure 3.10. The results show that the processing of p100 to p52 was noticeable after 2 hours with significant levels achieved at 24 hour (for p52), the protein expression levels of p100 also started to increase (significant levels achieved from 4 to 24 hour (for p100)) and the levels of p52 increased consistently until 24 hours. This indicated that TNFα can activate cytoplasmic processing of p100 to p52 in the non-canonical NF-κB pathway in the T98G glioblastoma cell line.

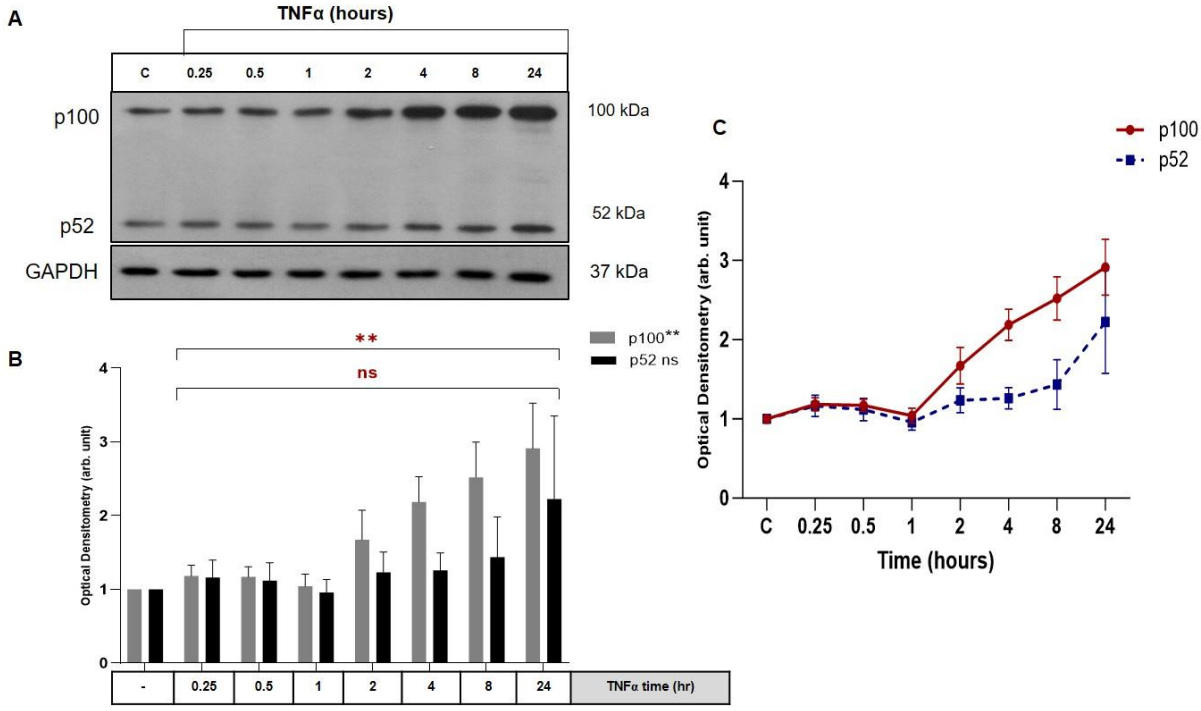


Figure 3. 9 The effect of TNFα on the expression of p100/p52 in T98G glioblastoma cells.

T98G cells were grown in 12 well plates to near confluence and rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cells were then treated with 10 ng/ml of TNFα over time (0-24 hours). Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western Blots showing the processing of p100 to p52 over time following the exposure to 10ng/ml TNFα. GAPDH was used as a loading control. **(B and C)** A bar chart and a line chart that shows the blots assessment using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

3.5.3 The effect of TNFα on nuclear translocation of p52/RelB in the T98G glioblastoma cell line

The results obtained in previous figures demonstrate that exposure to TNFα induced the formation of p52 in whole cell extracts from T98G cells. In order to investigate the ability of TNFα to induce p52/RelB translocation to the nucleus of T98G cells, nuclear extracts from cells treated with 10 ng/ml of TNFα were prepared as outlined in methods section. The results in Figure 3.11 show that 30 minutes after TNFα stimulation, a continuous increase in the level of nuclear p52, as well as RelB, was observed. This increase was followed by a sharp decrease of RelB after 4 hours and until the 24 hour time point. However, p52 presence in the nuclear compartment was maintained until peaking at the 24 hour time point. The highest nuclear accumulation of p52 was noticed at 24 hours post-stimulation with significant increase compared to control (P<0.05). These results suggested that TNFα can mediate the processing of p100 to p52/RelB and translocation to the nucleus.

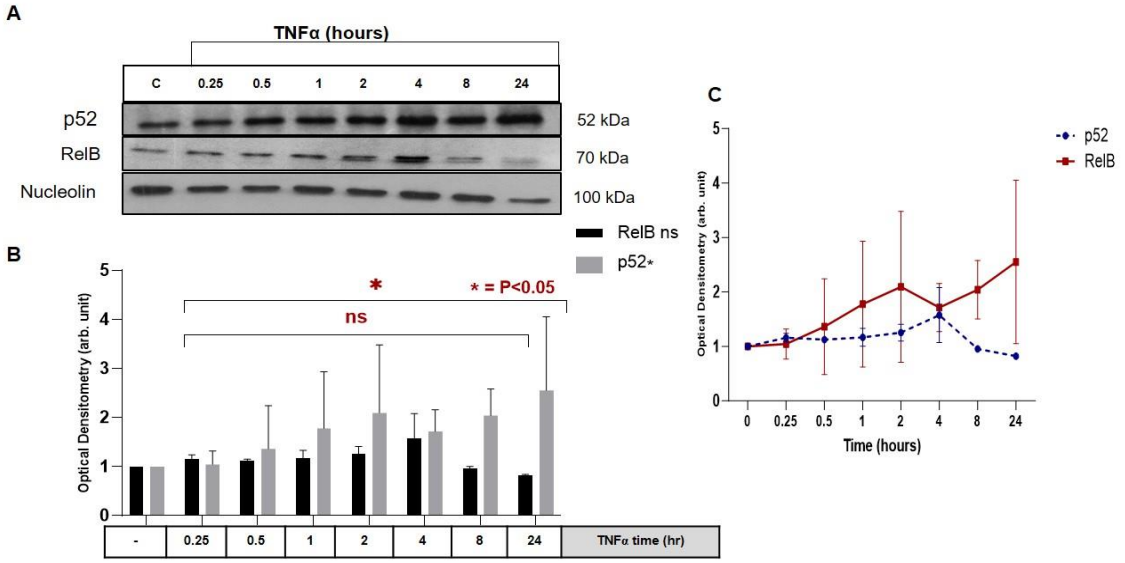


Figure 3. 10 The effect of TNFα on nuclear translocation of p52/RelB in T98G glioblastoma cells.

T98G cells were grown to near confluency in a 6 well plates (in triplicates) rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cells were then treated with 10 ng/ml of TNFα over time (0-24 hours). Nuclear extracts were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western Blots representing the nuclear translocation of p52 and RelB over time following the exposure to 10ng/ml of TNFα. Nucleolin was used as a loading control. **(B and C)** A bar chart and a line chart that show the blots assessment using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance (P<0.05).

3.5.4 The effect of TNFα on IKKα and IKKβ protein expression in the T98G glioblastoma cell line

As levels of p100 change in response to TNFα stimulation, the levels of cellular IKKα and IKKβ in T98G cells were investigated. Cells were exposed to TNFα for the same time points used in previous experiments. IKKα and IKKβ levels were assessed by Western blot. The results showed no variation in the levels of protein expression for both IKKα and IKKβ over all time points examined (Figure 3.12).

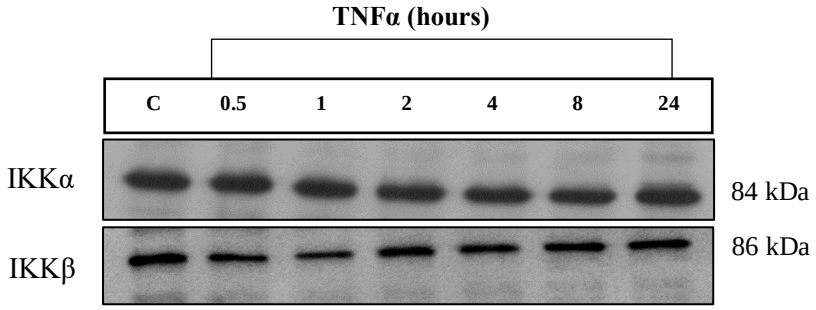


Figure 3. 11 The effect of TNFα on expression of IKKα and IKKβ in T98G glioblastoma cells.

T98G cells were grown in 12 well plates to near confluence and rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cell were then exposed to TNFα (10ng/ml) for the times indicated, whole cell extracts were prepared and analysed by Western blotting for expression of IKKα and IKKβ. The blot is representative of two independent experiments.

3.6 Characterisation of TNFα-mediated activation of the canonical NF-κB pathway in the T98G glioblastoma cell line

3.6.1 The effect of TNFα on the degradation of IκB-α in T98G glioblastoma cells

To examine the effect of TNFα on the degradation of IκB-α over time, T98G cells were exposed to TNFα over a 24 hours time course, as shown in Figure 3.13. Degradation of IκB-α was noticeable from 15 minutes (0.25 h) and maintained until 24 hours (with statistical significance compared to control), with only very limited-synthesis by- and 24 hours. This indicated that the canonical NF-κB pathway is functional and can be activated robustly by TNFα in the T98G glioblastoma cell line.

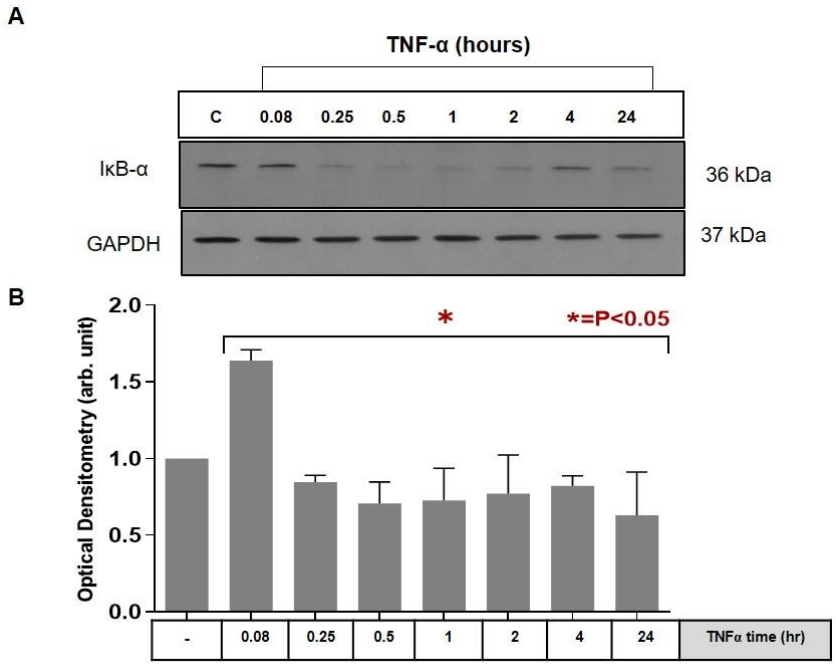


Figure 3. 12 The effect of TNFα on the degradation of IκB-α in T98G glioblastoma cells.

T98G cells were grown in 12 well plates to near confluence and rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cell were then treated with 10 ng/ml of TWEAK over time (0-24 hours as indicated). Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western Blots representing the degradation of IκB-α over time following the exposure to 10ng/ml of TNF-α. GAPDH was used as a loading control. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

3.6.2 The effect of TNFα on the phosphorylation of p65 (Ser536) in T98G glioblastoma cells

To examine the effect of TNFα on p65 (Ser536) phosphorylation over time, T98G cells were exposed to TNFα over a 24 hours time course, as shown in Figure 3.14. Phosphorylation of p65 was noticeable from 15 minutes (0.25 h) with the maximum at 2 hours followed by a gradual decrease (significant increase achieved from 30 minutes to 2 hours). This again supported the observation that the canonical NF-κB pathway is functional and active in response to TNFα in the T98G glioblastoma cell line.

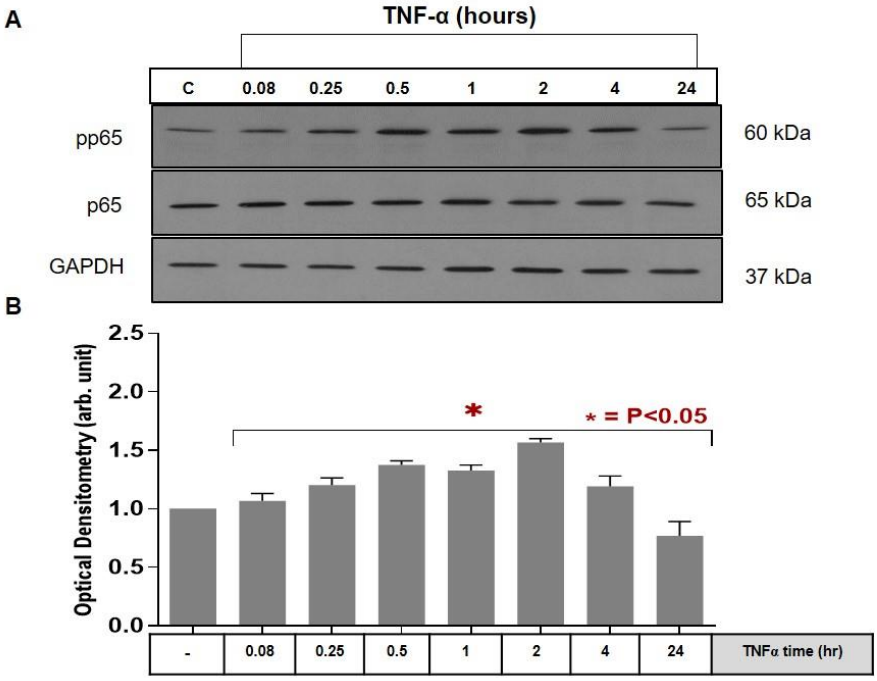


Figure 3. 13 The effect of TNFα on the phosphorylation of p65 in T98G glioblastoma cells.

T98G cells were grown in 12 well plates to near confluence and rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cell were then treated with 10 ng/ml of TNFα over time (0-24 hours as indicated). Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots representing the phosphorylation of p65 over time following the exposure to 10ng/ml of TNF-α. GAPDH was used as a loading control, p65 was used as a control for the total amount of p65. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

3.6.3 The effect of TNFα on nuclear translocation of p65 in T98G glioblastoma cells

After showing the effectiveness of TNFα at increasing p65 phosphorylation, it was obvious to examine the effect on p65 (RelA) nuclear translocation. According to the figure below, the general trend was an increase in p65 translocation into the nucleus over time from 0-4 hours and a significant accumulation was achieved from 2 to 4 hours. After 4 hours, the concentration of p65 started to decline all the way to 24 hours.

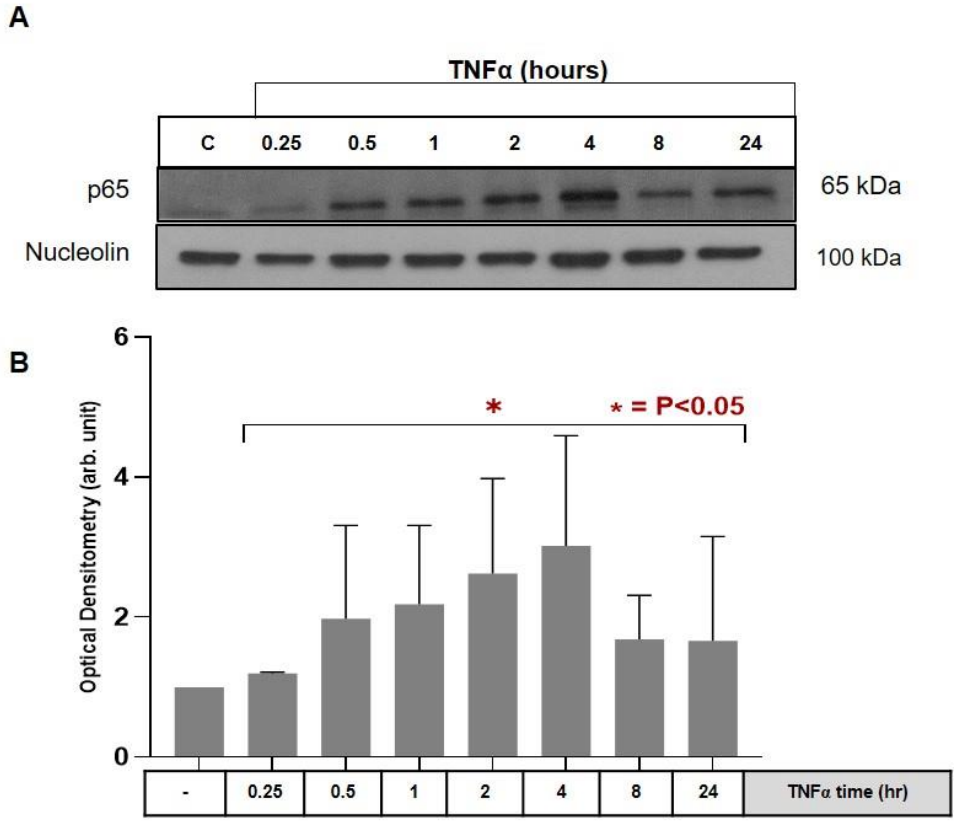


Figure 3. 14 The effect of TNFα on nuclear translocation of p65 in T98G cells over time.

T98G cells were grown in 12 well plates to near confluence and rendered quiescent by incubation in media containing 0.1% FCS for 24h. Cell were then treated with 10 ng/ml of TNFα over time (0-24 hours). Nuclear Extracts were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western Blots bands representing the nuclear translocation of p65 over time following the exposure to 10ng/ml of TNFα. Nucleolin was used as a loading control. **(B)** A bar chart that show the blots assessment using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

3.7 Discussion

The NF-κB pathways play an important role in many cellular processes that lead to cancer progression (Baud and Karin, 2009; Hoesel and Schmid, 2013). *Glioblastoma multiforme* is no exception since it is one of the most aggressive types of cancer with a mean progression-free survival period of approximately 6 months (Sunit & Philip, 2013). Recurrence after surgical resection, chemotherapy and radiation is almost definite since aggressive surgical resection reduces tumour cells to about 100 million and adjuvant chemotherapy with an agent such as temozolomide reduces the burden to about 10 million cells (Sunit & Philip, 2013). Recent evidence linked increased NF-κB signalling with GBM, but very few studies have investigated the association of the non-canonical NF-κB signalling pathway with such an aggressive cancer. As the IKK complex is recognised to regulate both the canonical and non-canonical NF-κB pathway, dependent on which dimeric structure is formed between the two catalytic IKK proteins (Perkins 2007; Gamble et al., 2012), examining and understanding the ways in which the IKK complex affects GBM is of great importance.

