Neuroblastoma: Clinical Outcomes and Experimental Studies on Cell Signalling

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master of Philosophy by

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Abstract

Neuroblastoma (NBL) is a solid childhood malignancy associated with survival rate of <50%. The most frequently diagnosed extracranial tumour in the paediatric age group, NBL is widely renowned for its heterogeneity as the tumour exhibits a spectrum of clinical behaviour from chemotherapy resistance to spontaneous regression. In this thesis, firstly, clinical outcomes for NBL are reviewed at a leading UK Children's Cancer and Leukaemia Group (Alder Hey Children's Hospital, Liverpool) in the context of evolving therapies for this enigmatic disease. In the second part of this work, building on previous knowledge linking the transcription factor nuclear factor kappa B (NF-κB) with the development of chemoresistance in NBL, this thesis highlights a series of laboratory experiments where a selection of novel pharmacological compounds are screened for their effects on the NF-κB pathway and their ability to induce cell death in NBL cells. A potential synergistic interaction between an NF-κB inhibitor and conventional chemotherapeutic agent is also investigated.

The past two decades have observed dramatic intensification of therapy for moderate to high-risk NBL. The clinical outcomes study has highlighted that 5-year survival for advanced stage 3 NBL patients has improved from 25 to 80% over two comparative treatment eras 1985-1994 and 1995-2005. Current opinion is widely divided in the international community over the role of aggressive surgery in high-risk NBL. This study has shown that although we observed a trend towards improved clinical outcomes by achieving complete resection, the benefits were marginal.

Experiments on NF-κB manipulation yielded the discovery that inhibition of NF-κB through various pharmacological compounds induced NBL cell death. A potentially synergistic interaction between cancer chemotherapy agent etoposide and an NF-κB inhibitor, H26(S), was observed. Further mechanistic investigations will be required to exploit the therapeutic potential herein described in this work.

Abbreviations

AF	Auranofin
AIDS	Acquired immune deficiency syndrome
CsA	Cyclosporin A
CsD	Cyclosporin D
CT	Computed tomography
DFOM	Deferoxamine mesylate
DiGG	1, 2-di-galloyl-glucose
DNA	Deoxyribonucleic acid
ECACC	European collection of cell cultures
EG	Epigallocatechin gallate
FasL	Fas ligand
FISH	Fluorescent in-situ hybridisation
FK506	Tacrolimus
H25(R)	2-amino-3-cyano-4, 6-diarylpyridine, right isomer
H26(S)	2-amino-3-cyano-4, 6-diarylpyridine, left isomer
Igκ	Immunoglobulin kappa light chain
IκBα	Inhibitor of NF-κB alpha
IKK	IκB kinase
IKK-ß	IκB kinase, ß sub-unit
INPC	International neuroblastoma pathology classification
INRG	International neuroblastoma risk group

INSS	International neuroblastoma staging system
LPS	Lipopolysaccharide
MDR-1	Multi-drug resistance 1 gene
MKI	Mitotic-karyorrhexis index
MRP	MDR-related protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBL	Neuroblastoma
NES	Nuclear export sequence
NF-κB	Nuclear factor kappa beta
NGF	Nerve growth factor
NLS	Nuclear localisation sequence
NSAID	Non-steroidal anti-inflammatory drug
PCD	Programmed cell death
PGG	1, 2, 3, 4, 6-penta-O-galloyl-ß-$_D$-glucose
Pgp	P-glycoprotein
PI	Propidium iodide
ROS	Reactive oxygen species
SAH	Sodium aurothiomalate hydrate
TAD	Transactivation domain
TNF-α	Tumour necrosis factor alpha
TLCK	N-α-p-tosyl-$_L$-lysine chloromethyl ketone
TPCK	N-Tosyl-$_L$-phenylalanine chloromethyl ketone
TRAIL	TNF-related apoptosis-inducing ligand
TRK	Tyrosine kinase
w/v	Weight/volume

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CHAPTER 1

Introduction

1.1 Neuroblastoma

Neuroblastoma is a neuroendocrine tumour arising from the sympathetic chain of the neural crest. It is the commonest extracranial solid tumour in childhood and each year approximately 1500 new cases are diagnosed in Europe, representing 7% of malignancies in patients younger than 15 years, or 28% of malignancies in infants in Europe and the US (Ries LAG 1999; Spix, Pastore et al. 2006). The incidence of neuroblastoma is highest in the first year of life and significantly drops thereafter, and cases are rare beyond the age of 10.

It is an enigmatic disease known for its remarkable spectrum of clinical behaviour. A subset of tumours will spontaneously regress whilst others are highly resistant

despite intensive multimodal therapy. Although there have been modest improvements in outcomes amongst certain subsets of patients, long-term survival in the high-risk group remains low at less than 40% (Matthay, Villablanca et al. 1999; De Bernardi, Nicolas et al. 2003).

1.1.1 Embryology

Neural development starts approximately at the beginning of the third week. As the neural folds develop, they turn inwards and form the neural tube. The lumen of the neural tube is lined by neuro-ectodermal cells, from which neurons develop. Detaching from the edges of the folds, these cells migrate laterally and become neural crest cells (Figure 1.1).

Neural crest cells will differentiate into several different lineages, e.g. melanocytes, sympathetic ganglia, enteric ganglion cells, sensory neurons, etc. (Figure 1.2). The first signal which triggers this differentiation are the bone morphogenetic proteins (BMPs) (Huber, Combs et al. 2002). Other key transcription factors which coordinate the direction of differentiation into sympathetic neurons are: *MASH1 (hASH1)*, *MYCN*, HIF1α, HuD, *PHOX2a*, *PHOX2b*, and p73 (a family member of p53) (Nakagawara 2004). Correct regulation of these genes are essential for normal differentiation into sympathetic neurons.

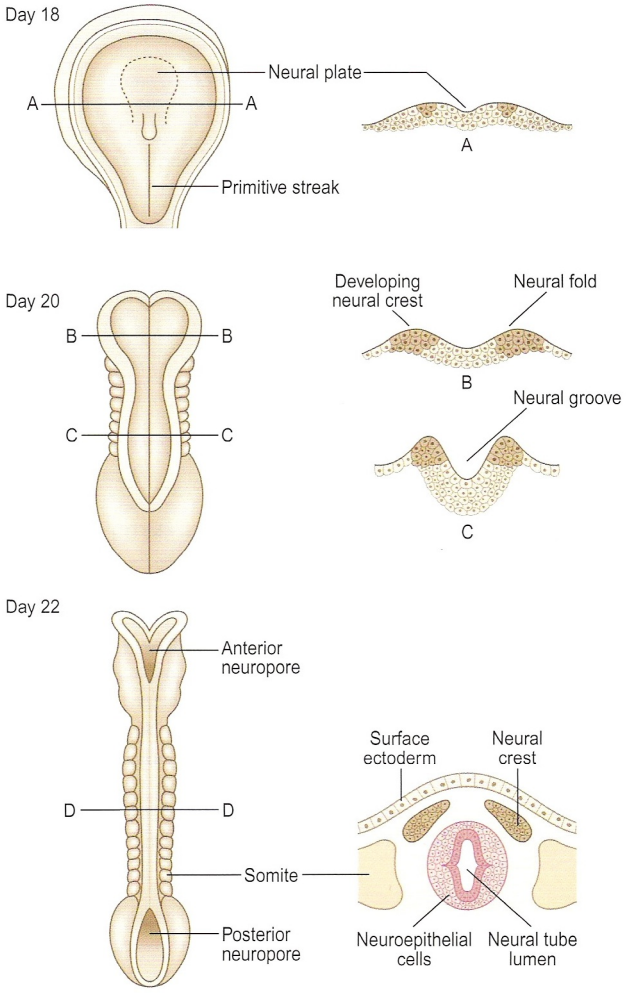


Figure 1.1: Neural crest development between days 18 and 22

By day 22, neural crest cells have migrated laterally and ready to differentiate to form various structures. Image taken from *Embryology* (Mitchell and Sharma 2005).

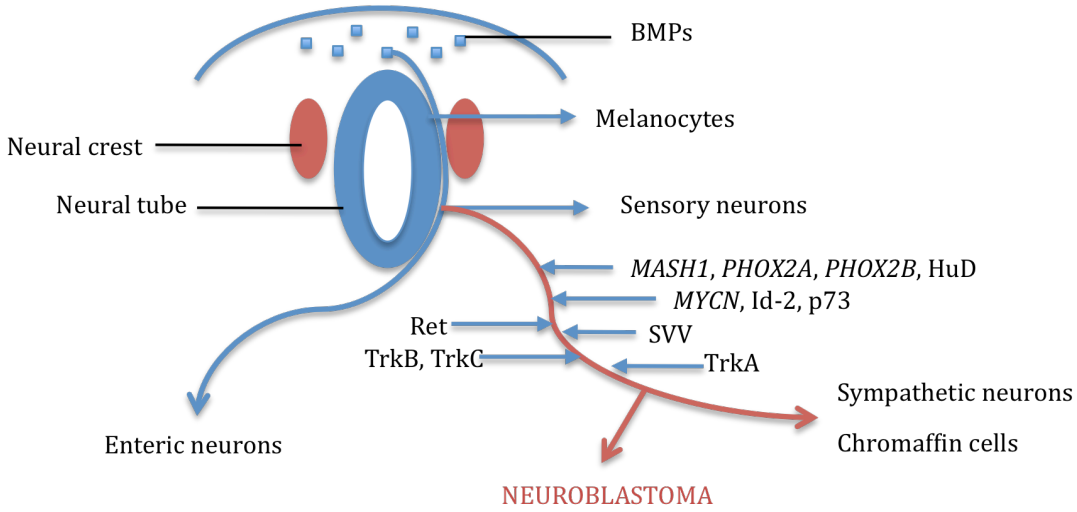


Figure 1.2: Pathways of neural crest differentiation

The bone morphogenetic proteins (BMPs) are involved in early stage of differentiation. Other important transcription factors listed above may also be involved in determination of cell fate. Often, upregulation or amplication of these genes are associated with aggressive neuroblastomas. Terminal differentiation of sympathetic neurons involved the tyrosine kinase receptors, e.g. Ret, TrkB, TrkC, and TrkA. Upregulation of these genes are found in favourable neuroblastomas (Nakagawara 2004). Image adapted from *Molecular and developmental biology of neuroblastoma* (Nakagawara 2005).

Development of neuroblastoma might be triggered by genetic events which lead to genomic aberrations such as amplification of *MYCN* gene, 1p and 11q chromosomal deletion, or unbalanced gain in chromosome 17q. Maris has postulated a genetic threshold for neuroblastoma development, in which malignant transformation might be a result of interactions between multiple common DNA variations or polymorphisms, i.e. excessive inheritance of "risk" variants increases an individual's susceptibility to developing the disease, or on the other hand, a mutation in *ALK* or *PHOX2b* gene results in highly penetrant risk allele that exceeds the threshold for malignant transformation (Figure 1.3) (Maris 2010).