In this study, the T98G cell line was selected for initial experiments and other brain cancer cell lines, such as UVW, were added to the study at later experiments for more robust testing and comparison. T98G cells were isolated from human *Glioblastoma multiforme*. Compared to normal human diploid cells (WI-38), T98G cells can undergo an unlimited number of population doublings *in vitro*, they can proliferate independent of anchorage, however, they share the same G_1 arrest under stationary conditions as the WI-38 cells (Stein, 1979). Previous studies have shown that TWEAK could activate the non-canonical NF-κB pathway (Cherry et al., 2015). A study by Li and co-workers published in 2015 tested briefly the ability of TWEAK to activate this pathway in T98G cells. The study looked primarily at the effect of TWEAK on *TERT* expression in T98G cells. Our study, though, primarily confirmed that TWEAK could induce both phosphorylation of p100 and the formation of the p52/RelB subunit and its translocation into the nucleus of T98G cells (see figures 3.2 to 3.4). Furthermore, the study showed that TWEAK had a minimal effect on the degradation of IκB-α and the phosphorylation of p65 in T98G cells (see figure 3.6).

There were, however, some interesting features of activation of the non-canonical NF-κB pathway. A noticeable signal for p100 phosphorylation was seen at very low concentrations of TWEAK starting from 1 ng/ml, which was not examined in previous studies. As expected, a concentration dependent increase in p100 phosphorylation was seen with 20 ng/ml showing the highest effect. The recorded EC_{50} was 8.65ng/ml, which meant that half of the maximal phosphorylation was achieved at 8.65 ng/ml (see Figure 3.1 C). To show the best inhibition using selective IKKα inhibitors (SU1433 and SU1644), an EC_{75} was calculated, which suggested that 10 ng/ml was the best concentration to use in further examinations. This is also supported by the study of Cherry et al in which the authors confirmed that 10 ng/ml TWEAK was able to stimulate the activation of the non-canonical NF-κB pathway in the GBM cell lines they utilised. In this study, it was noted that there was a significant difference in the effect between the concentrations 20, 30 and 50 ng/ml compared to control, with a p value less than 0.05 (p value of the 10 ng/ml was also calculated and also showed significant difference to control). Interestingly, there was a significant decrease in the effect at 100 ng/ml compared to the 20 ng/ml (p value less than 0.05, results not shown). Some older studies, though, have used 100 ng/ml TWEAK to stimulate GBM cell lines (Tran et al., 2005; Fortin et al., 2009) and so may simply reflect varying cellular expression of Fn14, the receptor for TWEAK.

3.7.1 Characterisation of TWEAK-mediated non-canonical NF-κB pathway activation in T98G glioblastoma cells

The primary aim of this study was to investigate the kinetics of activation of both non-canonical and canonical NF-κB pathway in GBM cell lines. The investigation involved the use of two agonists, namely TWEAK and TNFα, which have the ability to activate both the canonical and non-canonical NF-κB pathway (Paul et al., 2018). TWEAK was reported to activate the canonical NF-κB pathway in different cell types (Wand et al., 2017). However, the results achieved in this study showed that TWEAK predominantly activates the non-canonical side of the NF-κB pathway in the T98G GBM cells (see results above; Figure 3.2-3.8). Similar outcomes reported by Li et al. (2015). Li and his co-workers suggested that physiological concentrations of TWEAK (less than 30 ng/ml) have predominantly stimulated the non-canonical side of the NF-κB pathway (Li et al., 2015).

3.7.1.1 The effect of TWEAK on p100 (Ser866/870) phosphorylation and subsequent p52/RelB nuclear translocation in T98G glioblastoma cells

Over time, exposure of T98G GBM cells to TWEAK stimulated p100 phosphorylation as early as 30 minutes and showed significant phosphorylation at 4 hours. This was similar to that observed in Cherry et al., 2015. However, this effect started to reduce after that and dipped sharply at 24 hours. To better understand the stimulation pattern, an earlier time and longer intervals might be interesting to examine in the future along with expression of other upstream and downstream proteins in the signalling cascade, such as NF-κB-inducing kinase (NIK) and matrix metalloproteinases (MMPs), respectively. It is worth noting that Cherry and co-workers examined the influence of TWEAK on NIK expression and found that TWEAK, but not TNFα, increased NIK mRNA expression significantly in different GBM cells (T98G cells were not included) (Cherry et al., 2015).

When the processing of p100 to mature p52 was tested, the effect of TWEAK was very clear (see Figure 3.3). Processing of p100 started from 5 minutes post exposure to TWEAK and was maintained up to 24 hours. TWEAK activated p52 formation from 1 hour, which gradually increased over the time scale with a parallel decrease in p100 concentration. However, there was a drop in p52 formation at 24 hours suggesting that signalling via processing of p100 NF-κB2 was returning to basal levels, however, further study at times beyond 24h treatment with agonist are required to confirm this. It is worth noting that such an experiment was performed several times, nonetheless, the cells could not cope with the stress of serum starvation beyond the 24 hours time point (despite the use of low serum percentage media e.g. 0.2-1% FCS (v/v)).

A very similar pattern was also seen when the expression and nuclear translocation of p52/RelB was examined over a period of time using 10 ng/ml of TWEAK. Expression and translocation of p52/RelB to the nucleus of T98G cells was noticeable at 30 minutes followed by a decrease and then p52/RelB concentrations started to increase gradually for 24 hours (see Figure 3.4). A similar pattern of p52/RelB accumulation in the nuclear content has been reported by Ghosh and Karin (2002).

In this study, it was observed that despite the overall increase of RelB alongside p52, that increase of RelB was not as evident as with p52. The results of this experiment on p52/RelB nuclear translocation

suggest that earlier time points and longer exposure time to the agonist (TWEAK) would help to confirm the fuller kinetics of p52/RelB nuclear translocation linked to nuclear transcription of genes.

3.7.1.2 The effect of TWEAK on IKKα and IKKβ protein expression in T98G glioblastoma cells

With increasing levels of phosphorylated p100 in response to TWEAK stimulation, it was important to investigate the levels of cellular IKKα and IKKβ in T98G cells to identify whether the increase in p100 phosphorylation was an agonist stimulated event or reflected possibly an increase in IKK isoform expression and associated basal catalytic activity. Upon exposing the cells to TWEAK no variation in the levels of both IKKα and IKKβ over all time points was recorded (Figure 3.5 & 3.12). This suggested that the expression of the IKKs were not under the control of TWEAK stimulated transcriptional activation and as such were not inducible in response to this TNF superfamily member. The same response was observed with the use of TNFα as an agonist. Similar results to TNFα as well as LTα1β2 were reported on a pancreatic cell lines (Panc-1) (Al-Obaidi PhD thesis).

3.7.2 Characterisation of TWEAK-mediated canonical NF-κB pathway activation in T98G glioblastoma cells

The main objective to examining the potential effect of TWEAK on the canonical side of the NF-κB pathway was to confirm any selectivity of this agonist for the activation of the non-canonical NF-κB pathway only. As no previous study to date has been performed to thoroughly investigate TWEAK selectivity and efficacy for the non-canonical side of the NF-κB pathway, experiments were constructed with a focus on assaying key markers in the canonical NF-kB pathway. These tests allowed for better understanding of the NF-κB pathways and TWEAK behaviour in activating both the canonical and/or the non-canonical NF-κB pathway, which can be regulated differently in various cell types. For example, Panc-1 cells showed a different behaviour to TNFα stimulation, which did not activate the non-canonical NF-κB pathway, a feature that could be attributed to low NIK protein expression levels upstream in the pathway (Al-Obaidi, PhD thesis).

3.7.2.1 The effect of TWEAK on the degradation of IκBα, p65 phosphorylation and p65 nuclear translocation in T98G glioblastoma cells

To examine whether TWEAK selectively activated the non-canonical NF-κB pathway in T98G cells over the canonical NF-κB pathway, the potential effect of TWEAK on the degradation of IκBα and phosphorylation of p65 as markers of activation of the canonical NF-κB pathway were assayed.

TWEAK did not show any effect on IκBα protein expression, it did not stimulate proteolytic degradation and protein levels remained stable without any significant difference to the control (see Figure 3.6) with a p value of 0.534 (P less than 0.05 is considered significant). In contrast, TWEAK showed variable ability to stimulate p65 phosphorylation at the 15 minutes, 2 hour and 4 hour time points, exhibiting significant difference to control (P value less than 0.05) as shown in Figure 3.7. In this regard, no previous studies had thoroughly examined TWEAK effects on both IκBα and p65 in T98G cells. Some studies, such as Tran et al., (2005) have only examined the treatment of cells with TWEAK up to 4 hours using Western blotting, but notably without any quantification of the detected proteins. Cherry and co-workers, in contrast, did not study activation of the canonical NF-κB pathway in T98G cells in response to treatment with TWEAK, only in response to TNFα. These results, from this study, identified that TWEAK did not affect cytoplasmic IκBα compared to that observed in treatment of cells with TNFα (Cherry et al., 2015). Moreover, TWEAK did not induce nuclear translocation of RelA as compared to that observed with TNFα (Cherry et al., 2015). These experimental outcomes of Cherry and co-workers were later confirmed in further studies (see below) with no observed changes in RelA (p65) nuclear translocation following exposure to TWEAK compared to control untreated cells (see Figure 3.8).

3.7.3 Characterisation of TNFα mediated activation of the non-canonical NF-κB pathway in T98G glioblastoma cells

In order to both confirm the activation of NF-κB pathway and compare that to TWEAK, the use of a well-established standard such as TNFα was necessary. To do that, we examined the TNFα effect on both the canonical and non-canonical NF-κB pathway biomarkers.

3.7.3.1 The effect of TNFα on p100 phosphorylation and subsequent p52/RelB nuclear translocation in T98G glioblastoma cells

It has been established that TNFα stimulates the phosphorylation of p100 and the formation of mature p52 in different cell lines and tissues (Paul et al., 2018). This finding is supported by studies in RIP$^{-/-}$ MEFs cells, which demonstrated stimulation of non-canonical NF-κB 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). However, the effect of TNFα on p100 phosphorylation in T98G cells fluctuated over time as the response dropped in the first hour and showed a sharp increase at 2 hours' time point. Following that, the stimulation started to decrease gradually over 24 hours. On the other hand, the formation of p52 was consistent and gradual from 2 hours until 24 hours' time point (see Figure 3.10). This effect was reflected in the nuclear translocation of p52 and RelB, where p52 showed a steady increase over the time course. However, despite the gradual increase of RelB over the same time course, RelB nuclear concentration started to drop after 4 hours (see Figure 3.11). These results are supported by a study by Liu and co-workers who demonstrated the same findings, with TNFα increasing non-canonical NF-κB biomarkers and nuclear translocation of p52/RelB over 0, 24 and 48 hours in breast cancer cells (Liu et al., 2020).

3.7.3.2 Characterisation of TNFα-mediated activation of the canonical NF-κB pathway in T98G glioblastoma cells

In this section, the aim was to show that TNFα can activate the canonical NF-κB pathway. This finding is consistent with many other studies which have demonstrated TNFα activation of the canonical NF-κB pathway in many types of cell lines, such as hypopharyngeal cancer cells (Yu et al., 2014) and mouse lung epithelial (MLE) cells (Schwingshackl et al., 2013).

3.7.3.3 The effect of TNFα on the degradation of IκBα, p65 phosphorylation and nuclear translocation in T98G glioblastoma cells

Phosphorylation of p65 at Ser536 is considered a key marker for activation of the canonical NF-κB pathway. In the resting state, p65 is sequestered in cytoplasm by binding to IκB that upon stimulation it

is phosphorylated by the IκB kinases to catalyse its degradation allowing the release and nuclear translocation of p65 for further transcriptional activities (Orlowski et al., 2002). It is well recognised that cytokines such as TNFα, which in the majority of cell types is a non-selective activator of both the canonical and non-canonical NF-κB pathway, can stimulate the canonical NF-κB pathway in various cancer cell types such as GBM cells (Tran et al., 2005, Lee et al., 2007).

The effects of TNFα on the degradation of IκB-α and phosphorylation of p65 in T98G cells were very clear as the latter started to increase significantly after 5 minutes of the exposure to TNFα which continued steadily until 24 hours (see Figure 3.13-3.15). Maximum phosphorylation of p65 was evident after 2 hours, after that, the levels started to decrease gradually. Significant effect of TNFα on RelA (p65) nuclear translocation was evident from 2-4 hours compared to the control. The overall impact of TNFα on the kinetics of IκBα degradation and p65 phosphorylation are almost identical to that reported in other studies with GBM cells (Cherry et al., 2015) or other cancer cell types such as hypopharyngeal cancer cells (Yu et al., 2014) and mouse lung epithelial (MLE) cells (Schwingshackl et al., 2013) as mentioned earlier.

3.8 Conclusion

The aforementioned investigation revealed that TWEAK exhibited selective activation of the non-canonical NF-κB pathway, while TNFα activated both the canonical and non-canonical NF-κB pathway. Therefore, these studies have identified the appropriate experimental conditions in which pharmacological inhibitors, specifically isoform selective small molecule IKK kinase inhibitors, can be introduced and investigated for their effects on signalling responses in T98G glioblastoma cells. This approach offers a pathway to explore the involvement of IKKs in transcriptional and phenotypic responses.

Chapter 4

The effect of IKKα selective inhibitors (SU1433 and SU1644) on the activation of NF-κB pathways in the T98G glioblastoma cell line

4.1 Introduction

The NF-κB pathways have been implicated as tumour promoters in a variety of different cancer types (Hanahan, 2022). Breast cancer (Chua et al., 2007), lung cancer (Tew et al., 2008), leukaemia (Vilimas et al., 2007), lymphoma (Zou et al., 2007), and brain cancer (Torre et al., 2015) have all been shown to display elevated expression levels of NF-κB proteins. Moreover, numerous studies have linked elevated NF-κB expression and NF-κB activation and chemotherapy resistance in cancer cell lines (Goodenberger and Jenkins, 2012). Inhibition of NF-κB pathways or deletion of NF-κB subunits also inhibited the growth of tumour cells (Li et al., 2005; Meylan et al., 2009) thus reiterating these pathways may be good targets for drug intervention.

The IKKs are regulatory proteins considered essential for NF-κB activation. They play crucial functions in activating both NF-κB pathways, including the non-canonical NF-κB pathway (Liang et al., 2006). Muller and Siebenlist (2003) reported that phosphorylation of IKKα in response to cell stimulation with specific agonists resulted in the phosphorylation and processing of p100, which in turn regulates several genes. In addition, IKKβ played a role in the phosphorylation of p65 NF-κB, which is an important component to the activation of the canonical NF-κB pathway (Muller and Siebenlist, 2003). Numerous studies suggest that IKKα may function as a tumour suppressor; however, Park and colleagues discovered that a decrease in IKKα expression 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 by maintaining skin homeostasis (Liu et al., 2008).

Similarly, research on the effect of IKKβ-targeted inhibitors on pancreatic cancer cell lines found that TPCA-1, an IKKβ-selective inhibitor, decreased cell proliferation and chemotherapy resistance (Cataldi et al., 2015). However, due to the lack of available compounds, no IKKα-selective inhibitors have been examined in similar studies to date.

In this regard, several first class IKKα-selective inhibitors (SU compounds) have been developed by medicinal chemists in the Pharmaceutical Science Research Group at the University of Strathclyde, Glasgow to be examined thoroughly for potential anti-cancer

activities. Determining the potency and selectivity of these novel SU compounds as IKKα inhibitors in glioblastoma brain cancer cell lines was thus the objective of this chapter. This was accomplished through assaying markers of non-canonical NF-κB pathway activation; including p100 phosphorylation, the processing of p100, the formation of p52, and its subsequent nuclear translocation in association with RelB. In addition, selectivity for the IKKα-dependent non-canonical NF-κB pathway over the IKKβ-dependent canonical NF-κB pathway was assessed using IκB-α degradation, p65 phosphorylation and p65 nuclear translocation as markers of the canonical NF-κB pathway activation.

Experiments conducted in the previous chapter of this thesis demonstrated that TWEAK could activate the non-canonical NF-κB pathway and that TNFα can activate the canonical NF-κB pathway. Therefore, it was hypothesised that if SU compounds could be validated as selective IKKα inhibitors able to impact agonist stimulated non-canonical NF-κB activation in T98G glioblastoma cells they could be taken forward to investigate their potential impact on any IKKα-mediated regulation of downstream development of phenotypic outcomes representative of cancer hallmarks, e.g. cell viability/growth, replication potential etc.

4.2 Materials and Methods

Cell lines and routine cell maintenance

All routine maintenance of cell lines was performed as described in Section 2.5.

Cell stimulations were performed using TNFα and TWEAK as described in section 2.6.

Cell treatments were performed using IKKα small molecule inhibitors SU1433, SU1644 and IKKβ small molecule inhibitor ML120B as described in section 2.6.

Western Blotting

Western Blotting was performed using different antibodies (details in table below) as described in methods section 2.6.

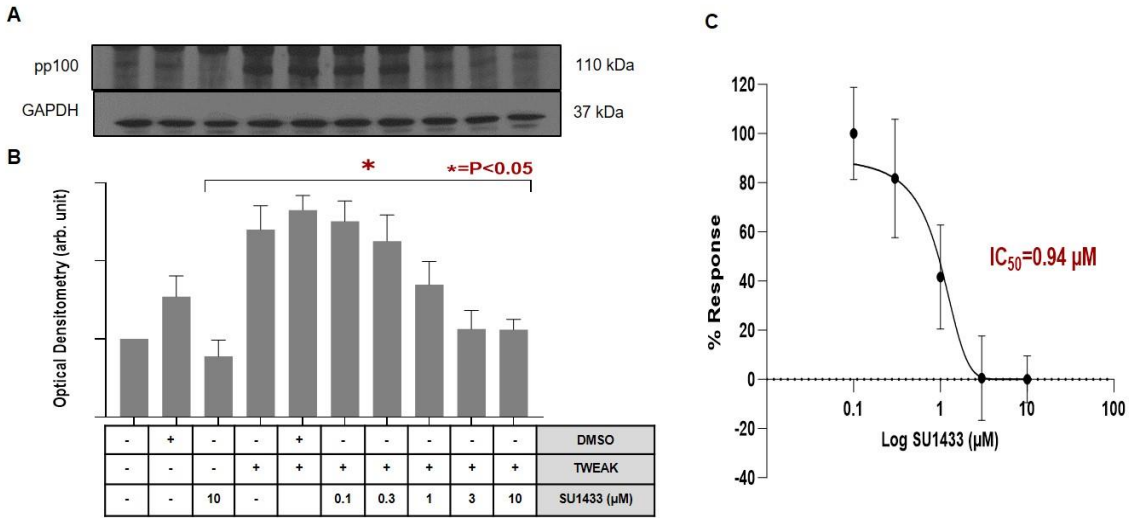
Table 4. 1 Antibodies and inhibitors used throughout chapter 4

Antibody/inhibitor	Type	Dilution	Remarks
Anti-p-NF-kappa B2 p100	Rabbit	1:1000	Cell signalling Technology Inc. (USA)
Anti-NFκB p52	Mouse	1:20,000	Millipore (U.K.) Limited (UK)
Anti-RelB	Rabbit	1:3000	Cell signalling Technology Inc. (USA)
Anti-IκB-α	Rabbit	1:2000	Cell signalling Technology Inc. (USA)
Anti-p-NF-kappa B2 p65	Rabbit	1:2000	Cell signalling Technology Inc. (USA)
Anti-NFκB p65 (C-20)	Rabbit	1:2000	Santa Cruz Biotechnology Inc (USA)
SU1433, SU1644	-	-	SIPBS (Prof. S. Mackay)
ML120B	-	-	MedChemExpress (MCE), USA.