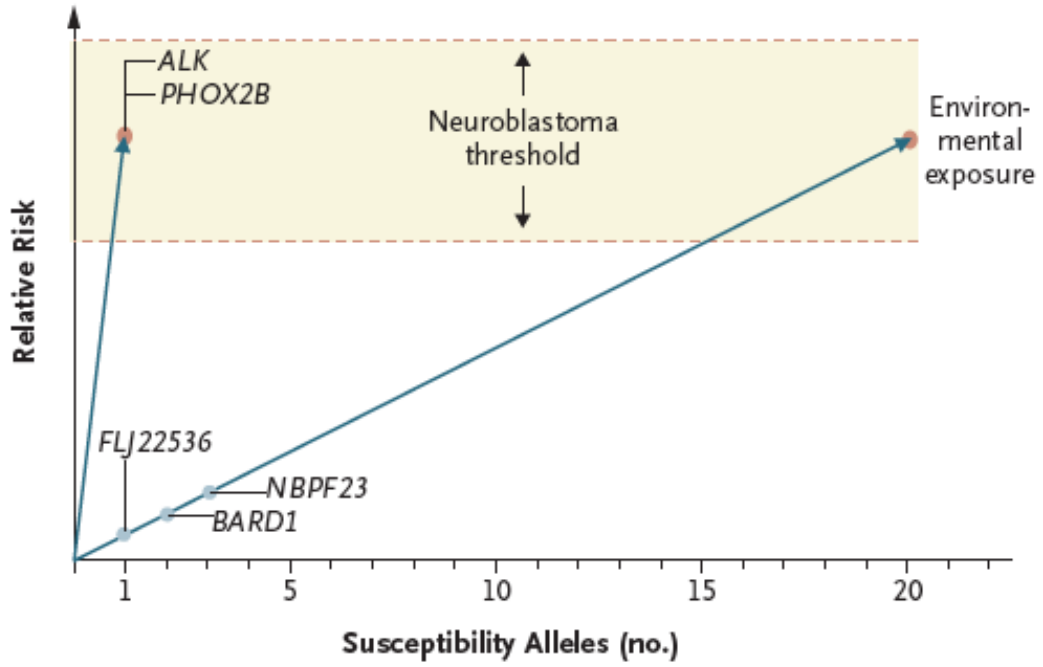


Figure 1.3: Genetic model of neuroblastoma susceptibility (Maris 2010)

1.1.2 Epidemiology and risk factors

Neuroblastoma is the most common malignancy amongst children under one year of age, with incidence rate approximately double that of leukaemia (Ries LAG 1999). Age plays a critical role in neuroblastoma as these perinatal tumours generally have a benign course and is associated with spontaneous regression (Evans, Gerson et al. 1976).

There have been several attempts to investigate the role of maternal lifestyles and reproductive history in the aetiology of neuroblastoma, although most of these studies lack statistical power to be able to conclude a definitive link. A possible association between maternal alcohol consumption and subsequent fetal alcohol syndrome has been described in several studies (Kinney, Faix et al. 1980; Schwartzbaum 1992). An elevated risk for the fertility drug Clomid was found amongst male offspring (Michalek, Buck et al. 1996; Olshan, Smith et al. 1999),

other maternal medications used during pregnancy which appeared to have an increased risk are amphetamines, antidepressants, phenytoin, diuretics, and antibiotics (Schwartzbaum 1992).

Approximately 1 to 2% of patients have a family history of neuroblastoma, suggesting a dominant pattern with incomplete penetrance (Kushner, Gilbert et al. 1986). Associations with other congenital abnormalities have been reported such as Hirschsprung's disease and congenital central hypoventilation syndrome (Ondine's curse) (Stovroff, Dykes et al. 1995), possibly linked by a shared mutation in the *PHOX2b* gene. Further connections with neurofibromatosis type 1 (von Recklinghausen's disease) and hypomelanosis of Ito have also been reported (Kushner, Hajdu et al. 1985; Oguma, Aihara et al. 1996).

1.1.3 Histopathology and genetic features

Neuroblastoma tumour cells are small round blue cells with hyperchromatic nuclei and a scant amount of cytoplasm (La Quaglia and Rutigliano 2008). Neuritic processes and Homer-Wright pseudo rosettes are often seen. Other small round blue cell tumours which must be differentiated from neuroblastoma include Ewing's sarcoma, lymphoma, and rhabdomyosarcoma. Within one tumour specimen, different stages of differentiation of neuroblastoma may be found, from undifferentiated neuroblastoma to mature ganglioneuroma.

The new International Neuroblastoma Pathology Classification (INPC) system (Table 1.1) has been developed from the initial Shimada classification of neuroblastoma (Shimada, Chatten et al. 1984), taking into account the age of the

patient, the mitotic-karyorrhexis index (MKI), the amount of Schwann cells, and degree of cellular differentiation (Shimada, Umehara et al. 2001). The INPC system has been proven a useful prognostic indicator in recent years (Lau 2002; Sano, Bonadio et al. 2006).

Table 1.1: Prognostic evaluation of neuroblastoma according to the INPC system (Shimada, Umehara et al. 2001)

Age	Pathology	Prognostic group
<1.5 years	Poorly differentiated or differentiating Low or intermediate MKI	Favourable
1.5 – 5 years	Differentiating Low MKI tumour	Favourable
<1.5 years	Undifferentiated High MKI	Unfavourable
1.5 – 5 years	Undifferentiated or poorly differentiated Intermediate or high MKI	Unfavourable
>5 years	All tumours	Unfavourable

The association between certain biological features of neuroblastoma tumours and disease progression has been extensively studied. The discovery of *MYCN* oncogene, found on chromosome 2p, has led to its development to become the most important biological prognostic factor in neuroblastoma (Brodeur, Pritchard et al. 1993). It is amplified in 5-10% of infants and in 20-30% of childhood and adolescent cases and

it is strongly correlated to rapid disease progression and poor outcome in patients, regardless of age and stage (Conte, Parodi et al. 2006; Kaneko, Kobayashi et al. 2006). It is postulated that overexpression of *MYCN* activates angiogenesis pathways and thereby increasing tumour growth and metastasis (Benard 1995). *MYCN* level can be detected by fluorescent in-situ hybridisation (FISH), where 10 or more copy numbers detected is generally accepted as genomic amplification (La Quaglia and Rutigliano 2008). Other methods to detect *MYCN* include polymerase chain reaction (PCR), Southern blot, and immunohistochemistry.

The ratio of DNA amount found within the nucleus of a cell compared to the expected amount (DNA index/ploidy) can be measured by flow cytometry or cytogenic analysis. Hyperdiploidy (DNA index >1) has been associated with better prognosis, particularly in infant population, and conversely diploid tumour is associated with aggressive tumour behaviour and poorer outcome (Look, Hayes et al. 1984).

Nerve growth factor (NGF) and its receptor tyrosine kinase (TRK) have also been implicated in neuroblastoma disease progression. Three subtypes of TRK, a transmembrane protein, are present: TRK-A, TRK-B, and TRK-C. Expression of TRK-A and TRK-C are associated with favourable prognosis and no *MYCN* amplification (Brodeur, Nakagawara et al. 1997; Yamashiro, Liu et al. 1997). Conversely, lack of TRK-A is seen in *MYCN* amplified tumours and carries poor prognosis. The presence of TRK-B is associated with chromosomal aberrations such as gain of 17q and loss of heterozygosity for 14q, *MYCN* amplification, and overall poor outcome (Brodeur, Nakagawara et al. 1997).

Several chromosomal aberrations found in neuroblastoma tumours are associated with poor prognosis, including deletion of chromosome 1p, which occurs in 25-35% of cases, and gain on chromosome 17q, a region which contains the antiapoptotic gene Survivin (Bown, Lastowska et al. 2001; Lastowska, Cotterill et al. 2002; White, Thompson et al. 2005). Other chromosomal abnormalities linked with poor prognosis include loss of heterozygosity for 14q, allelic loss of 11q, bcl-e overexpression, ras expression, ret expression, and telomerase activity (Guo, White et al. 1999; La Quaglia and Rutigliano 2008).

Some of these biological abnormalities have been incorporated in the most recent neuroblastoma staging system to enable a more accurate risk assessment and treatment strategy (Cohn, Pearson et al. 2009).

1.1.4 Clinical presentation and diagnosis

1.1.4.1 Clinical presentation

Neuroblastoma tumours can develop anywhere along the sympathetic ganglia, giving rise to a spectrum of clinical presentations depending on tumour location. Most primary tumours occur in the abdomen, in particular the adrenal medulla (40%), other common sites are the neck, chest, and pelvis (Ries LAG 1999).

Abdominal lesions might be detected incidentally, i.e. prenatal ultrasound, but most remain asymptomatic until significant enlargement of the tumour has occurred. These patients commonly present with abdominal pain and distension, and

examination will reveal palpable abdominal mass.

Thoracic tumours might be detected incidentally, i.e. chest radiographs, or patients can present with persistent cough or difficulty breathing due to compression by tumour mass. Cervical masses are often associated with Horner's syndrome (Figure 1.4) (Cardesa-Salzmann, Mora-Graupera et al. 2004; Mahoney, Liu et al. 2006; Zafeiriou, Economou et al. 2006). Paravertebral tumours (5-15% patients) can invade the spinal canal causing symptoms of spinal cord compression including motor weakness, sphincter dysfunction, and sensory deficits (De Bernardi, Balwierz et al. 2005; Gunes, Uysal et al. 2009).

Figure 1.4: Horner's syndrome in a child with neuroblastoma

Source: Prof P D Losty. Photograph reproduced with parental permission.

Paraneoplastic syndromes, although rare, can be seen at initial presentation. Vasoactive intestinal peptide secretion by neuroblastoma cells leads to intractable watery diarrhoea, weight loss, and metabolic disorders. For these patients, surgery remains the first-line treatment (Bourdeaut, de Carli et al. 2009). Opsoclonus-myoclonus syndrome (OMS), characterised by rapid eye movements and ataxia, affects 2-3% of patients with neuroblastoma. It occurs almost exclusively to patients in the low-risk group, and survival is nearly 100%. Nevertheless, many patients will have persistent neurological and developmental deficits (Rothenberg, Berdon et al. 2009). Occasionally patients develop hypertension due to compromised renal vasculature or excess production of catecholamines. It is often severe and requires specific antihypertensive therapy, but resolves following tumour treatment, i.e. chemotherapy and/or surgical resection (Wolff, Bauch et al. 1993; Shinohara, Shitara et al. 2004; Madre, Orbach et al. 2006).