4.3 The effect of SU1433, SU1644 and ML120B on TWEAK-mediated stimulation of the non-canonical NF-κB pathway in T98G glioblastoma cells

4.3.1 The effect of SU1433 on TWEAK-stimulated phosphorylation of p100 in T98G glioblastoma cells

SU1433 is a potent selective inhibitor of IKKα (K_i IKKα vs IKKβ: is 11 vs. 2,295 nM) that has been developed by the medicinal chemistry team by Professor Simon MacKay in SIPBS, the University of Strathclyde. In order to examine the pharmacological effects of SU1433 on the non-canonical NF-κB pathway in T98G glioblastoma cells, T98G cells were initially incubated with vehicle (DMSO) or SU1433 prior to exposure to TWEAK and phosphorylation of p100 examined by Western blotting (Figure 4.1 A & B) and the processing of p100 to p52 (Figure 4.2). Cells were treated with 10 ng/ml of TWEAK at 4 hours following the treatment with vehicle or varying concentrations of SU1433 (as described in the methods section). TWEAK considerably increased the phosphorylation of p100 with



minimal additive effect observed in the presence of vehicle (0.05% (v/v) DMSO). SU1433 however, inhibited TWEAK-stimulated p100 phosphorylation in a concentration-dependent manner (from 0.3 to 10 μM) with a significant inhibition observed at 3 and 10 μM (p value less than 0.05). This indicated that SU1433 can inhibit the non-canonical NF-κB pathway in T98G glioblastoma cells. Furthermore, SU1433 showed high potency by producing an IC_{50} of 0.94 μM (see Figure 4.1 C).

4.3.2 The effect of SU1433 on TWEAK-stimulated p100/52 processing in the T98G glioblastoma cells

In order to further examine the pharmacological effect of SU1433 on TWEAK-stimulated activation of the non-canonical NF-κB pathway in T98G glioblastoma cells, the processing of p100 to p52 was examined (see Figure 4.2). Following the treatment with varying concentrations of SU1433 for 1 hour (as mentioned in the methods section), cells were stimulated with 10 ng/ml of TWEAK at 4 hours.

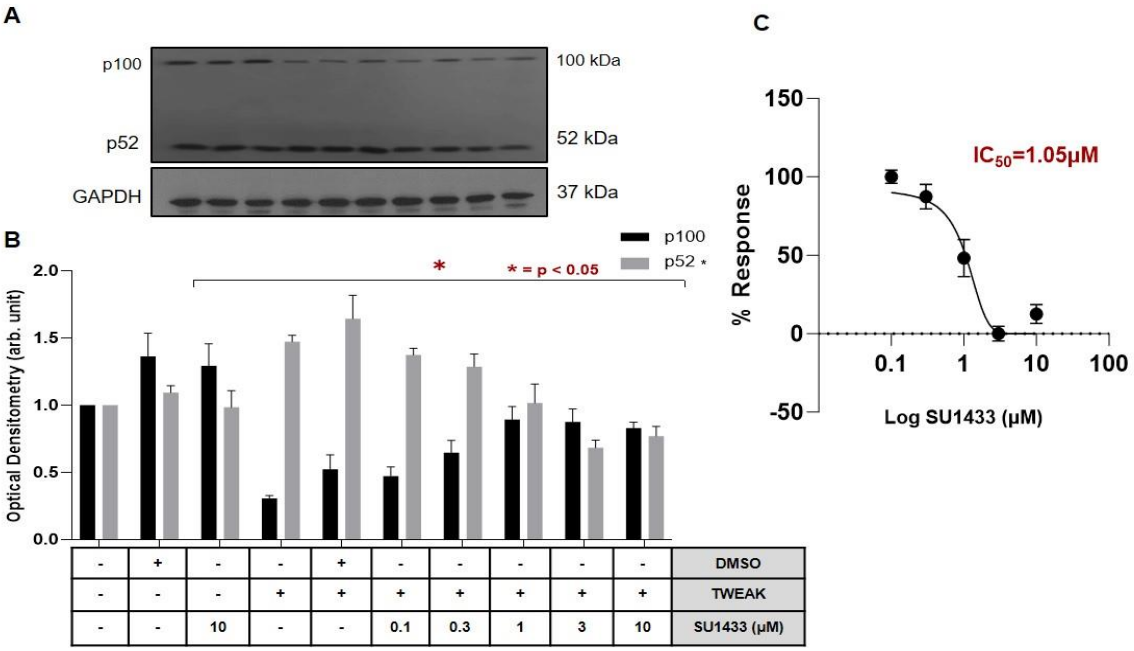


Figure 4. 1 The effect of SU1433 on TWEAK-stimulated p100/52 processing in T98G glioblastoma cell line.

T98G cells were grown in 12 well plates, rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with vehicle or increasing concentrations of SU1433 (0.1-10μM) for 1h prior to exposure to10 ng/ml TWEAK for 4 hours. Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots representing the processing of p100 to p52. GAPDH was used as a loading control. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. **(C)** An inhibition curve that represents p52 formation following increasing concentrations of SU1433 (IC_{50}=1.05μM). Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance (P<0.05).

SU1433 inhibited processing of p100 to p52 in a concentration dependent manner (from 0.3 to 10 μM) with significant inhibition observed at 3 and 10 μM (P value less than 0.05) and an IC_{50} of 1.05 μM. This result identified the ability of SU1433 to inhibit the non-canonical NF-κB pathway in T98G glioblastoma cells, by targeting IKKα.

4.3.3 The effect of SU1433 on TWEAK-stimulated nuclear translocation of p52 and RelB in T98G glioblastoma cells.

The results obtained in previous experiments demonstrated that exposure of T98G cells to SU1433 reduced the formation of p52 in whole cell extracts following TWEAK stimulation. In order to investigate the ability of SU1433 to inhibit p52/RelB translocation to the nucleus of T98G cells, nuclear

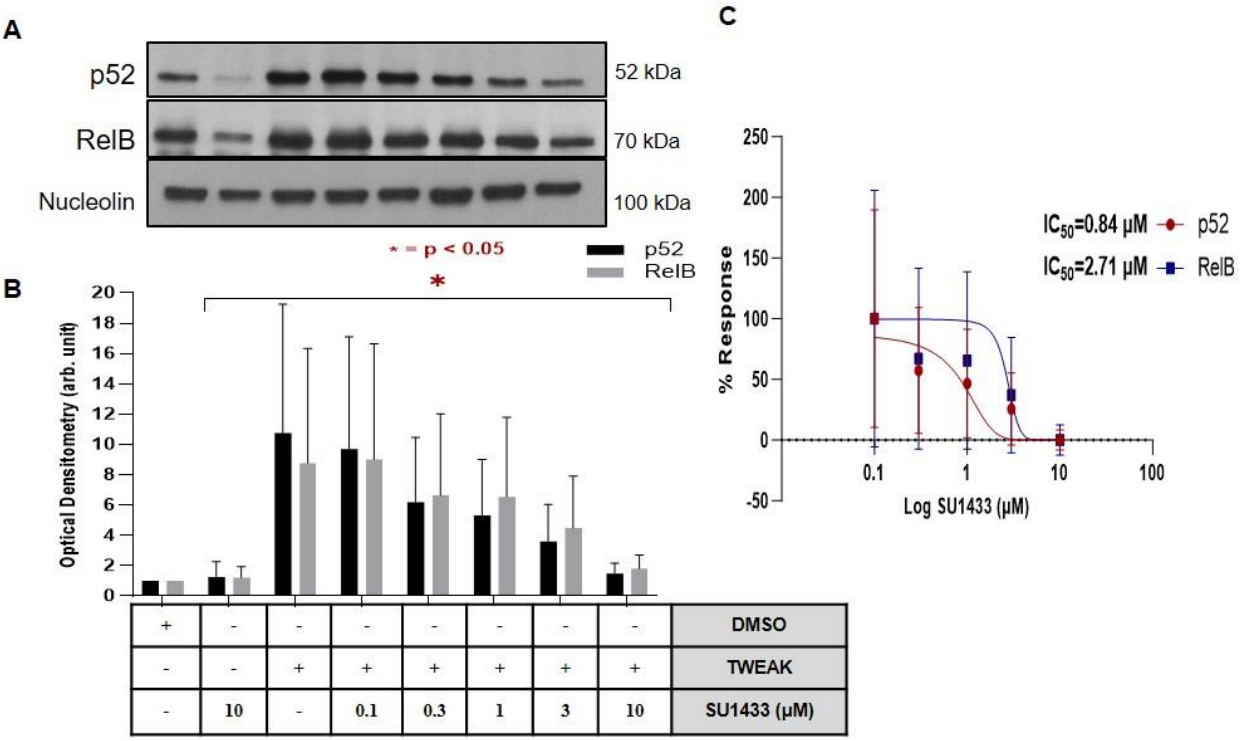


Figure 4. 2 The effect of SU1433 on TWEAK-stimulated nuclear translocation of p52 and RelB in the T98G glioblastoma cell line.

T98G cells were grown in a 6 well plates (in triplicates), rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with vehicle or increasing concentration of SU1433 (0.1-10μM) for 1 hour prior to exposure to 10 ng/ml TWEAK for 4 hours Nuclear Extracts were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots representing the nuclear translocation of p52 and RelB. Nucleolin was used as a loading control. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. Results expressed as fold stimulation relative to control. **(C)** An inhibition curve that represents p52/RelB following increasing concentrations of SU1433 (IC_{50} for p52 and RelB as indicated in the figure). Each value represents the mean ± SEM of three independent experiments. Statistical significance (P<0.05).

extracts from cells treated with varying concentrations of SU1433 for 1 hour (as mentioned in the methods section), followed by treatment with 10 ng/ml of TWEAK at 4 hours were analysed. SU1433 inhibited the TWEAK-stimulated nuclear translocation of p52 and RelB in a concentration dependent manner (from 0.3 to 10 μM) with significant inhibition observed at 3 and 10 μM. This result identified the ability of SU1433 to inhibit the downstream nuclear translocation of p52 and RelB within the non-canonical NF-κB pathway in T98G glioblastoma cells, by targeting IKKα. This would likely have implications for further downstream DNA transcription. Calculations were made for the IC_{50} of both NF-κB isoforms (Figure 4.3C). The IC_{50} value for p52 is determined to be 0.84 μM, whereas the IC_{50} value for RelB is found to be 2.71 μM. These findings indicate the existence of two distinct dimers for the NF-κB isoforms.

4.3.4 The effect of SU1644 on TWEAK-stimulated phosphorylation of p100 in the T98G glioblastoma cell line.

Another small molecule IKKα-selective kinase inhibitor, SU1644, was developed in SIPBS (again, kindly provided by Prof. Mackay) as an SU1433-related, later generation molecule with comparable *in vitro* potency against IKKα and with similar selectivity over IKKβ but approximately 10-fold higher potency in a cell-based setting (IC_{50} in cell-based studies IKKα vs. IKKβ: 0.05μM vs. >10 μM). In order to examine the pharmacological effect of SU1644 on TWEAK-stimulated activation of the non-canonical NF-κB pathway in T98G glioblastoma cells, the phosphorylation of p100 (Figure 4.4) and the processing of p100 to p52 (Figure 4.5) were examined. Cells were treated with vehicle or increasing concentration of SU1644 for 1 hour prior to exposure to 10 ng/ml TWEAK for 4 hours (as mentioned in the methods section). SU1644 inhibited TWEAK-stimulated p100 phosphorylation in a concentration dependent manner with the highest inhibition observed at 1 μM ($p < 0.05$). This indicated that SU1644

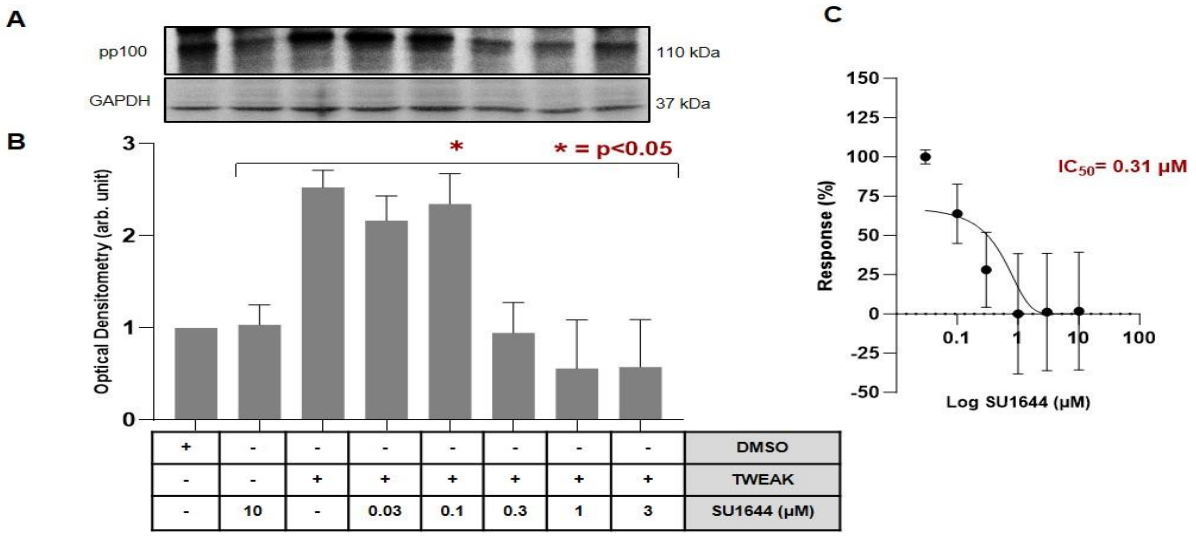


Figure 4. 3 The effect of SU1644 on TWEAK-stimulated phosphorylation of p100 in the T98G glioblastoma cell line.

T98G cells were grown in 12 well plates, rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with vehicle or increasing concentrations of SU1644 (0.03-3μM) for 1 hour prior to exposure to 10 ng/ml TWEAK for 4 hours (as indicated). Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots representing the phosphorylation of p100. GAPDH was used as a loading control. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. Results expressed as fold stimulation relative to control. **(C)** An inhibition curve that represents p100 phosphorylation following increasing concentrations of SU1644 (IC_{50}= 0.31 μM). Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

can inhibit the non-canonical NF-κB pathway in T98G glioblastoma cells, again via targeting IKKα. Furthermore, SU1644 showed high potency by producing an IC_{50} of 0.31 μM (see Figure 4.4 C).

4.3.5 The effect of SU1644 on TWEAK-stimulated p100/p52 processing in the T98G glioblastoma cell line

In order to further examine the pharmacological effect of SU1644 on TWEAK stimulated activation of the non-canonical NF-κB pathway in T98G glioblastoma cells, processing of p100 to p52 was examined (see Figure 4.6). Cells were treated with vehicle or increasing concentrations of SU1644 (0.03-3 mM) for 1 hour prior to exposure to 10 ng/ml TWEAK for 4 hours. SU1644 inhibited TWEAK-stimulated processing of p100 to p52 in a concentration dependent manner (from 0.3 to 3 μM) with the highest inhibition observed at 3 μM yet without statistical significance in p52 inhibition an IC_{50} of 0.46 μM

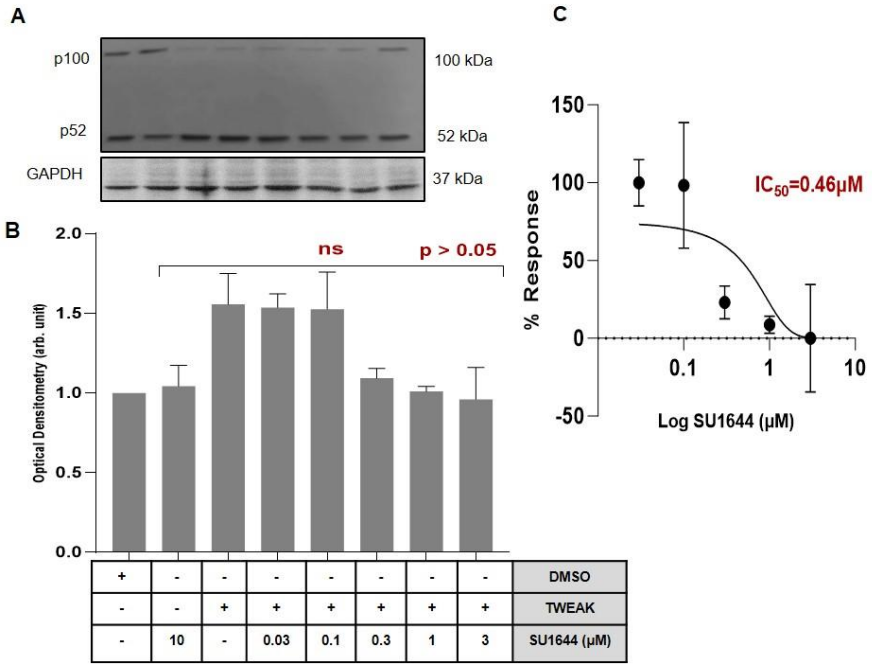


Figure 4. 4 The effect on of SU1644 on TWEAK-stimulated p100/52 processing in the T98G glioblastoma cell line.

T98G cells were grown in 12 well plates, rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with vehicle or increasing concentrations of SU1644 (0.03-3μM) for 1h prior to exposure to10 ng/ml TWEAK for 4 hours. Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots representing the processing of p100 to p52. GAPDH was used as a loading control. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. **(C)** An inhibition curve that represents p52 formation following increasing concentrations of SU1644 (IC_{50}= 0.46 μM). Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

achieved. This result supports that SU1644 can inhibit the non-canonical NF-κB pathway in T98G glioblastoma cells.

4.3.6 The effect of SU1644 on TWEAK-stimulated nuclear translocation of p52 and RelB in the T98G glioblastoma cell line

Previous results demonstrated that exposure to SU1644 reduced TWEAK-stimulated formation of p52 in whole cell extracts from T98G cells. In order to investigate the ability of SU1644 to inhibit TWEAK-stimulated p52/RelB translocation to the nucleus of T98G cells, nuclear extracts from cells treated with vehicle or increasing concentration of SU1644 prior to exposure to 10 ng/ml TWEAK for 4 hours were prepared and examined by Western blotting. SU1644 inhibited TWEAK-stimulated nuclear translocation of p52 and RelB in a concentration-dependent manner (from 0.1 to 3 μM) with significant

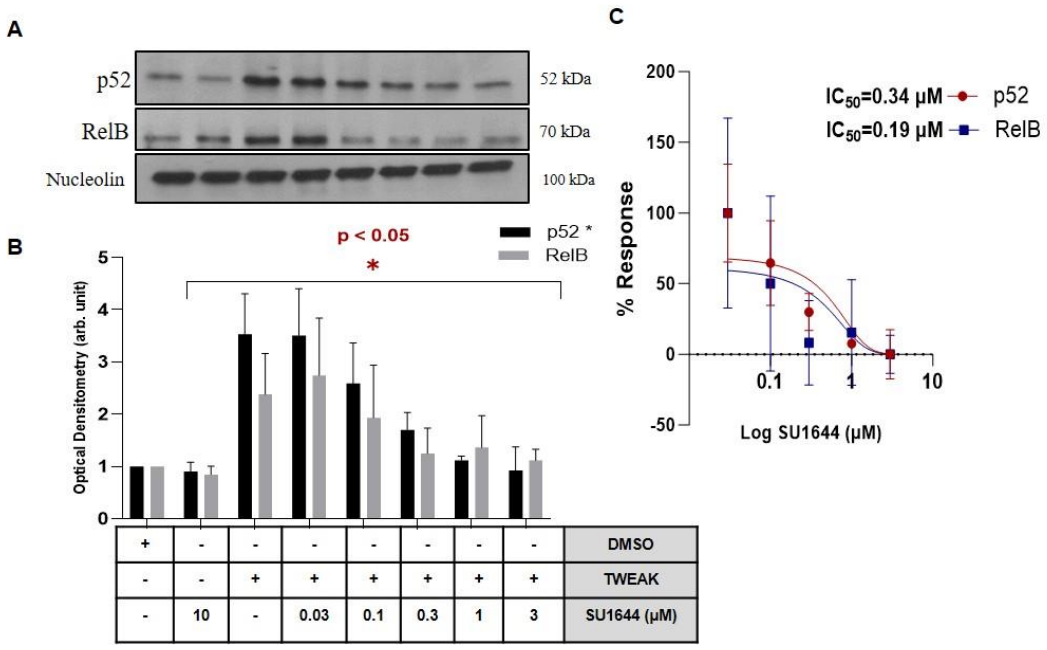


Figure 4. 5 The effect of SU1644 on TWEAK-stimulated nuclear translocation of p52 and RelB in the T98G glioblastoma cell line.