Approximately half of patients present with metastatic disease. Widespread bone and bone marrow metastases would cause bone pain and limping. Orbital metastases commonly present with periorbital ecchymosis (panda eyes) and proptosis (Ahmed, Goel et al. 2006). Infiltration of the bone marrow by tumour cells can cause symptoms of bone marrow failure.

1.1.4.2 Diagnosis

A combination of radiological (including nuclear medicine), biochemical, histopathological, and molecular biology investigations are used to aid diagnosis.

1.1.4.2.1 Radiology

Computed tomography (CT) is the preferred method for determining tumour consistency, location, and metastasis, in particular with lesions in the abdomen, pelvis, or mediastinum (Figure 1.5). Magnetic resonance imaging (MRI) is better for paravertebral lesions particularly in assessing spinal canal extension of tumour in patients presenting with cord compression. Specific assessment for metastatic disease is performed using ^{99}mTc-diphosphonate scintigraphy (bone scan) or metaiodobenzylguanidine (MIBG) scintigraphy using ^{123}I or ^{131}I isotope.

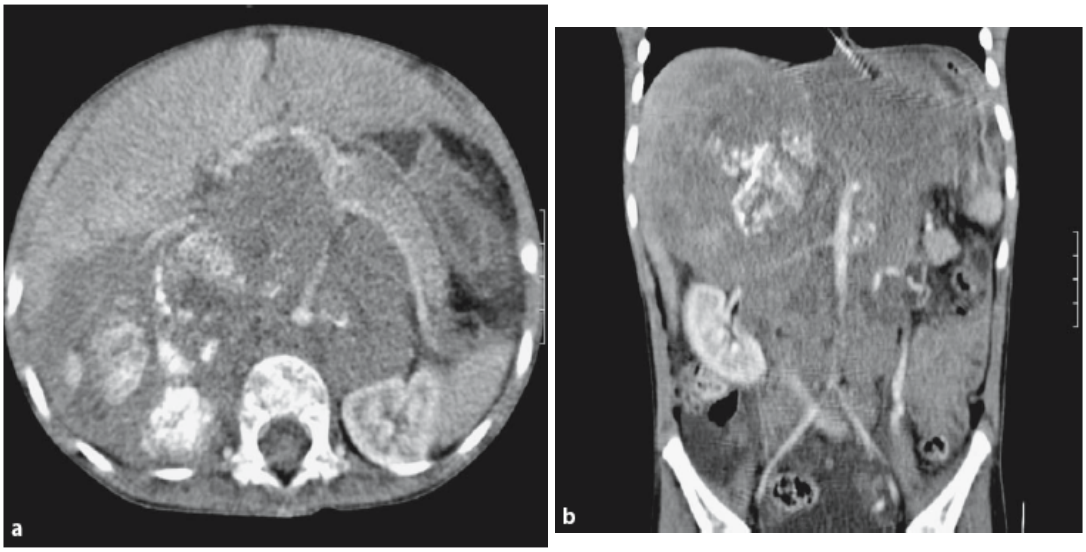


Figure 1.5: CT of an infant with neuroblastoma

A: The tumour has extended throughout the retroperitoneum, classically engulfing the blood vessels and pushed them anteriorly. B: Coronal view, the tumour appeared to have arisen from the right suprarenal area and contained typical areas of calcification. The renal artery was stretched by the mass effect of the tumour. Image taken from *The Surgery of Childhood Tumors (Hiorns 2008)*.

1.1.4.2.2 Biochemistry

Elevated levels of catecholamines and their metabolites including vanillylmandelic acid (VMA), homovanillic acid (HVA), dopamine, adrenaline, and noradrenaline are found in the plasma or urine of many neuroblastoma patients. In the 1980s, screening

programmes based on urinary catecholamines were designed in Japan. However, despite its apparent short-term success (Sawada, Kidowaki et al. 1984; Nishi, Miyake et al. 1987), prospective clinical trials in Germany and North America have shown that they do not improve overall survival as almost all tumours detected by screening had favourable biological features (Woods, Gao et al. 2002; Schilling, Spix et al. 2003), and the screening programme is no longer practised nowadays. Screening programmes for neuroblastoma are discussed further in Section 1.1.6.

Elevated levels of serum ferritin (Silber, Evans et al. 1991), lactate dehydrogenase (Joshi, Cantor et al. 1993), and neuron-specific enolase (NSE) (Berthold, Engelhardt-Fahrner et al. 1991), although non-specific, are often seen in high-risk patients and associated with poor prognosis.

1.1.4.2.3 Histopathology

Histological assessment of tumour biopsy is crucial in risk stratification and adequate tissue biopsy at the time of diagnosis is strongly encouraged. Results from Canada have shown that a significantly higher proportion of patients who underwent image-guided needle biopsy had insufficient tissue for complete histological and molecular assessment despite similar rate of procedural complications between needle and open biopsy (Gupta, Kumar et al. 2006). The current INPC system is used for tumour biopsy classification to enable evaluation of clinical behaviour and prognosis (Table 1.1).

Bone marrow aspirate and/or biopsy looking at the presence of tumour cells should be investigated at the time of diagnosis, as well as during therapy to assess tumour responsiveness to treatment.

1.1.4.2.4 Biological markers

Routine diagnostic procedure should now include investigation of biological markers such as *MYCN* amplification, DNA ploidy, and chromosomal abnormalities which are correlated with clinical behaviour and disease progression of neuroblastoma tumours (Section 1.1.3).