T98G cells were grown in a 6 well plate (in triplicates), rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with vehicle or increasing concentrations of SU1644 (0.03-3μM) for 1 hour prior to exposure to 10 ng/ml TWEAK for 4 hours. Nuclear Extracts were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots representing the nuclear translocation of p52 and RelB. Nucleolin was used as a loading control. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. **(C)** An inhibition curve that represents p52/RelB following increasing concentrations of SU1644 (IC_{50} for p52 and RelB as indicated in the figure) Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

inhibition observed at 0.3-3 μM on p52 and 3 μM on RelB. The IC_{50} for p52 was 0.34 μM and for RelB it was 0.19 μM. This result identified the ability of SU1644 to inhibit the downstream nuclear translocation of p52 and RelB within the non-canonical NF-κB pathway in T98G glioblastoma cells, by targeting IKKα.

4.4 Establishing the selectivity of SU1433 and SU1644 for targeting IKKα-mediated non-canonical NF-κB signalling versus the selectivity of ML120B (MLN120B), a commercially available IKKβ-selective inhibitor, in the T98G glioblastoma cell line

ML120B is a potent well-established IKK inhibitor with defined selectivity for the IKKβ isoform (Newton et al., 2007), for laboratory use with a reported IC_{50} *in vitro* (versus purified enzyme) of 60 nM. The effect of ML120B on agonist-stimulated non-canonical (using p100 phosphorylation and p52 formation and nuclear translocation as markers) and canonical (using IκB-α degradation and p65 phosphorylation and nuclear translocation as markers) NF-κB pathways were examined. These experiments sought to define and confirm the selectivity of the SU compounds against the IKKα-mediated non-canonical NF-κB pathway. Furthermore, the use of ML120B as a recognised IKKβ-selective inhibitor aimed to confirm IKKβ did not contribute to the regulation of the non-canonical NF-κB pathway whilst inhibiting clearly the canonical NF-κB of the pathway in T98G glioblastoma cells.

4.4.1 The effect of ML120B on TWEAK-stimulated phosphorylation of p100 in the T98G glioblastoma cell line

In order to study the pharmacological effect of ML120B on TWEAK-stimulated activation of the non-canonical NF-κB pathway in T98G glioblastoma cells, the effect of ML120B on TWEAK-stimulated phosphorylation of p100 (Figure 4.8) and the processing of p100 to p52 (Figure 4.9) were examined. Cells were treated with vehicle or increasing concentration of ML120B prior to exposure to 10 ng/ml TWEAK for 4 hours. TWEAK significantly increased the phosphorylation of p100 with no additive effect observed with DMSO. Moreover, pretreatment with ML120B did not inhibit TWEAK-stimulated p100 phosphorylation at concentrations examined (from 0.3 to 10 μM) (without statistical significance in inhibition of p100 phosphorylation). This indicated that ML120B cannot inhibit the non-canonical NF-κB pathway in T98G glioblastoma cells, suggesting targeting of IKKβ plays no contributing

regulatory role to stimulated phosphorylation of p100 nor activation of the non-canonical NF-κB pathway.

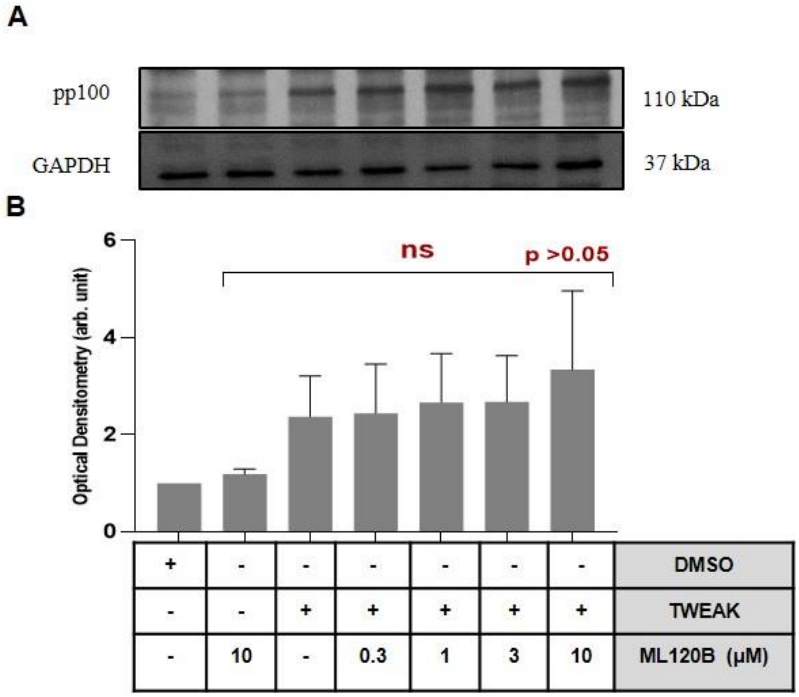


Figure 4. 6 The effect of ML120B on TWEAK stimulated phosphorylation of p100 in the T98G glioblastoma cell line.

T98G cells were grown in 12 well plates, rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with vehicle or increasing concentrations of ML120B (0.3-10μM) for 1 hour prior to exposure to 10 ng/ml TWEAK for 4 hours. Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots representing the phosphorylation of p100. GAPDH was used as a loading control. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

4.4.2 The effect of ML120B on TWEAK-stimulated p100/52 processing in the T98G glioblastoma cell line

To further examine the pharmacological effect ML120B on TWEAK-stimulated activation of the non-canonical NF-κB pathway in T98G glioblastoma cells, the processing of p100 to p52 was examined (see Figure 4.9). Cells were treated with varying concentrations of ML120B for 1 hour followed by treatment with 10 ng/ml TWEAK for 4 hours (as mentioned in the methods section). Relative to TWEAK stimulation alone, ML120B did not inhibit TWEAK-stimulated processing of p100 to p52 at all concentrations examined (from 0.3 to 10 μM; p> 0.05). However, ML120B managed to inhibit IKKβ regulated synthesis of p100. Again, this result supported the conclusion that as ML120B, did not inhibit

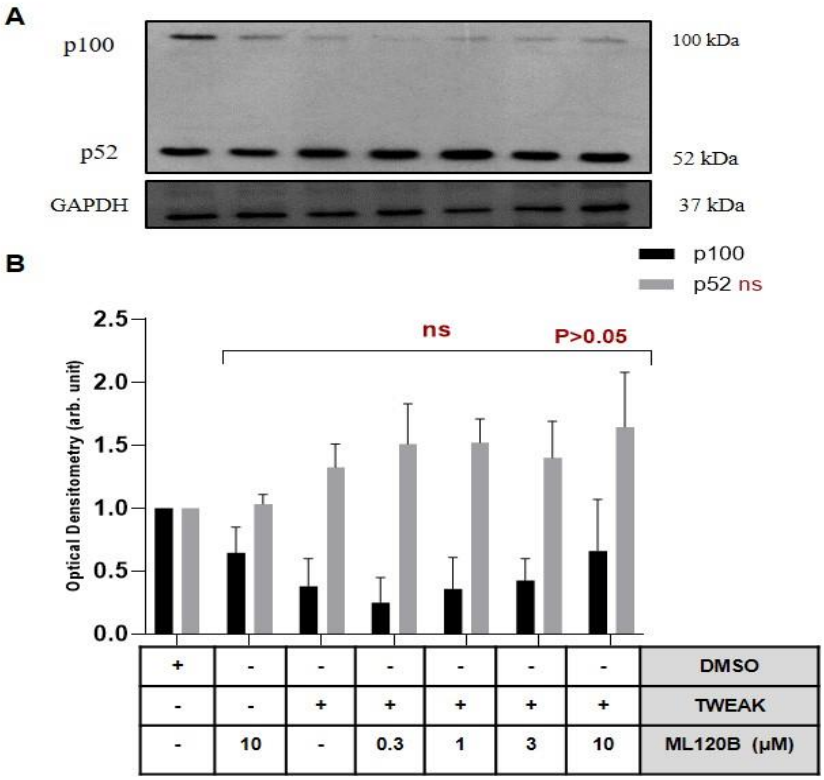


Figure 4. 7 The effect of ML120B on TWEAK-stimulated p100/52 processing in the T98G glioblastoma cell line.

T98G cells were grown in 12 well plates, rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with vehicle or increasing concentrations of ML120B (0.3-10μM) for 1 hour prior to exposure to 10 ng/ml TWEAK for 4 hours. Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots representing the processing of p100 to p52. GAPDH was used as a loading control. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Statistical significance (P<0.05).

the non-canonical NF-κB pathway in T98G glioblastoma cells, IKKβ played no role in regulating this axis of the NF-κB signalling.

4.5 Effect of SU1433, SU1644 and ML120B on the canonical NF-κB pathway

After establishing the ability of SU1433 and SU1644 in inhibiting the non-canonical NF-κB pathway and that ML120B is not able to inhibit the pathway, it was important to examine their effect on the canonical side of the NF-κB pathway to prove the selectivity of SU compounds. This was achieved by examining their effect on IκB-α degradation and p65 phosphorylation. TNFα was used to stimulate the canonical NF-κB pathway in T98G glioblastoma cells.

4.5.1 Effect of SU1433 on TNFα-stimulated degradation of IκB-α in the T98G glioblastoma cell line

In order to examine the pharmacological effect of SU1433 on TNFα-stimulated activation of the canonical NF-κB pathway in T98G glioblastoma cells, the effect of SU1433 effect on TNFα-stimulated IκB-α degradation was examined (Figure 4.10). Cells were treated with varying concentrations of SU1433 followed by 10 ng/ml of TNFα after 1 hour following the treatment (as mentioned in the methods section). TNFα noticeably increased IκB-α degradation with minimal effect observed with DMSO. However, pretreatment with SU1433 did not inhibit TNFα-stimulated degradation of IκB-α at all concentrations examined (from 0.3 to 10 μM) (without significant inhibition) (Figure 4.10). This indicated that SU1433 does not inhibit the canonical NF-κB pathway in T98G glioblastoma cells.

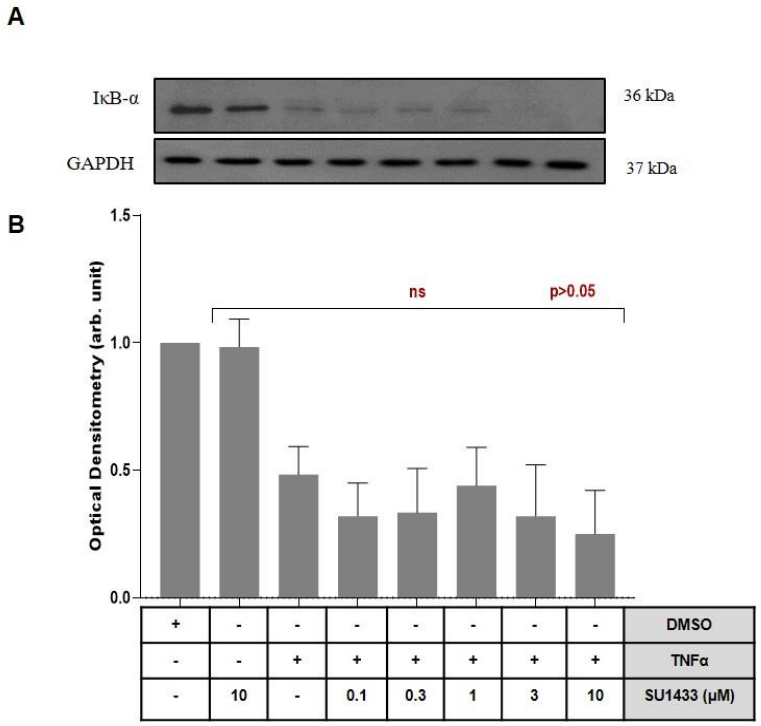


Figure 4. 8 The effect of SU1433 on TNFα-stimulated the degradation of IκB-α in the T98G glioblastoma cell line.

T98G cells were grown in 12 well plates, rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with vehicle or increasing concentration of SU1433 (0.1-10μM) for 1 hour prior to exposure to 10 ng/ml TNFα after 1 hour. Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots bands representing the degradation of IκB-α. GAPDH was used as a loading control. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

4.5.2 The effect of SU1644 on TNFα-stimulated degradation of IκB-α in the T98G glioblastoma cell line

To determine SU1644 pharmacological effect on the canonical NF-κB pathway in T98G glioblastoma cells, the effect of SU1644 was examined on the IκB-α degradation (Figure 4.11). Cells were treated with varying concentrations of SU1644 followed by 10 ng/ml of TNFα after 1 hour following the treatment (as mentioned in the methods section). TNFα significantly increased IκB-α degradation with minimal effect observed with DMSO. However, SU1644 did not inhibit the degradation of IκB-α in all

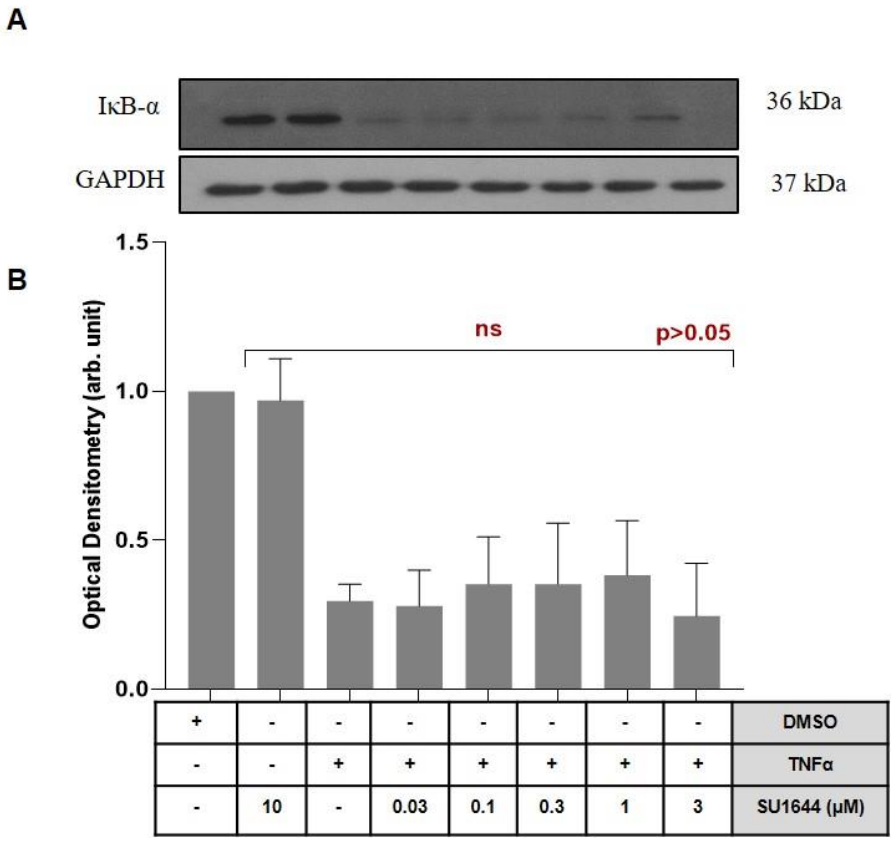


Figure 4. 9 The effect of SU1644 on TNFα-stimulated degradation of IκB-α in the T98G glioblastoma cell line.

T98G cells were grown in 12 well plates, rendered quiescent by incubation in media containing 0.1% FCS for 24h and t treated with vehicle or increasing concentration of SU1644 (0.03-3μM) for 1 hour prior to exposure to 10 ng/ml TNFα after 1 hour (as indicated). Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots bands representing the degradation of IκB-α. GAPDH was used as a loading control. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

the concentration range (from 0.03 to 3 μM). This indicates that SU1644 does not inhibit the canonical NF-κB pathway in T98G glioblastoma cells.

4.5.3 The effect of SU1433 on TNFα-stimulated phosphorylation of p65 in the T98G glioblastoma cell line

To further examine SU1433 pharmacological effect on the canonical NF-κB pathway in T98G glioblastoma cells, SU1433 effect was examined on p65 phosphorylation (Figure 4.12). Cells were treated with varying concentrations of SU1433 followed by 10 ng/ml of TNFα after 1 hour following the treatment (as mentioned in the methods section). TNFα significantly increased p65 phosphorylation with minimal effect observed with DMSO. SU1433 did not inhibit the phosphorylation of p65 at all

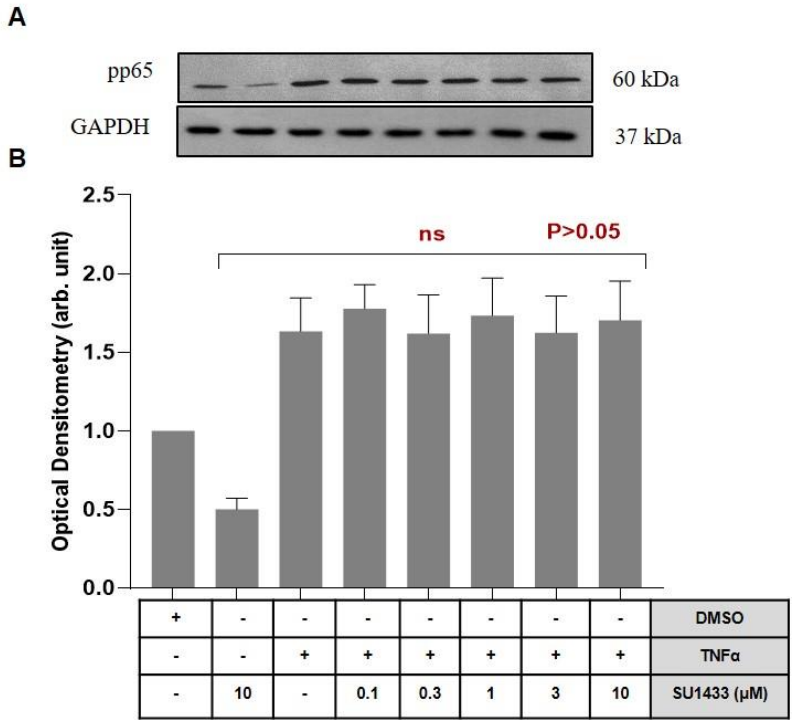


Figure 4. 10 The effect of SU1433 on TNFα-stimulated phosphorylation of p65 in the T98G glioblastoma cell line.

T98G cells were grown in 12 well plates, rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with vehicle or increasing concentration of SU1433 (0.1-10μM) for 1 hour prior to exposure to 10 ng/ml TNFα after 1 hour (as indicated). Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots bands representing the phosphorylation of p6. GAPDH was used as a loading control**. (B)** A bar chart that shows the blots assessed using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

concentrations (from 0.3 to 10 μM). Again, this indicates that SU1433 does not inhibit the canonical NF-κB pathway in T98G glioblastoma cells.

4.5.4 The effect of SU1644 on TNFα-stimulated phosphorylation of p65 in the T98G glioblastoma cell line

In addition, to examine SU1644 pharmacological effect on the canonical NF-κB pathway in T98G glioblastoma cells, SU1644 effect was examined on p65 phosphorylation (Figure 4.13). Cells were treated with varying concentrations of SU1644 followed by 10 ng/ml of TNFα after 1 hour following the treatment (as mentioned in the methods section). TNFα increased p65 phosphorylation with minimal effect observed with DMSO. However, SU1644 did not inhibit the phosphorylation of p65 in all the

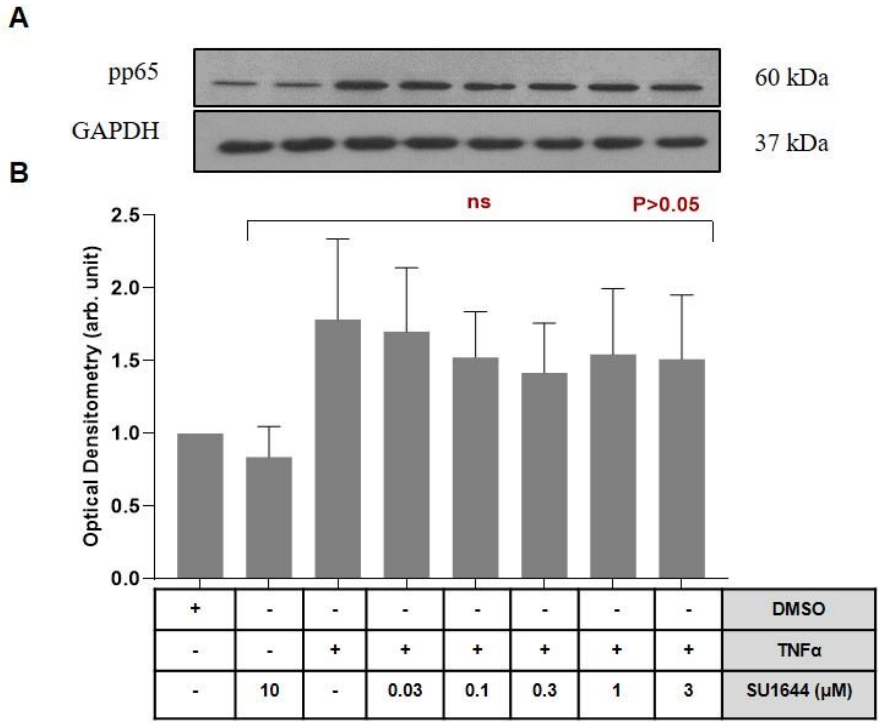


Figure 4. 11 The effect of SU1644 on TNFα-stimulated phosphorylation of p65 in the T98G glioblastoma cell line.

T98G cells were grown in 12 well plates, rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with vehicle or increasing concentration of SU1644 (0.03-3μM) for 1 hour prior to exposure to 10 ng/ml TNFα after 1 hour (as indicated). Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots bands representing the phosphorylation of p65. GAPDH was used as a loading control. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. Results expressed as fold stimulation relative to control. GAPDH was used as a loading control. Each value represents the mean ± SEM of three independent experiments. Statistical significance ($P<0.05$).

concentration range (from 0.03 to 3 μM) (without statistical significance). Again, this indicates that SU1433 SU1644 does not inhibit the canonical NF-κB pathway in T98G glioblastoma cells.

4.5.5 The effect of ML120B on TNFα-stimulated degradation of IκB-α and the phosphorylation of p65 in the T98G glioblastoma cell line

In order to determine ML120B pharmacological effect on the canonical NF-κB pathway in T98G glioblastoma cells, ML120B effect was examined on the IκB-α degradation and p65 phosphorylation (Figure 4.14). Cells were treated with varying concentrations of ML120B followed by 10 ng/ml of TNFα at 1 hour following the treatment (as mentioned in the methods section). TNFα increased IκB-α degradation and p65 phosphorylation with minimal effect observed with DMSO. However, ML120B inhibited the degradation of IκB-α with minimal effect on p65 phosphorylation from 1 μM to 10 μM. However, the inhibition was not statistically significant and that would be attributed to a low concentration range used. Higher concentrations could yield better results. This indicates that ML120B can inhibit the canonical NF-κB pathway in T98G glioblastoma cells.

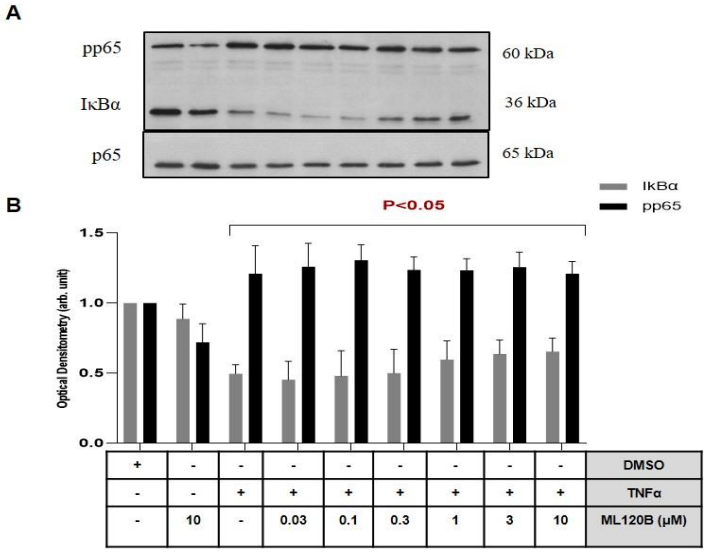


Figure 4. 12 The effect of ML120B on TNFα-stimulated degradation of IκB-α and phosphorylation of p65 in the T98G glioblastoma cell line.

T98G cells were grown in 12 well plates, rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with vehicle or increasing concentration of ML120B (0.03-10μM) for 1 hour prior to exposure to 10 ng/ml TNFα after 1 hour (as indicated). Whole cell lysates were prepared for separation using SDS-PAGE and analysis by Western Blotting using the above antibodies. **(A)** Western blots bands representing the IκB-α degradation and p65 phosphorylation. p65 was used as a loading control. **(B)** A bar chart that shows the blots assessed using semi-quantitative analysis. Results expressed as fold stimulation relative to control. Each value represents the mean ± SEM of three independent experiments. Statistical significance (P<0.05).

4.6 Discussion

4.6.1 The effect of IKKα selective inhibitors (SU1433 and SU1644) on the NF-κB pathways in the T98G glioblastoma cell line

As described previously, the NF-κB pathway has been identified as a potential facilitator of tumorigenesis in various forms of cancer (Hanahan, 2022). In addition, the growth of cancer cells was shown to be decreased by either inhibiting NF-κB pathways or deleting NF-κB subunits, as demonstrated in studies conducted by Li et al. (2005) and Meylan et al. (2009).

IKKs are widely acknowledged as crucial components in the activation of NF-κBs. Multiple studies have indicated that IKKα may serve as a tumour suppressor, however, Park et al. (2007) found that a reduction in IKKα expression facilitated the progression of squamous cell carcinomas. Liu et al. (2008) conducted a study that revealed the involvement of IKKα in the maintenance of skin homeostasis, hence contributing to the prevention of skin cancer.

In a similar vein, a study conducted by Cataldi et al. (2015) investigated the impact of IKKβ inhibitors on pancreatic cancer cell lines. The findings revealed that the administration of TPCA-1, an IKKβ inhibitor, resulted in a reduction in both proliferation and chemotherapy resistance in pancreatic cells. Nevertheless, to date, the absence of sufficient molecules targeting IKKα selectively has hindered the investigation of the contributory role(s) of cellular IKKα in comparable settings.

The Medicinal Chemistry Team within the Pharmaceutical Sciences Group, SIPBS at the University of Strathclyde, Glasgow has created a number of high-quality IKKα-selective inhibitors (referred to as SU compounds) for the purpose of comprehensive investigation into the potential targeting in tumour cells and potential validation of these molecules as anti-cancer agents. The purpose of this chapter was to evaluate the potency and selectivity of the novel SU compounds as inhibitors of IKKα in a glioblastoma brain cancer cell line. This was achieved by examining key pharmacodynamic markers associated with the activation of the non-canonical NF-κB pathway which encompassed; the phosphorylation of p100 (Ser866/870), the proteolytic processing of p100 thus producing p52, and the subsequent translocation of p52-RelB into the nucleus. Furthermore, the evaluation of selectivity was

conducted by measuring key pharmacodynamic markers associated with the activation of the canonical NF-κB pathway; the degradation of IκB-α and the phosphorylation of p65 (Ser536).

4.6.1.1 The effect of SU1433, SU1644 and ML120B on agonist-stimulated activation of the non-canonical NF-κB pathway in the T98G glioblastoma cell line

4.6.1.1.1 Effect of SU1433 on TWEAK-stimulated phosphorylation of p100 and p100/52in the T98G glioblastoma cell line

To examine the regulatory role of IKKα in non-canonical NF-κB signalling in T98G glioblastoma cells, the cells were exposed to one SIPBS-generated proprietary selective IKKα kinase inhibitor (SU1433). SU1433 was designed to be a potent selective small molecule inhibitor of IKKα (K_i IKKα vs. IKKβ: 11 vs 2250nM) and so potentially abrogate agonist-stimulated activation of the non-canonical NF-κB pathway.

To examine the effect and potential potency of SU1433 against IKKα-related signalling in T98G cells, its effects were first tested on the phosphorylation of p100 following TWEAK activation of the pathway after 4 hours. Pre-treatment of cells with SU1433 inhibited TWEAK-stimulated p100 phosphorylation in a concentration-dependent manner with maximum inhibition observed at a concentration of 3 μM. The IC_{50} was calculated to be 0.94 μM.

For further confirmation of the effect of SU1433, its effect against other relevant pharmacodynamic markers of the non-canonical NF-κB pathway, such as the processing of p-p100 to mature p52 were examined. SU1433 inhibited TWEAK-stimulated formation of p52 in a concentration-dependent manner with a concentration of 3 μM showing the highest effect, which is complementary to what was observed in the previous experiment. This effect was also noticeable on the nuclear translocation of p52 and RelB markers, as SU1433 inhibited TWEAK-stimulated translocation in a concentration-dependent manner, with the highest inhibitory effect observed at 10 μM concentration on both p52 and RelB. It was also noticeable that the effect of SU1433 was more pronounced on p52 nuclear translocation than on RelB (see Figure 4.3).

Examination of the impact of inhibiting IKKα using SU1433 and/or other SU small molecules on downstream transcriptional events will enable the assessment of the status of key regulatory proteins such as MMP-2 and MMP-9, linked with migratory and invasive phenotypes displayed in glioblastoma. In this regard, Cherry and co-workers have investigated the effect of TWEAK on the expression of MMP-9 and found that TWEAK promotes MMP-9 expression and subsequent cell invasion in glioblastoma-derived cells (T98G cells were not included in the study) (Cherry et al., 2015).

4.6.1.1.2 The effect of SU1644 on TWEAK-stimulated phosphorylation of p100 and p100/52 processing in the T98G glioblastoma cell line

SU1644 is another selective IKKα small molecule inhibitor that has been recognised to act as a more potent inhibitor of agonist stimulated non-canonical NF-κB signalling in a cell based setting (e.g. prostate and pancreatic cancer cell lines), approximately 10-fold more potent that the related SU compound, SU1433 (Paul & Mackay, personal communication). In order to examine the effect of SU1644 on the non-canonical NF-κB signalling in T98G glioblastoma cells, the cells were exposed to varying concentrations of the compound and specific biomarkers were tested accordingly.

To examine the effect and potential potency of SU1644 against IKKα-related signalling in T98G cells, its effects were first tested on the TWEAK-stimulated phosphorylation of p100 after 4 hours. Pre-treatment of cells with SU1644 inhibited TWEAK-stimulated p100 phosphorylation in a concentration-dependent manner with maximum inhibition observed at a concentration of 1 μM. The IC_{50} was calculated to be 0.31 μM. This result showed SU1644 to be approximately 4.7 times more potent than SU1433 in inhibiting TWEAK stimulated non-canonical NF-κB pathways activation (Figure 4.5).

For further confirmation of the potent effect of SU1644, the impact of SU1644 against other relevant pharmacodynamic markers of the non-canonical NFκ-B pathway such as the processing of p-p100 to mature p52 were examined. SU1644 inhibited the formation of p52 in a concentration-dependent manner with a concentration of 3 μM showing the highest effect. This effect was also noticeable on the nuclear translocation of p52 and RelB markers as SU1644 inhibited their translocation showing a

concentration-dependent pattern with the highest inhibitory effect observed at 3 μM concentration on both p52 and RelB (Figure 4.7).

It was noticeable that SU1433 and SU1644 achieved very similar IC_{50} against all the markers examined (inhibition of p100 phosphorylation, p52 processing and nuclear translocation off p52/RelB). The findings of this study provide support for the notion that IKKα is positioned upstream of all other components and serves as the primary regulator of the interconnected cascade of signalling events.

4.5.2 Comparing the selectivity of SU1433 and SU1644 against IKKα versus ML120B (MLN120B), a commercially available IKKβ-selective inhibitor in theT98G glioblastoma cell line

ML120B is a well-established potent selective IKKβ inhibitor for laboratory use with a reported IC_{50} of 60 nM (Newton et al., 2007; reviewed in Gamble et al., 2012). It was important to compare the effect of SU compounds with an IKKβ-selective inhibitor in order to establish their selectivity and confirm the regulatory roles for both IKKα and IKKβ in modulating different aspects of downstream NF-κB activation. First, ML120B was tested against the non-canonical NF-κB pathway pharmacodynamic markers (pp100 and p100/52 processing) and showed no inhibitory effect on either marker (see Figures 4.8 and 4.9) (With each of these results, examining the nuclear translocation of p52/RelB was redundant and of no added value). The absence of any effect on non-canonical NF-κB signalling by ML120B, supports earlier findings that the NF-κB signalling mediated by IKKβ is separate from the non-canonical pathway.

4.5.3 The effects of SU1433, SU1644 and ML120B on TNFα-stimulated activation of the canonical NF-κB pathway in the T98G glioblastoma cell line

Following the determination of the inhibitory potential of SU1433 and SU1644 on the non-canonical NF-κB pathway, and the lack of inhibitory activity exhibited by ML120B, it became imperative to investigate the potential impact of these compounds on the canonical arm of the NF-κB pathway in order to validate the selectivity of the SU compounds. This was accomplished through the investigation of their potential impact on TNFα-stimulated degradation of IκB-α and the phosphorylation of p65.

4.5.3.1 The effect of SU1433 and SU1644 on TNFα-stimulated degradation of IκB-α and p65 phosphorylation in the T98G glioblastoma cell line

Pretreatment of cells with varying concentrations of either SU1433 or SU1644 showed no effects on TNFα-stimulated degradation of IκB-α nor p65 (Ser536) phosphorylation in the glioblastoma cells. This confirmed the selectivity of the compounds for IKKα and not IKKβ (which is the predominant regulatory factor on the canonical side of the NF-κB pathway) (Paul et al., 2018) (See Figure 4.10 & 4.13).

4.5.3.2 The effect of ML120B on TNFα-stimulated degradation of IκB-α and the phosphorylation of p65 in the T98G glioblastoma cell line

In contrast, ML120B, a widely recognised and effective inhibitor of the canonical NF-κB pathway (Newton et al., 2007), exhibited a concentration-dependent inhibition pattern for both TNFα-stimulated IκB-α degradation and p65 phosphorylation, compared to control. The greatest level of inhibition was detected at a concentration of 10 μM (see Figure 4.14). The concentrations used in this study were based on previous studies conducted on multiple myeloma and on lung cells where 10 μM showed significant inhibition of NF-κB2 signalling (Hideshima et al., 2006; Ansaldi et al., 2016). However, conducting a concentration curve for ML120B was deemed essential to enhance comprehension of the inhibitory effects of this chemical on T98G glioblastoma cells. This analysis was driven by the observation that higher concentrations of ML120B yielded more pronounced inhibition.

4.6 Conclusion

Given the confirmed selectivity of SU1433 and SU1644 as inhibitors of the non-canonical NF-κB pathway through specific inhibition of IKKα, it is crucial to investigate the downstream events mediated by IKKα and the resulting phenotypic outcomes. These investigations include NF-κB-associated protein-DNA binding as well as to assess the effects of these inhibitors on cell viability and clonogenic survival.

Chapter Five

The effect of SU compounds on agonist stimulated protein-DNA binding, cell viability and clonogenic survival in glioblastoma cells

5.1 Introduction

The confirmation of NF-κB pathway activation in T98 GBM cells through the use of TWEAK and TNFα aligns with the results obtained in earlier chapters. Moreover, it was demonstrated that the non-canonical side of the pathway can be stimulated by TWEAK and inhibited by specific small molecule inhibitors of IKKα (SU1433 and SU1644), while having no impact on the canonical side of the pathway. It was imperative to advance the progress of this study by investigating other downstream events and their influence on the phenotypic characteristics of *Glioblastoma multiforme* (GBM) cell lines.

In this chapter, different experiments were done aiming to observe and explain the outcome after introducing SU compounds to specific cancer cell characteristics (cell viability and clonogenic survival) and protein-DNA interaction.

1) Electrophoretic Mobility Shift Assay (EMSA) was conducted in order to examine TNFα & TWEAK stimulated DNA binding activity by using NF-κB oligonucleotide and p52-C250T *TERT* promoter; to then examine the effect of SU1433 and SU1644 on such interactions.

2) The same thing applies to cell viability and clonogenic survival, where MTT and clonogenic assays were performed with T98G (and UVW for MTT assay) glioblastoma cell lines. Both cell viability and clonogenicity were examined against SU1433, SU1644 and ML120B as a point of comparison.

5.2 Materials and Methods

Cell lines and routine cell maintenance

All routine maintenance of cell lines was performed as described in Section 2.5.

Cell stimulations were performed using TNFα and TWEAK as described in section 2.7 and 2.8.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed using different oligonucleotides (EMSA oligo NFκB IRDye700) and (C250T TERT promoter) as descried in the methods section 2.7.