1.1.5 Staging

Staging is crucial in predicting outcome as well as planning treatment modality. Standard methods of tumour staging such as the TNM method is largely not applicable in neuroblastoma due to its heterogeneity and in 1971, Evans and colleagues (Evans, D'Angio et al. 1971) proposed a practical staging system which takes into account tumour site, lymph node involvement, distant metastases, as well as bone marrow infiltration (Table 1.2). It also recognises the special IVs group of disease in which prognosis is good despite widespread metastases in liver, skin, or bone marrow.

Table 1.2: Evans staging system for neuroblastoma (Evans, D'Angio et al. 1971)

Stage	Description
I	Tumour confined to the organ or structure of origin
II	Tumour extends beyond organ of origin but not crossing the midline. Ipsilateral lymph nodes may be involved.
III	Tumour extends beyond the midline. Bilateral lymph nodes may be involved.
IV	Remote disease involving skeleton, organs, soft tissues, or distant lymph nodes.
IVs	Patients who would otherwise be stage I or II but with remote disease confined only to the liver, skin, or bone marrow (without bone metastasis).

In an effort to make results comparable throughout the world, the International Neuroblastoma Staging System (INSS) (Table 1.3) was established in 1988 and subsequently revised in 1993 (Brodeur, Pritchard et al. 1993), taking into account tumour resectability, which was deliberately omitted in the Evans staging system.

Table 1.3: INSS staging system (Brodeur, Pritchard et al. 1993)

Stage	Description
1	Localised tumour with complete gross excision, ipsilateral lymph nodes negative for tumour.
2a	Localised tumour with incomplete gross excision, ipsilateral lymph nodes negative for tumour.
2b	Localised tumour with or without complete gross excision, ipsilateral lymph nodes positive for tumour, contralateral lymph nodes negative.
3	Unresectable unilateral tumour crossing the midline with or without regional lymph node involvement; or localised unilateral tumour with contralateral lymph node involvement; or midline tumour with bilateral extension by infiltration or by lymph node involvement.
4	Dissemination to distant lymph nodes, bone, bone marrow, liver, skin, or other organs.
4S	Localised primary tumour in infants younger than 1 year (stage I, IIA, or IIB) with dissemination limited to liver, skin, or bone marrow.

Nevertheless, as surgical approaches differ between one institution and another, a working group representing the major paediatric groups around the world met in 2005 to develop the International Neuroblastoma Risk Group (INRG) classification system, which takes into account radiological as well as molecular characteristics of the tumour (Table 1.4) (Ambros, Ambros et al. 2009; Cohn, Pearson et al. 2009).

Table 1.4: INRG Pre-treatment classification system (Cohn, Pearson et al. 2009)

INRG Stage	Age (months)	Histologic Category	Grade of Tumor Differentiation	*MYCN*	11q Aberration	Ploidy	Pretreatment Risk Group
L1/L2		GN maturing; GNB intermixed					A Very low
L1		Any, except GN maturing or GNB intermixed		NA			B Very low
				Amp			K High
L2	< 18	Any, except GN maturing or GNB intermixed		NA	No		D Low
					Yes		G Intermediate
	≥ 18	GNB nodular; neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermediate
			Poorly differentiated or undifferentiated	NA			
				Amp			N High
M	< 18			NA		Hyperdiploid	F Low
	< 12			NA		Diploid	I Intermediate
	12 to < 18			NA		Diploid	J Intermediate
	< 18			Amp			O High
	≥ 18						P High
MS	< 18			NA	No		C Very low
					Yes		Q High
				Amp			R High

Ploidy: diploid (DNA index = 1.0); hyperdiploid (DNA index > 1.0 and includes near-triploid and near-tetraploid tumors). Risk: very low risk (5-year EFS > 85%); low risk (5-year EFS > 75% to <= 85%); intermediate risk (5-year EFS >= 50% to <= 75%); high risk (5-year EFS < 50%). Histology: GN, ganglioneuroma; GNB, ganglioneuroblastoma. *MYCN*: Amp, amplified; NA, not amplified. Stage: L1, localized tumor confined to one body compartment and with absence of image-defined risk factors (IDRFs); L2, locoregional tumor with presence of one or more IDRFs; M, distant metastatic disease (except stage MS); MS, metastatic disease confined to skin, liver and/or bone marrow in children < 18 months of age; EFS, event-free survival.

1.1.6 Spontaneous regression and screening programme

A well-known feature of neuroblastoma is the ability of a small proportion of cases to undergo complete regression in the absence of therapeutic intervention (Evans, Gerson et al. 1976). This phenomenon is usually associated with infants in stage 4S and rarely occurs in patients over one year of age (D'Angio, Evans et al. 1971), thereby raising the possibility that epigenetic regulations and development of sympathetic neurons might be attributable to neuroblastoma regression (Nakagawara 2005). During the perinatal period, a physiological neuronal programmed cell death occurs which results in a massive death of sympathetic neurons. This death mechanism has been observed in infant neuroblastomas, and correlated with their induction of spontaneous regression (Nakagawara 1998).

Neuroblastoma possesses unique biochemical characteristics as the tumour is involved in catecholamine synthesis and metabolisms. This allows sensitive and convenient markers for neuroblastoma as two metabolites, homovanillic acid (HVA), the main metabolite of dopamine, and vanillylmandelic acid (VMA), the main metabolite of adrenaline and noradrenaline, are excreted in excess in a patient’s urine (Woods, Gao et al. 2002).