MTT assay

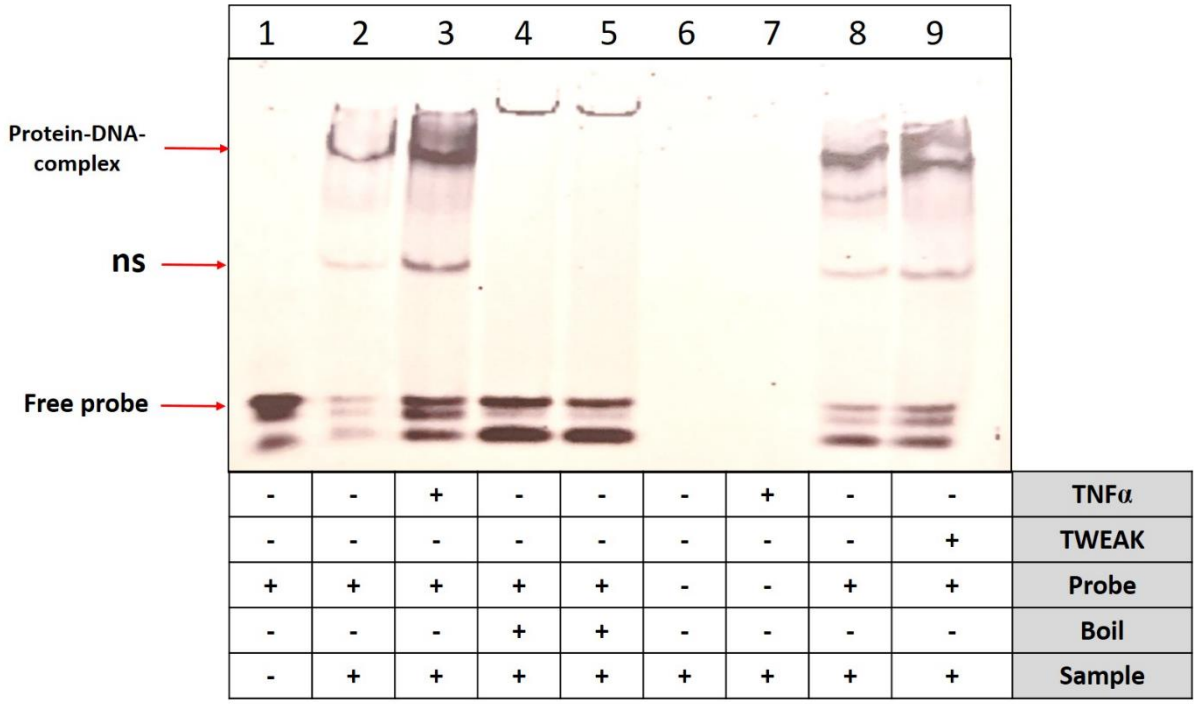
MTT assay was performed using SU1433 and SU1644 provided by SIPBS (Prof. S. Mackay) and ML120B (MedChemExpress (MCE), USA).

Clonogenic Assay

A clonogenic survival assay was performed using SU1433 and SU1644 provided by SIPBS (Prof. S. Mackay) and ML120B (MedChemExpress (MCE), USA).

5.3 Analysis of NF-κB protein-DNA binding activity in T98G glioblastoma cells

The Electrophoretic Mobility Shift Assay (EMSA) is a widely employed technique in the field of molecular biology for investigating interactions between proteins and nucleic acids, specifically protein-DNA or protein-RNA interactions. The aforementioned methodology has the capacity to ascertain the binding capability of a protein or a combination of proteins towards a specific DNA or RNA sequence. Additionally, it may provide insights into the potential involvement of multiple protein molecules in the binding complex. In this study two different probes were used that were fluorescently labelled to enable imaging and detection of any protein-DNA complexes relative to free probe in nuclear extracts prepared from T98G GBM cells exposed to TNFα or TWEAK.



-	-	+	-	-	-	+	-	-	TNFα
-	-	-	-	-	-	-	-	+	TWEAK
+	+	+	+	+	-	-	+	+	Probe
-	-	-	+	+	-	-	-	-	Boil
-	+	+	+	+	+	+	+	+	Sample

Figure 5. 1 NF-κB protein-DNA binding activity in T98G glioblastoma cells by EMSA.

T98G cells were grown in 6 well plate (in triplicates), rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with vehicle or 10 ng/ml of TNFα or TWEAK for 1 hour. Nuclear Extracts (sample) were prepared for separation using EMSA assay using a labelled-NF-κB oligonucleotide and analysed by Li-Cor scanning as described in the methods section. Lane1: DNA probe alone, Lane 2: control untreated plus probe. Lane 3: TNFα stimulated plus probe. Lanes 4 &5: boiled control and TNFα-treated (+ is missing in the table) samples plus probe. Lane 6 & 7 control and TNFα treated samples without probe. Lanes 8 & 9: control and TWEAK treated samples plus probe. The red arrows indicate where the DNA complex, non-specific binding (ns) and free probe should be. This assay is representative of 3 independent experiments.

In the initial experiment, the objective was to observe any agonist-stimulated NF-κB protein-DNA binding. It was observed that TNFα relative to control increased NF-κB protein-DNA binding activity in nuclear extract from T98G glioblastoma cells (red arrow in Figure 5.1, lane 3). However, this binding activity was not observed when cells were stimulated by TWEAK (Figure 5.1, lane 9).

5.2.1 The effect of TNFα-stimulated vs TWEAK-stimulated NF-κB-DNA binding activity in the T98G glioblastoma cell line

After the initial observation in Figure 5.1 that TWEAK did not induce NF-κB-DNA binding, a more thorough investigation was conducted to validate this discovery. An 8-hour time course was carried out, In order to validate the finding, a study was done to determine the NF-κB-DNA binding activity in T98G cells upon TNFα stimulation and TWEAK stimulation. A time course experiment of 8 hours was conducted, employing a concentration of 10 ng/ml of TNFα and TWEAK independently. As expected, with TNFα, a progressive trend of activation was found over the duration of the study while no discernible binding activity was seen for the duration of the experiment when TWEAK was used (Figure 5.2).

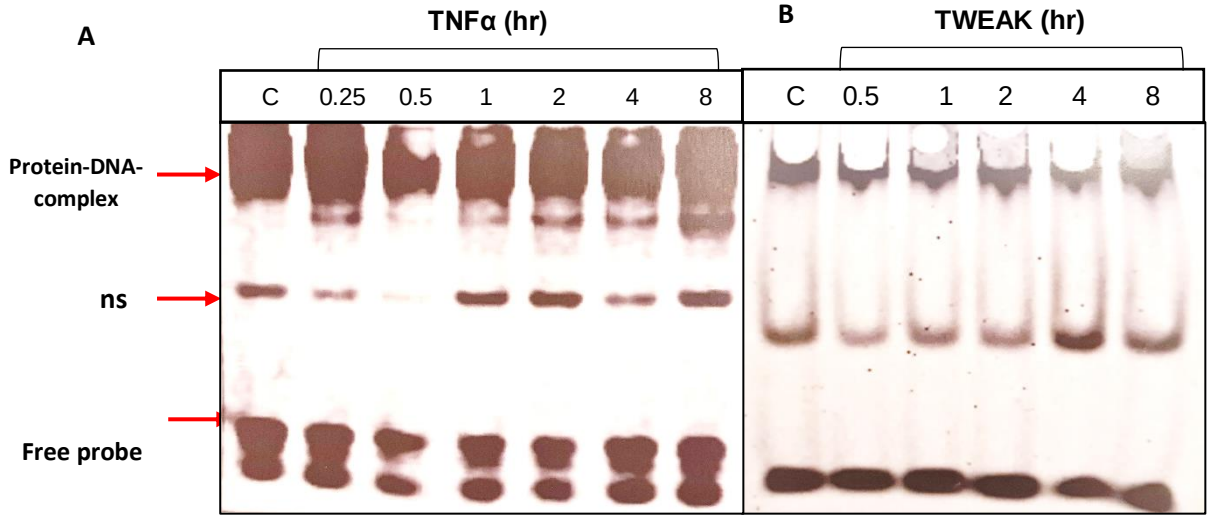


Figure 5. 2 TNFα and TWEAK-stimulated NF-κB-DNA binding activity in T98G cells by EMSA.

T98G cells were grown in a 6 wells plate (in triplicates) rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with 10 ng/ml of TNFα over time **(A)** and 10 ng/ml of TWEAK over time **(B)**. Nuclear Extracts were prepared for separation using EMSA assay and analysis by Li-Cor scanning machine using the NF-κB oligonucleotide. The red arrows indicate where the DNA complex, non-specific binding (ns) and free probe should be. This assay is representative of 3 independent experiments.

5.3 Analysis of p52-DNA binding activity in T98G glioblastoma cells by EMSA

The aim of this experiment was to investigate the binding of the p52 protein to DNA by employing both TWEAK and TNFα.

5.3.1 TWEAK and TNFα-stimulated p52-DNA binding activity in the T98G glioblastoma cell line

After the observation in Figure 5.1 and 5.2, that TWEAK did not induce NF-κB-DNA binding, compared to TNFα. Further investigation on the ability of TWEAK and TNFα to activate p52-DNA (which as described ealier, can be overexpressed by TWEAK stimulation) binding, a 24-hour time course was carried out, in order to validate the finding. A time course experiment of 24 hours was conducted, employing a concentration of 10 ng/ml of TNFα and TWEAK independently. As expected, with TWEAK, a progressive trend of activation was found from 8-24 hours compared to control while no discernible binding activity was seen for the duration of the experiment when TNFα was used (Figure 5.3 and 5.4).

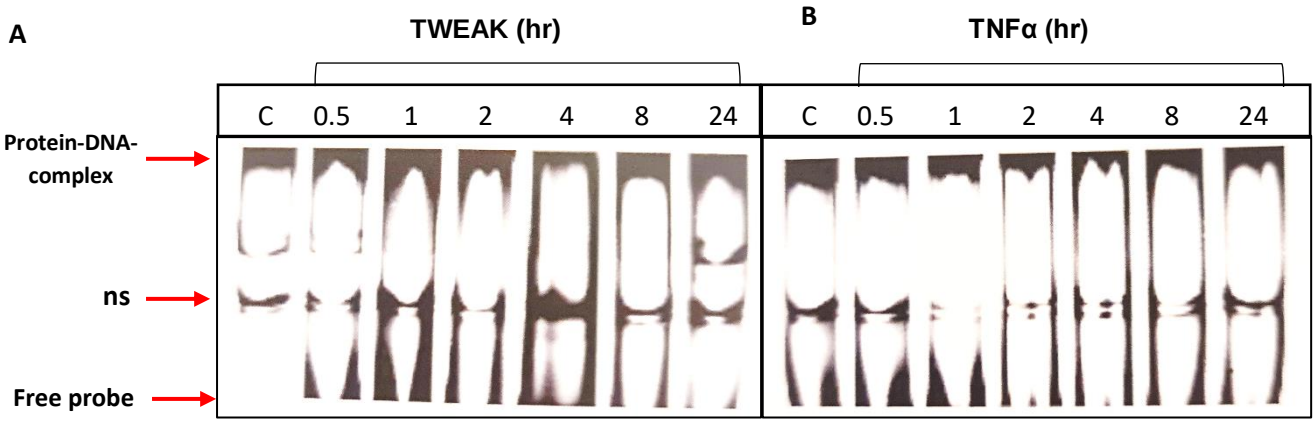


Figure 5. 3 TWEAK and TNFα-stimulated p52-DNA binding activity in T98G cells by EMSA.

T98G cells were grown in a 6 wells plate (in triplicates) rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with 10 ng/ml of TWEAK over time **(A)** and 10 ng/ml of TNFα over time **(B)**. Nuclear Extracts were prepared for separation using EMSA assay and analysis by Li-Cor scanning machine using the *TERT* C250T oligonucleotide. The red arrows indicate where the DNA complex, non-specific binding (ns) and free probe should be.

5.4 The effect of SU1433 and SU1644 on TWEAK-stimulated p52-DNA binding activities in the T98G glioblastoma cell line

In the next stage of experimentation, the effectiveness of the two selective IKKα small molecule inhibitors, SU1433 and SU1644 were examined in relation to potential p52-DNA binding activity. Given the effectiveness of SU1433 and SU1644 in inhibiting the non-canonical NF-kB pathway demonstrated previously (refer to Chapter 3), it was anticipated that both SU1644 and SU1433 compounds would exhibit inhibitory effects on TWEAK-stimulated p52-DNA binding activity in T98G cells. However, this yielded inconclusive results and due to time constraints, the experiment was only conducted twice. Yet, An inhibition of p52-C250T DNA binding was noticeable by increasing the concentration of the

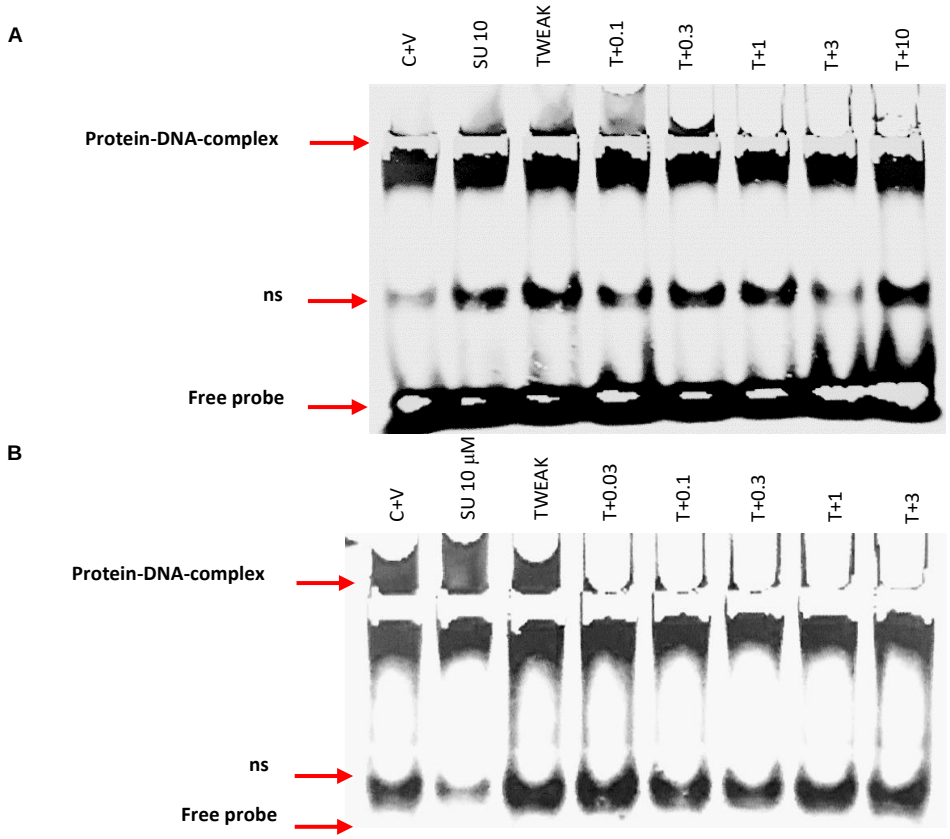


Figure 5. 4 Effect of SU1433 and SU1644 on TWEAK-stimulated p52-DNA binding activities in T98G by EMSA.

T98G cells were grown in a 6 wells plate (in triplicates) rendered quiescent by incubation in media containing 0.1% FCS for 24h and treated with varying concentrations of SU1433 (0.1-10 µM) **(A)** and SU1644 (0.03-3 µM) **(B)** for 1 hour followed by 10 ng/ml of TWEAK for 8 hours. Nuclear extracts were prepared for separation using EMSA assay and analysis by Li-Cor scanning machine using the TERT C250T oligonucleotide. The red arrows indicate where the DNA complex, non-specific binding (ns) and free probe should be. This assay is representative of two independent experiments. (C=Control, V=Vehicle, SU10=SU 10µM, T=TWEAK)

both SU compounds with SU1433 starting inhibtion at 1 μM concentration and SU1644 at 0.03 μM. Therefore, further experiments are required to achieve a more precise understanding. The acquired preliminary results (Figures 5.6 A & B) are presented for reference purposes.

5.5 Analysis of the effect of SU compounds of the phenotypic characteristics of T98G and UVW glioblastoma cells

By establishing the ability of SU compounds to inhibit p52-DNA binding activity, it was necessary to examine the impact of such inhibition on the phenotypic characteristics of GBM cell lines that can express C250T or C228T TERT promoter mutation (T98G and UVW, respectively). Some of the cancer hallmarks that can be examined in an *in vitro* setting include sustained proliferative signalling using an MTT viability assay and enabling replicative potential through examining clonogenic survival.

In this section, different studies were carried out to investigate the effectiveness of SU1433 and SU1644 on some of the phenotypic characteristics of two GBM cell lines (T98G and UVW). These characteristics include cell viability and cell clonogenic survival. It is hypothesised that upon the achieved results in previous chapters of this study, SU1433 and SU1644 could impact and inhibit GBM cancer cell viability as well as clonogenic survival by inhibiting IKKα activity within cancer cells.

5.5.1 The effect of SU1433, SU1644 and ML120B on cell viability in T98G glioblastoma cells

Experiments to examine the potential effect of the SU compounds and ML120B on cell viability were developed by means of an MTT assay (as described in the methods section). The results depicted in (Figure 5.5 A) demonstrated the impact of SU1433 on the vitality of T98G cells, generating a significant concentration-dependent inhibition of cell viability over the concentration range examined (0.1-10 µM) compared to control and an IC_{50} value calculated for the effects of SU1433 of 1.21 µM (Figure 5.5 D). Similar observations were made for SU1644, where the greatest decrease in cell viability was observed

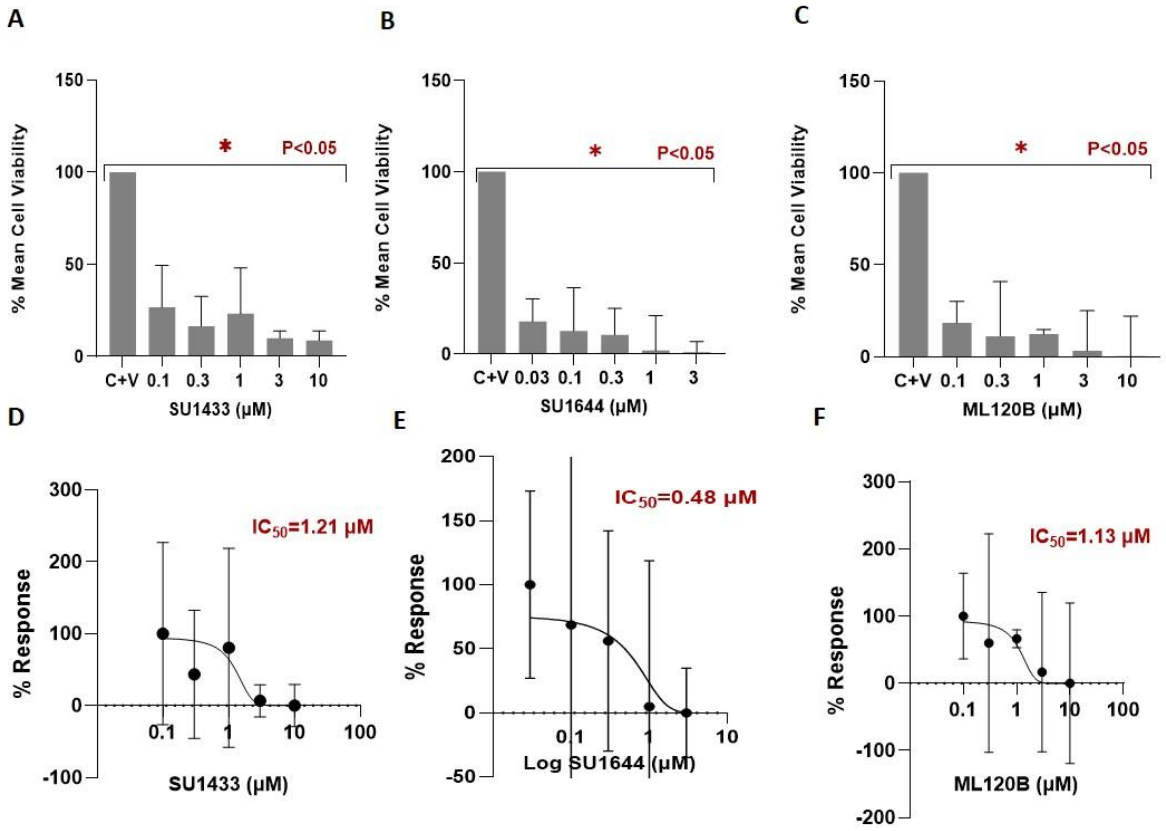


Figure 5. 5 The effect of SU1433, SU1644 and ML120B on viability of the T98G glioblastoma cell line.