The idea that childhood cancer can be detected pre-clinically by screening, coupled with the significantly better outcome for younger children and patients with localised disease, led researchers in Japan, Europe, and North America to conduct extensive screening programmes based on urinary HVA and VMA analysis (Sawada, Kidowaki et al. 1984; Nishi, Miyake et al. 1987; Schilling, Spix et al. 2002; Woods, Gao et al. 2002).

However, these studies reported marked increase in incidence of the disease with no reduction in its mortality due to the vast majority of tumours detected by screening have favourable clinical and biological features. It was demonstrated that screening did not reduce the prevalence of advanced disease over one year of age or the overall death rate (Schilling, Spix et al. 2003; Spix, Michaelis et al. 2003). Moreover, screening programmes were associated with long-term psychological anxiety among parents whose infants were referred to cancer centres because of elevated catecholamines but were found not to have neuroblastoma upon more thorough investigation (Woods 2005). Screening programme was eventually abandoned by 2004.

1.1.7 Treatment

Localised tumours with favourable biology can be successfully treated with surgical resection alone. In a subset of cases, the tumours will spontaneously regress; they can be safely observed without needing any treatment. If residual tumour remains after surgical resection, assessment of biological parameters becomes important in predicting its behaviour as adjuvant therapy may not be indicated in cases with favourable biological features. The treatment of localised tumours with unfavourable biological features (*MYCN* amplification) remains controversial, some may need intensive adjuvant therapy but conversely a subset of cases could achieve long-term remission with surgery alone (Perez, Matthay et al. 2000).

Stage 3 tumours with favourable biology have been treated with adjuvant therapy to aid subsequent surgical resection. Nevertheless, those with unfavourable biological

features often required intensive multimodal therapy.

In metastatic disease (stage 4), initial surgery is aimed at obtaining adequate tissue biopsy for analysis. Chemotherapy aiming for tumour downstaging follows and in many cases allows complete surgical resection. High-dose chemotherapy with autologous stem cell rescue has been shown to improve event-free survival among high-risk cases, and the use of differentiating agents such as 13-cis-retinoic acid has been shown beneficial too (Matthay, Villablanca et al. 1999).

Most patients with stage 4S disease fall into the low-risk group, however a small group will have unfavourable biology and they often present with rapidly progressive tumour similar to the classic stage 4 disease (Nakagawara, Sasazuki et al. 1990). Those who present before the age of 2 months are also more susceptible to respiratory compromise due to rapid progression of hepatomegaly.

Surgical resection of neuroblastoma is technically challenging and due to its infiltrative nature, it is not always possible to acquire a microscopically negative resection margins, and therefore the term “gross total resection” is often used in literature (Figure 1.6). Complication is often associated with neuroblastoma resection, a Japanese report has discovered up to 15% occurrence of renal infarction and atrophy as a result of aggressive surgery for abdominal neuroblastoma (Kubota, Yagi et al. 2004). Kiely reported incidences of aortic injuries following a subadventitial approach of resection (Kiely 2007).