T98G cells were counted and seeded into a 96-well plate at a density that allowed for exponential growth. The cells were then cultured to approximately 30% confluency in full media and exposed to vehicle or increasing concentrations of SU1433 (0.1-10µM), SU1644 (0.03-10µM) or ML120B (0.1-10µM) for 48 hours and viability assessed by means of a colorimetric MTT assay as described in the methods section. A, B and C are bar charts of the MTT assay results for SU1433, SU1644 and ML120B, respectively. D, E and F are line charts of the MTT assay results for SU1433, SU1644 and ML120B, respectively (IC_{50} for each compound represented in the figure. Each value represents the means ± SEM of three independent experiments. P value for statistical significance (* = $P < 0.05$).

at 3 μM concentration and the IC_{50} of 0.48 μM. ML120B has also siginificantly redued cell viability and achieved an IC_{50} of 1.13 μM.

5.5.2 The effect of SU1433, SU1644 and ML120B on cell viability in UVW glioblastoma cells

The findings illustrated in Figure 5.6 demonstrate the influence of SU1433, SU1644, and ML120B on the viability of UVW cells. UVW cells are a GBM cell line which possesses a C228T hTERT promoter mutation rather than C250T with different genetic background. This would suggest that SU compounds would have less impact as C250T is the mutation driven by the IKKα-p52 signalling pathway (Li et al., 2015).

A consistent and significant decline in cell viability was noted when cells were treated with increasing concentrations of SU1433, SU1644, and ML120B. SU1433 achieved an IC_{50} of 1.09 μM and SU1644 0.38 μM while ML120B achieved an IC_{50} of 0.34 μM (Figure 5.6). However, the effect of the compounds on UVW cells was generally less pronounced than that produced on T98G cells. This suggested that in the UVW cells being derived and established from an anaplastic astrocytoma with a different h*TERT* genetic background (C228T mutation) reacts differently to IKKα selective inhibitors than T98G GBM cells (h*TERT* C250T mutation). This finding requires further study and examination to idenfitify why such variation occurs.

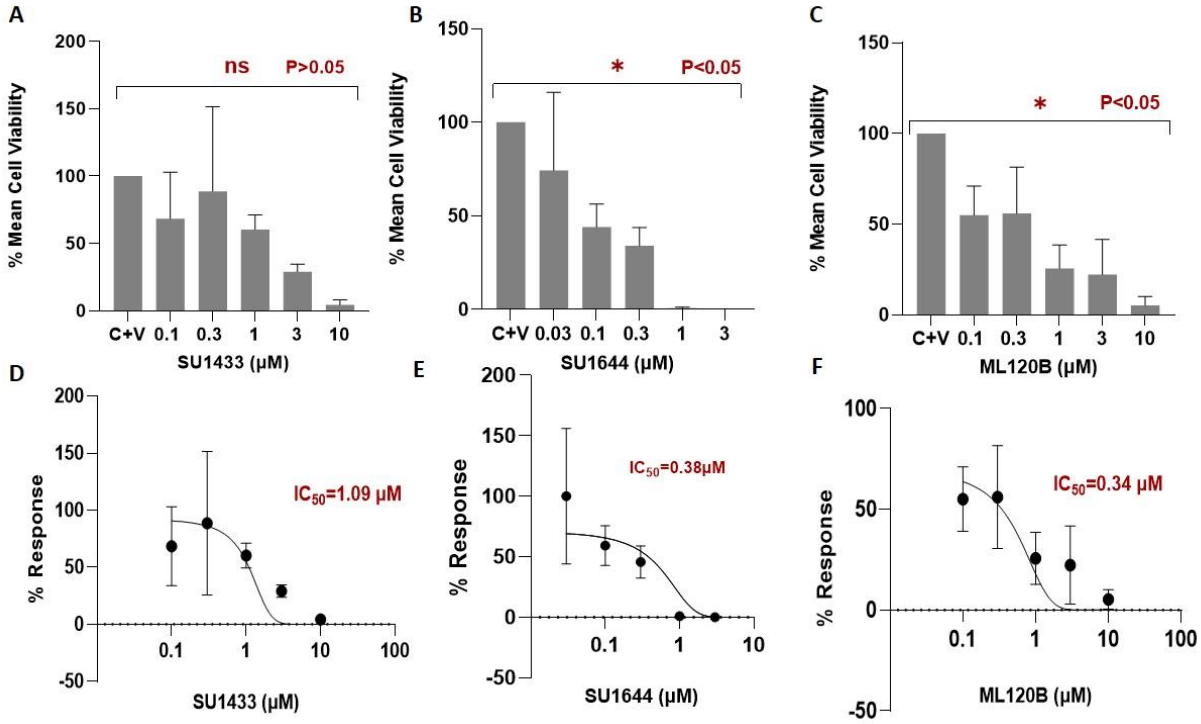


Figure 5. 6 The effect of SU1433, SU1644 and ML120B on viability of the UVW glioblastoma cell line.

UVW cells were counted and seeded into a 96-well plate at a density that allowed for exponential growth. The cells were then cultured to approximately 30% confluency in full media and exposed to vehicle or increasing concentrations of SU1433 (0.1-10μM), SU1644 (0.03-10μM) or ML120B (0.1-10μM) for 48 hours and viability assessed by mean of a colorimetric MTT assay as described in the methods section. A, B and C are bar charts of the MTT assay results for SU1433, SU1644 and ML120B, respectively. D, E and F are line charts of the MTT assay results for SU1433, SU1644 and ML120B, respectively (IC_{50} for each compound represented in the figure. Each value represents the means ± SEM of three independent experiments. P value for statistical significance ($* = P < 0.05$).

5.6 The effect of SU1433 and SU1644 on clonogenic survival of T98G glioblastoma cells

After establishing the effect of SU compounds on cell viability which represents the effect on the sustained proliferative signalling hallmark, next was to examine the effect of the compounds on clongenic survival assay that is a representative of 'enabling replicative potential' hallmark. The effect of the same compounds (SU1433 and SU1644) on clonogenic survival of the T98G cells was investigated by using the same concentrations that were used in the previous experiments considering cell viability. Exposure of cells to agents could affect their division and ability to form colonies. T98G cells were treated with vehicle or SU1433 compound (0.1- 10 µM) and SU1644 (0.03-3 µM) for 24 hours and their ability to produce colonies was then assessed by counting the number of colonies that

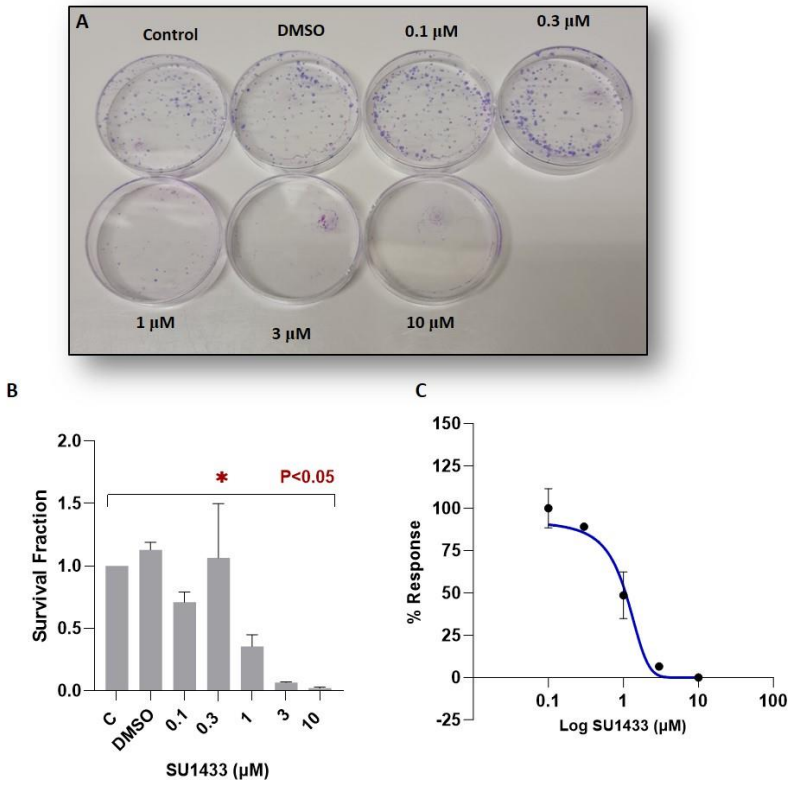


Figure 5. 7 The effect of SU1433 on the clonogenic survival of the T98G glioblastoma cell line.

SU1433 (0.1-10 µM) for 24 h and clonogenic survival was assessed after an incubation period of 14 days, as described in the methods section . **(A)** Clonogenic survival/colony formation in Giemsa stained cells at 14 days post-treatment. Data was fitted as outlined in the methods section; results were expressed as the average of Survival Fraction compared to control. **(B)** Data was fitted to a bar chart**. (C)** Data was fitted to a concentration response curve. Each value represented the mean ± SEM of triplicate samples from two independent experiments. Statistical significance (* = $P < 0.05$).

formed after 14 days of incubation and thereafter calculated as Survival Fraction (SF; see methods section). As shown in Figure 5.7, it was observed that 0.1 and 0.3 μM of SU1433 showed no significant inhibition of SF, relative to control cells (cells treated with DMSO vehicle), however, significant reductions with higher concentrations of the SU compounds (1-10 μM) were apparent.

On the other hand, T98G cells treated with different concentrations of SU1644 (0.03-3 μM) showed a significant inhibition of SF at earlier concentration between (0.3-3 μM) after 14 days of incubation. The survival fraction was significantly decreased reaching maximum reduction at 3 μM compared with control sample, see Figure 5.8.

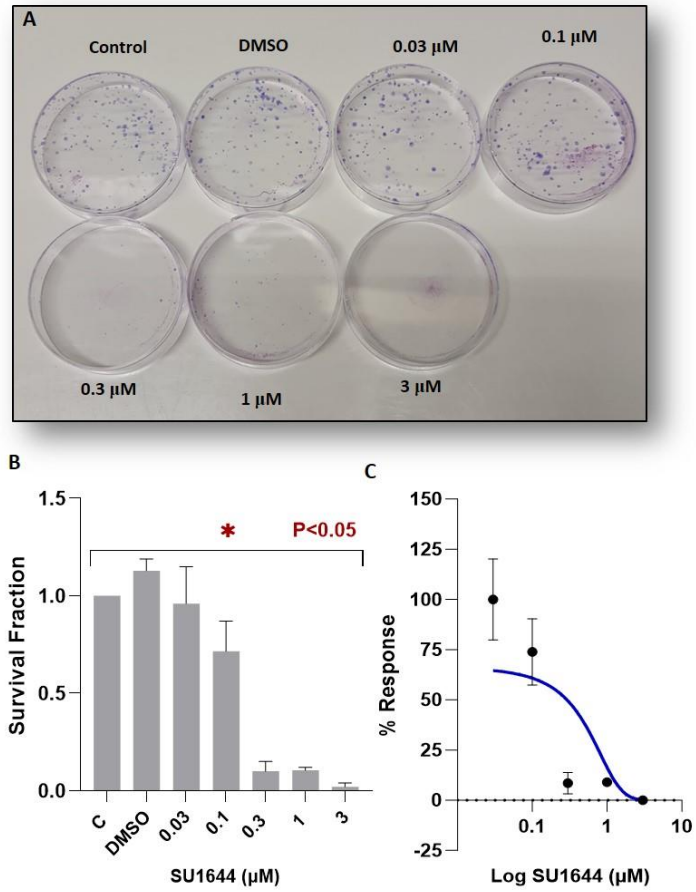


Figure 5. 8 The effect of SU1644 on the clonogenic survival of the T98G glioblastoma cell line.

T98G cells were counted, seeded and treated with vehicle (0.05% DMSO) or increasing concentrations of SU1644 (0.1-10 µM) for 24 h and clonogenic survival was assessed after an incubation period of 14 days, as described in the methods section . **(A)** Clonogenic survival/colony formation in Giemsa stained cells at 14 days post-treatment. Data was fitted as outlined in methods section, results were expressed as the average of Survival Fraction compared to control. **(B)** Data was fitted to a bar chart**. (C)** Data was fitted to a concentration response curve. Each value represented the mean ± SEM of triplicate samples from two independent experiments. Statistical significance (* = $P < 0.05$).

The figure below (figure 5.9) compares the potency of both SU1433 and SU1644 on the T98G cell clonogenic survival which shows that SU1644 is more potant inhibitor than SU1433.

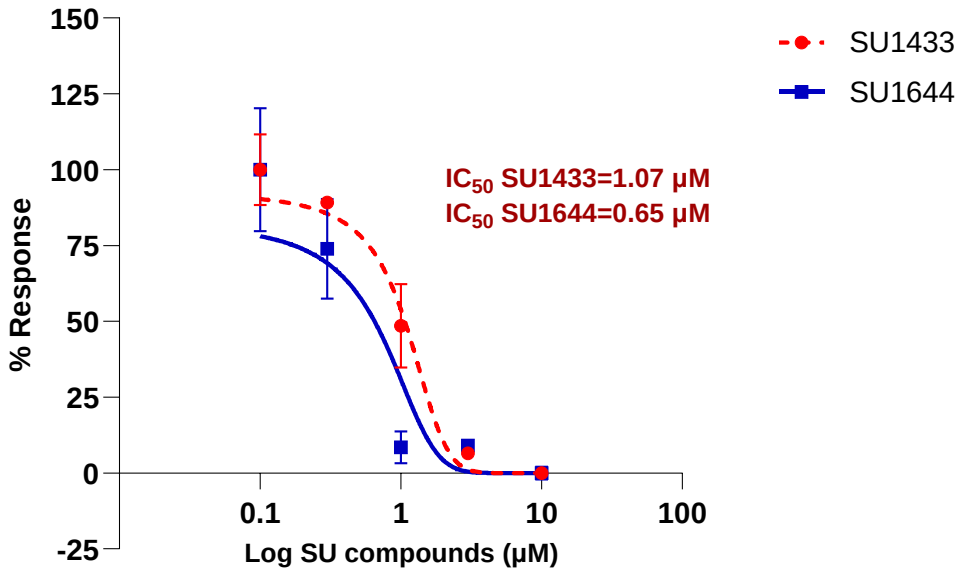


Figure 5. 9 The effect of SU1433 and SU1644 on clonogenic survival of T98G glioblastoma cell line.

T98G cells were counted, seeded and treated with vehicle (0.05% DMSO) or increasing concentrations of SU1433 (0.1-10µM) or SU1644 (0.03-3 µM) for 24 h and clonogenic survival determined after an incubation period of 14 days as described in the methods section. Data was fitted to a concentration response curve with IC_{50} of each compound. Each value represents the mean ± SEM of two independent experiments conducted in triplicates.

5.7 Discussion

This chapter presents a series of experiments conducted to investigate and elucidate the effects of adding SU compounds on, protein-DNA interactions and the impact on particular properties of cancer cells, namely cell viability and clonogenic survival. The study employed Electrophoretic Mobility Shift Assay (EMSA) to investigate NF-κB-DNA binding activity whether by use of general NF-κB-binding element or a suggested p52 specific sequence derived from the C250T mutated h*TERT* promoter. This investigation involved the use of TNFα and TWEAK as stimuli. Furthermore, the study aimed to assess the impact of SU1433 and SU1644 on these interactions. Furthermore, in the context of cellular responses and phenotypic outcomes representative of cancer hallmarks, cell viability and clonogenic survival assays were pursued to understand better the potential impact of the IKKα-targeting SU compound on the cellular processes that underpin tumour development/progression.

5.7.1 Analysis of NF-κB protein-DNA biding activity and p52-DNA binding activity in the T98G glioblastoma cell line

At the start of this chapter, Electrophoretic Mobility Shift Assays (EMSA) were employed to investigate the capacity of TWEAK to trigger specific protein-DNA binding events associated with the non-canonical NF-κB pathway— which consequently, should result in the transcription of DNA and the expression of specific genes. It was presumed that p52 expression can help drive human *TERT* augmentation, which leads to the immortalization of cancer cells and their ability to evade apoptosis (i.e. cancer progression). Li et al. (2015) reported the existence of two mutations, namely C250T and C228T, in the core region of *TERT* promoters and the C250T mutation in the h*TERT* promoter was reported to generate a p52-specific binding sequence, p52 binding supported by interaction with the ETS1/2-transcription factors. These data immediately identified a DNA binding sequence specific to the p52 NF-kB2 isoform, distinct from the other four NF-kB isoforms (described in Chapter 1) and also put forward a novel experimental tool to examine p52 non-canonical NF-κB pathway activation. This had until that point in time remained a major challenge in the NF-κB research field as there were no means of assaying NF-κB isoform-specific increases in cellular DNA-binding activity. This study therefore, aimed to utilise this more recently described p52-DNA binding/enhancer sequence (Li et al.,

2015) to examine whether TWEAK could generate a protein-C250T h*TERT* promotor binding activity in the nuclear extracts prepared from T98G cells, again driven by non-canonical NF-κB signalling.

By referring to Figure 5.1 and 5.3, it was observed that TNFα, as a well-recognised activator of both canonical and non-canonical NF-κB pathways could induce a DNA-binding activity. This was apparent when an oligonucleotide probe encompassing a general NF-κB-binding sequence (derived from the human immunoglobulin G (IgG) promoter) was utilised in the EMSA format. This sequence is widely acknowledged as a standard oligonucleotide that potentially binds to all NF-κB isoforms. Based on the findings presented in Chapter 3, which demonstrated that TWEAK had negligible impact on the canonical aspects of the NF-κB pathway, it was perhaps no surprise that relative to TNFα stimulation, TWEAK did not induce protein-DNA binding in an EMSA utilising the general NF-κB oligonucleotide (see Figure 5.2). Hence, the subsequent stage involves the evaluation of TNFα and TWEAK on p52-C250T *TERT* promoter expression. The observed protein-DNA binding reaction/activity favoured TWEAK stimulation over TNFα stimulation (Figure 5.4). However, it was first assumed that p52-C250T *TERT* promoter would respond to TNFα stimulation due to its ability to activate both the canonical and non-canonical sides of the NF-κB pathway. However, as seen in Chapter 3, the non-canonical NF-κB pathway was activated by TNFα, albeit to a lesser extent compared to the stimulation induced by TWEAK and that was supported by EMSA results (Figure 5.5). Li et al. (2015) provided evidence supporting the aforementioned findings, as they conducted an investigation into the effects of TNFα and TWEAK on NF-κB consensus oligonucleotides and that derived from the C250T *TERT* promoter. The researchers observed that the presence of TNFα resulted in an increase in NF-κB DNA binding, but no discernible effect was observed on the C250T *TERT* promoter. In the study conducted by Li et al. (2015), it was observed that TWEAK reactivated the C250T *TERT* promoter, while having no impact on NF-κB DNA binding. Moreover, this study aimed to investigate the impact of TWEAK stimulated protein binding of the oligonucleotide derived from the C250T *TERT* promoter region, explicitly focusing on selective IKKα small molecule inhibitors (SU1433 and SU1644) on the DNA binding at this site. Upon examination, no definitive outcomes were obtained regarding SU-mediated inhibition of TWEAK stimulated p52-C250T hTERT

promoter sequence binding (Figures 5.5 A and B). Due to the limitations on time, replicating and improving these experiment utilising EMSA are required to link the upstream liberation of p52-RelB complexes and their elevated nuclear translocation with that of modulation of nuclear protein-DNA-binding activity. Furthermore, in the absence of the opportunity to develop further EMSA 'super-shift' assays, using concentrated antibodies with affinity for individual NF-κB isoforms, the identification of the NF-κB subunits that contribute to the formation of individual protein-DNA complexes binding each of the two probes remain to be carried out.

5.7.2 The effect of SU1433, SU1644 and ML120B on viability of T98G glioblastoma cells using an MTT assay.

Additional investigations conducted in this study pertaining to investigate the consequences of inhibiting the activity of GBM cell lines expressing C250T or C228T *TERT* promoter mutation (T98G and UVW, respectively) on their phenotypic characteristics. Several cancer hallmarks can be assessed in an *in vitro* environment. For instance, persistent proliferative signalling can be evaluated using the MTT viability test. Therefore, identifying the potency of SU compounds and ML120B on cell viability was deemed necessary.

The experiment was conducted utilising three specific compounds, namely SU1433, SU1644, and ML120B, on two distinct glioblastoma cell lines, namely T98G and UVW. The results of SU1433 treatment demonstrated a consistent and concentration-dependent decline in cell viability in both T98G and UVW cell lines. The highest reduction of cell viability was found at concentrations of 10 μM and 1 μM for T98G and UVW, respectively (Figure 5.5 A and 5.6 A). The achieved IC_{50} was 0.98 μM in T98G cells and 0.59 μM in UVW cells. On the other hand, the experimental treatment SU1644 exhibited an overall decrease in cell viability in both cell lines. Nevertheless, the T98G cell line exhibited variability in the observed effect, as depicted in Figure 5.6 B. Conversely, the effect found in the UVWs cell line demonstrated a more linear relationship and was dependent on the concentration, as illustrated in Figure 5.6 B. The achieved IC_{50} were 0.55 μM in T98G cells and 0.38 μM in UVW cells. In contrast, ML120B exhibited only marginal inhibitory action and a limited reduction in cell viability in both T98G and

UVW cell lines, relative to the control (Figure 5.5 C and 5.6 C), potentially elucidating the predominant influence of IKKα over IKKβ in promoting cancer cell proliferation.

In order to enhance the reliability of future findings, it is imperative to conduct more tests utilising alternative SU compounds and diverse cell lines with variable genetic backgrounds. Moreover, a study conducted by Jo et al. (2015) revealed that the MTT assay lacks accuracy as a cytotoxicity test when applied to GBM cells. In 2015, a team of researchers from Korea, led by Hwa Yeon Jo, discovered that the MTT assay produced inaccurate outcomes when evaluating the impact of cytotoxicity on two distinct types of GBM cell lines (U87MG and U373MG) and two primary GBM cell types (GBL-13 and GBL15). The primary cause of this error is attributed by the researchers to an enhanced non-specific reduction of tetrazolium salt. In light of this, it is recommended by the researchers that additional tests such as western blotting, flowcytometric assay and siRNA transfection be conducted to ensure the reliability and validity of the data (Jo et al., 2015).

5.7.3 The effect of SU1433 and SU1644 on the clonogenic survival of T98G glioblastoma cells

The present investigation encompassed an additional series of studies involving clonogenic survival assays. These assays were conducted to evaluate the impact of SU1433 and SU1644 on the replicative potential of the T98G cell line, with the objective of determining the cellular survival rate. Treatment of cells with both compounds resulted in a decline in the survival of cells in a concentration-dependent manner, beginning at 0.1 μM and continuing up to the highest concentrations employed (Figure 5.7 and 5.8). SU1433 produced an IC_{50} of 1.07 μM while SU1644 was twice as potent with an IC_{50} of 0.65 μM (Figure 5.9). The results of this study provide evidence for the effectiveness of selective small molecule inhibitors that target IKKα in suppressing the proliferation of cancer cells and inducing a decrease in their survival capacity.

5.8 Conclusion

In conclusion, after a thorough examination of the effects of SU small molecule inhibitors of IKKα on various cellular features and phenotypic characteristics of *Glioblastoma multiforme*, it can be affirmed that the targeting of IKKα has promise as a possible opportunity for innovative cancer drug therapy.

Chapter 6

General Discussion

Chapter 6 General Discussion

6.1 The on-going impact of glioblastoma

According to Cancer Research UK (CRUK, 2015), in the United Kingdom in 2011, brain and central nervous system malignancies constituted 2.8% of all cancer diagnoses and 3.2% of all cancer-related fatalities. This category encompasses a wide range of cancer types affecting the brain and central nervous system. According to a report by CRUK in 2015, there was a consistent rise in the prevalence of brain cancer between 1979 and 2010. However, it is important to note that this upward trend can be partially ascribed to the progress made in diagnostic imaging techniques, which have facilitated more precise identification and evaluation of brain cancer cases (McKinney, 2004). An earlier comparison of the occurrence of all central nervous system (CNS) tumours identified over the periods of 1975-1979 and 1996-1999 demonstrated a consistent pattern, as reported by Hoffman et al. (2006) and Legler et al. (1999). The more recent findings of Torre et al. (2015) indicated that there is a higher mortality rate and occurrence of central nervous system (CNS) malignancies in both males and females residing in more developed regions compared to those in less developed regions. These results suggested that the adoption of a westernised lifestyle may potentially have a role in the aetiology of these types of cancers. Alternatively, the higher incidence of CNS malignancies in older patients may be a contributing factor to these figures, as these regions with more advanced healthcare systems tend to have populations with an increased life expectancy. Furthermore, it is plausible that underdeveloped areas may face a dearth of essential technology, resources, and clinical proficiency required for precise cancer diagnosis, hence potentially distorting the findings. According to data from the United Kingdom, it has been shown that a negligible proportion, namely less than one percent, of all diagnosed cases of brain, central nervous system (CNS), and intracranial tumours can be attributed to preventable causes (CRUK, 2015). According to Adamson et al. (2009), the only identified causes of the condition are high-dosage X-rays, commonly employed in radiotherapy, and chemicals utilised in the petrochemical industry. However, it is essential to note that these factors contribute to a minimal proportion of instances. According to McKinney (2004), there is a lack of empirical data supporting the notion that brain or central nervous system tumours are caused by mobile phones, smoking, alcohol consumption, or certain infections.

A limited set of genetic variables have been identified as potential causes of brain and central nervous system (CNS) cancers. Neurofibromatosis, tuberous sclerosis, Von-Hippel Landau disease, Li-Fraumeni syndrome, and Turcot syndrome, also referred to as mismatch repair cancer syndrome, are genetic disorders that have been linked to an elevated susceptibility to the development of brain and central nervous system tumours (Goodenberger and Jenkins, 2012). The occurrence of oncogenesis is attributed to the functional loss of tumour suppressor genes, heightened activity of oncogenes, or the failure to repair mutagenic DNA damage. Despite extensive research and clinical progress, the survival rates for brain and central nervous system (CNS) cancers have remained relatively low. Over the course of the previous four decades, the total 5-year survival rate has only increased from 7% to 19% (Cancer Research, 2014). The observed poor survival rate is concomitant with a rising occurrence and not surprisingly it highlights the need for better understanding of the cellular processes that underpin the initiation and development of disease. Furthermore, there is a clear need for new, effective therapeutics to treat GBM. A better understanding of disease characteristics may therefore identify suitable targets for drug intervention and enable development of alternative approaches to treating disease.

6.2 The molecular and cellular features of glioblastoma – a key role for IKKα-NF-κB signalling in driving tumour initiation and progression

Over the past two decades, various genetic alterations have been identified in association with *Glioblastoma multiforme* (GBM). As described previously, these alterations include the mutation and loss of tumour protein p53 (*TP53*), amplification and mutation of epidermal growth factor receptor (*EGFR*), mutation of cyclin-dependent kinase 4 (*CDK*) inhibitor p16/ADP-ribosylation factor (*INK4a/ARF*), mutation of phosphatase and tensin homolog (*PTEN*), and loss of heterozygosity (LOH) in chromosome 10p and 10q (Furnari et al., 2007). In a study conducted by Patil et al. (2015), the authors investigated the presence of telomerase reverse transcriptase (h*TERT*) promoter mutations and activation in six different *Glioblastoma multiforme* (GBM) cell lines, namely T98G, U87, U343, U373, LN 229, and LN18. The findings revealed that 55% of GBM tumour samples exhibited these mutations and activation, resulting in cell immortalisation through telomerase activation. Another mutation that was identified is in the *p53* gene, which plays a crucial role in various cancer cell attributes, including

apoptosis, differentiation, proliferation, and adhesion (Patil et al., 2015). According to Mollereau and Ma (2014), the presence of *NF1* mutations, a known suppressor of the *RAS* signalling pathway, was predominantly observed in U87 cells (Patil et al., 2015; Brennan et al., 2013). In addition, it was discovered by Patil et al. (2015) that none of the six cell lines examined exhibited mutations in mismatch repair (MMR) genes, specifically *MSH2, MSH3, MSH6, MLH1, PMS2, MSH4, MSH5, MLH3, PMS1,* and *PMS2L3*. Glioma, similar to numerous other malignancies, harbours a multitude of genes that are commonly mutated or overexpressed. Examples of proteins involved in DNA damage repair include *MGMT*, the transcription factor NF-κB with pro-inflammatory properties, the gene family *IDH* responsible for isocitrate dehydrogenase activity, and h*TERT*. Several transcription factors, including Myc, β-catenin, and NF-κB, have been identified as regulators of the *TERT* promoter due to the presence of specific binding sites for these factors on the *TERT* promoter (Greider et al., 2012). The transcription factor NF-κB is widely recognised for its role in regulating several biological processes, such as cancer (Shin et al., 2014). The study conducted by Ghosh et al. (2012) demonstrates that NF-κB signalling has the ability to exert an influence on *TERT* expression in an *in vitro* setting. Additionally, it has been observed by Yin et al. (2000) that the *TERT* promoter contains two potential NF-κB-binding motifs. These findings make the NF-κB pathway a potential target for cancer drug discovery.

Given the insights described above, this thesis considered the potential contribution of the IKKα-mediated non-canonical NF-κB signalling to the development of GBM. This pathway again is involved in regulating various biological processes, including inflammation, immunological response, cell growth, proliferation, apoptosis, and cell differentiation and development (Paul et al., 2018). The protein IKKα plays a critical role in the activation of various genes that control cellular processes associated with the development of cancer, including invasion, metastasis, proliferation, and resistance to chemotherapy (Park et al., 2005; Hirata et al., 2006; Fernandez-Majada et al., 2007a; Doppler et al., 2013; Mackay et al., 2018). The primary focus of numerous studies have been directed towards the targeting of IKKβ, which serves as the principal upstream activator of the canonical NF-κB pathway, in order to develop an effective strategy for anti-cancer treatment. Currently, in experimental settings, there are various inhibitors of IKKβ, including PS-1145, ML120B, and TPCA-1 (Gamble et al., 2012).

The efficacy of these inhibitors has been examined in various cancer cell lines and *in vivo* cancer models through experimental investigations. However, clinical use of these inhibitors has been associated with several severe side effects and toxicities in normal cells, as reported in studies conducted by Castro et al. (2003), Chariot (2009), Gamble et al. (2012b), and Nomura et al. (2016).

On the other hand, the inhibition of IKKα led to the suppression of the non-canonical NF-κB pathway, which relies on IKKα. This included inhibition of p100 phosphorylation, p52 processing and nuclear translocation of p52/RelB (see Chapter 4). Activation of this pathway is associated with a gradual and sustained NF-κB signal, in contrast to the canonical NF-κB pathway, which depends on IKKβ and elicits a quick and transient response. The observed distinction may potentially facilitate the application of selective inhibitors targeting IKKα in the context of disease intervention (Sun, 2012; Mackay et al., 2018).

This thesis examines the impact of IKKα inhibition on glioblastoma (GBM) cell lines. Specifically, two selective IKKα inhibitors were analysed to assess their effects on various parameters associated with proliferative outcomes, such as cell viability, cell growth, and the modulation of the protein-DNA binding measured using a sequence derived from the h*TERT* gene promoter.

Several factors need to be taken into account during the creation of selective IKKα inhibitors. First, because the N-terminal kinase domains of IKKα and IKKβ share more than 50% sequence homology, it is difficult to create inhibitors of the ATP binding site that are selective (Israel, 2010; Gamble et al., 2012b). Moreover, it is imperative that all newly synthesised molecules possess suitable physiochemical characteristics, such as solubility and resistance to degradation and clearance. The laboratory specialising in small molecule drug discovery at the University of Strathclyde supplied a collection of chemical substances (SU1433 and SU1644 are the ones examined in this study) to be evaluated in GBM cell lines (namely T98G and UVW).

The findings of this work provided novel evidence indicating that these inhibitors, in a cell-based setting, exhibited selectivity in inhibiting IKKα-dependent non-canonical NF-κB pathway signalling in GBM cells. This selectivity was determined by observing a decrease in the phosphorylation

of p100 and a reduction in the production and subsequent nuclear translocation of p52 NF-κB. Furthermore, it was revealed that these inhibitors possess the capability to halt the advancement of the cell viability, impede cell growth, and hinder the formation of colonies. This suggested that the SU compounds, SU1433 and SU1644, represent molecules with the desired pharmacological and pharmacodynamic characteristics that can be built upon, to then design-in the features of an appropriate pharmacokinetic profile for studies *in vivo* that may be translated to further pre-clinical studies in animal models of disease and longer term to patients, all with a focus of delivering a future viable therapeutic targeting IKKα.

6.3 Future work and advancing the approaches to treating GBM

Whilst a future goal is to deliver novel therapeutics targeting IKKα that may be applicable to intervening in GBM, there remains a number of experimental activities related to the work of this thesis that need to be addressed and consolidated:

1) As a link between p52-RelB nuclear translocation and p52-DNA-binding activity, further development of EMSA experiments (described in Chapter 5) to characterise DNA-binding activities generated in GBM cell lines with different genetic background h*TERT* promoter status. This can be achieved by completing the 'n' numbers of EMSA assay and the use of EMSA 'super-shift' assays, reliant on concentrated antibodies specific for NF-κB and ETS isoforms to identify clearly the distinct individual transcription factors that make up protein-DNA complexes capable of binding the C250T h*TERT* promoter specific for p52 engagement post-TWEAK-mediated activation of the non-canonical NF-κB pathway in GBM cell lines.
2) Generation of a linked/related C250T h*TERT*-based luciferase reporter assay to measure associated transcriptional activation. This would allow further investigations examining effectiveness of SUs against TWEAK-stimulated responses against WT, C250T and C228T-luciferase reporters', as positive and comparative experimental controls. This would also yield a rapid means of screening for inhibitors of the TWEAK-mediated non-canonical NF-κB activation in T98G cells (and any other relevant GBM and/or cancer cell type).

3) Consolidation of (n) numbers for the clonogenic assays as representative markers of cancer hallmarks. Additional cellular factors could be examined including investigating the impact on apoptosis, the expression of genes related to inflammation and cancer hallmarks, and crucial elements of the tumour microenvironment.
4) The consideration of combination studies *in vitro* using viability, clonogenic survival assays etc. as a precursor to potential studies *in vivo* e.g. TMZ (as Standard of care (SoC)) plus and minus SUs.
5) The pursuit of studies in animal models of GBM. Specifically through the employment of genetically engineered mouse models (GEMM) and/or primary xenografts.
6) Advancement to preclinical studies that are focused on local delivery of SU to the brain to address GBM *in situ* – using drug-eluting wafers and/or paintable polymers that are introduced into brain setting by surgical means (as described by Rahman et al., 2022).

In what manner might the discoveries elucidated in this thesis be extrapolated to other aspects within the realms of discovering novel cancer medications? The current consensus in the field acknowledges that the synergistic administration of many anti-cancer treatments surpasses the individual use of these drugs. This approach enhances the potency against cancerous cells while concurrently mitigating the likelihood of drug resistance development (Komarova and Boland, 2013). A study conducted by Avci et al., (2020) who examined the concurrent administration of NF-κB inhibitor (BAY 11-7082) and temozolomide (TMZ) resulted in that the concurrent administration of the treatments had a substantial impact on cell proliferation, resulting in a decrease in viability of *Glioblastoma multiforme* (GBM) cells. Additionally, the co-treatment was seen to suppress the NF-κB pathway and promote apoptosis. Furthermore, the study revealed that the concurrent administration of BAY 11-7082 and TMZ had a substantial impact on reducing the migratory behaviour of GBM cells generated from patients. This effect was achieved through the modulation of the actin cytoskeleton pathway (Avci et al., 2020).

One crucial approach for examining the possible impact of IKKα inhibitors is the utilisation of *in vivo* cancer models (as mentioned in point 4 above). Currently, there exists a total of 13 cell lines derived from human GBM cancer. These cell lines serve as valuable model systems due to their

numerous advantages. One notable advantage is the ability to readily manipulate signalling pathways through the use of pharmacological inhibitors and molecular tools like siRNA. However, it is important to note that there is a potential risk that certain models may lose key characteristics that resemble clinical diseases due to prolonged *in vitro* culture settings (Lee et al., 2006). Hence, the utilisation of animal models presents a viable approach for validating the prospective therapeutic effectiveness of IKKα inhibitors, specifically through the employment of genetically engineered mouse models (GEMM) and/or primary xenografts.

6.4 Concluding Summary

In summary, this study has demonstrated the potential application of novel proprietary selective inhibitors targeting the IKKα-mediated non-canonical NF-κB activation as a means to suppress survival, viability and replicative potential of GBM cancer cells. Further cell-based experimentation and investigations in murine models of GBM *in vivo* are required for further development towards potential clinical trials involving human subjects. However, whilst temozolomide remains the present standard of care for the treatment of aggressive GBM, perhaps in the future this may potentially be augmented by the incorporation of further refined SU molecules (with the appropriate pharmacokinetic characteristics) into future treatment strategies in the hope of gaining better patient outcomes.

Chapter Seven

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Chapter 7 References

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