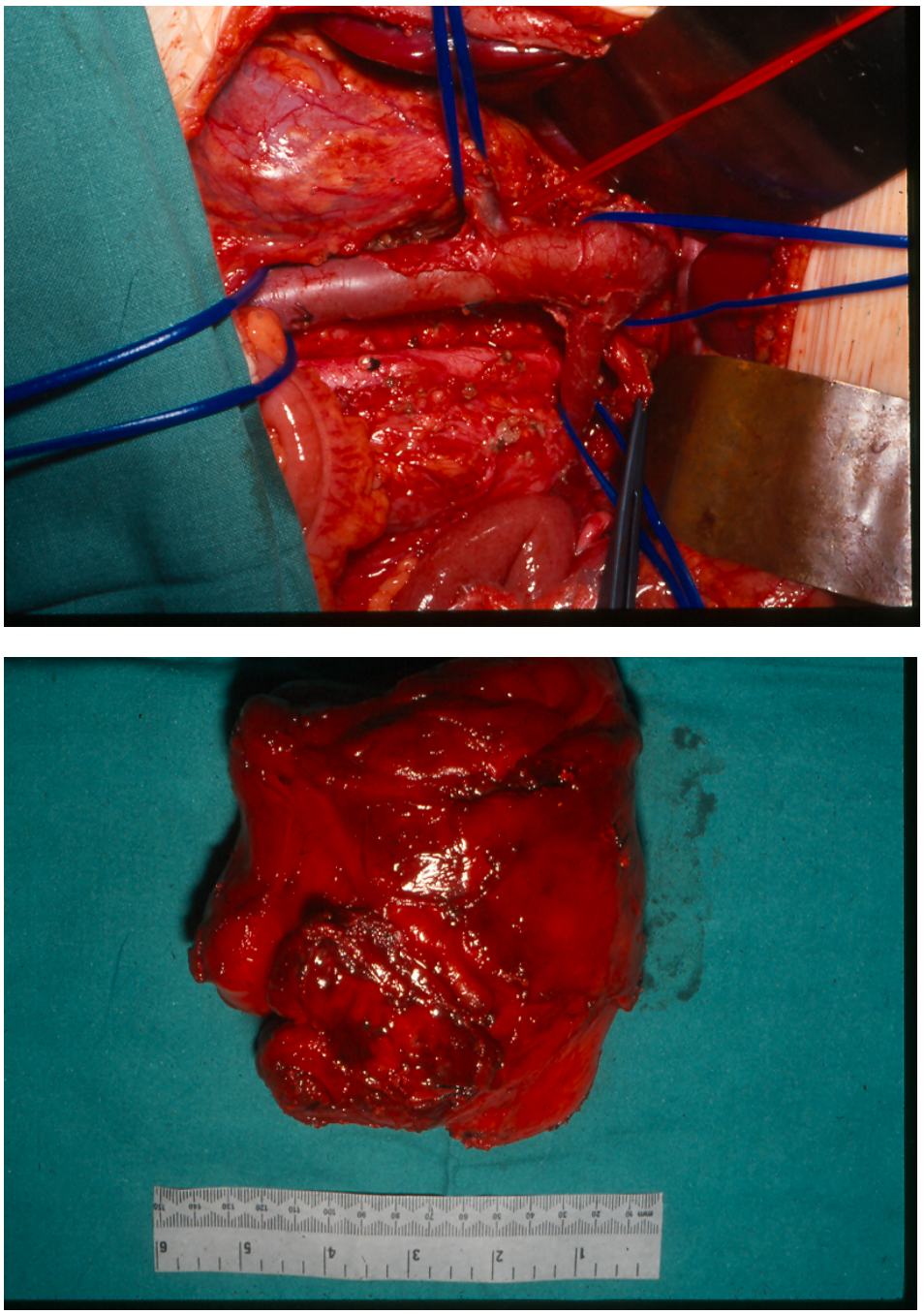


Figure 1.6: Surgical resection of retroperitoneal neuroblastoma

Source: Prof P D Losty. Photograph reproduced with permission from patient.

Although surgery remains the mainstay treatment for localised tumour, current opinion is divided over the role of aggressive surgery for stage 3 and 4 neuroblastoma. Several studies demonstrated that complete tumour resection is associated with significantly improved outcome (La Quaglia 2001; Tsuchida and Kaneko 2002), however others have shown that the radicality of surgery did not change the outcome (Losty, Quinn et al. 1993; Castel, Tovar et al. 2002; von Schweinitz, Hero et al. 2002).

1.1.8 Drug resistance

Despite intensive multi-modal treatment, the majority of high-risk neuroblastoma patients eventually progresses and to date, no salvage treatment regimen has been found curative (Maris 2010). The underlying mechanisms of tumour resistance are still poorly understood, however several ideas have been postulated including altered expression of drug resistance genes and the presence of cancer 'stem cells'.

The cancer stem cell hypothesis suggests the presence of stem cells which may escape cytotoxic agents through slower proliferation rate (Dick 2008). As a developmental malignancy, it is likely that the cell of origin is a stem cell, characterised by its self-renewal multipotent properties. Several markers for neuroblastoma stem cells have been developed including CD-133 and c-kit, Hnk1, and nestin (Tucker, Delarue et al. 1988; Thomas, Messam et al. 2004; Walton, Kattan et al. 2004). The presence of stem cells in neuroblastoma tumours is associated with worse prognosis (Ross and Spengler 2007).

Nevertheless, it appears that most high-risk neuroblastoma tumours were initially sensitive to chemotherapy at diagnosis, whereas relapsed tumours are highly resistant. A likely explanation for this phenomenon is alterations in expression of drug resistance genes, most notably involving the presence of p-glycoprotein (Pgp) and increased expression of its multi-drug resistance gene (MDR-1) and MDR-related protein (MRP) (Norris, Bordow et al. 1996; Haber, Bordow et al. 1999; Blanc, Goldschneider et al. 2003). Other mechanisms include mutations in the p53 gene affecting downstream effectors such as Bax and Caspase-8 resulting in decreased apoptotic activity (Keshelava, Zuo et al. 2001).

Despite the introduction of newer agents and increasing number of patients whose survival after relapse is prolonged, this remains a sensitive and delicate issue and it is hoped that recent advances in the understanding of molecular biology of high-risk neuroblastoma will eventually lead to the discovery of novel therapeutic targets. One possible target is the transcription factor, nuclear factor kappa B (NF-κB).

1.2 Nuclear Factor Kappa B (NF-κB)

Nuclear Factor Kappa B (NF-κB) is a family of dimeric protein complexes which regulate DNA transcription. Found in virtually all animal cell types, NF-κB is implicated in cellular responses to stress, immune and inflammatory processes, and regulation of cell cycle, differentiation, and death. First described as a B-cell factor which interacts with the immunoglobulin kappa (Igκ) enhancer (Sen and Baltimore 1986), it is now known that NF-κB plays a very important role in the control of life and death, and is one of the major molecular targets in cancer research.

1.2.1 Structure and family members of NF-κB

The NF-κB family is composed of five proteins, all characterised by the presence of amino-terminal sequence known as the Rel homology domain (RHD) (Gilmore 1999). The five family members are: p50/p105 (NF-κB1), p52/p100 (NF-κB2), c-Rel, RelB, and p65 (RelA) (Ghosh, May et al. 1998). They can be further classified into two groups based on the C-terminal sequences in the RH domain. The first group (p105 and p100) has long C-terminal domain containing the inhibitor ankyrin. Through limited proteolysis or arrested translation, they become shorter, active DNA-binding proteins p50 and p52 respectively (Gilmore 1999). The transcriptionally active second group (c-Rel, RelB, and p65 (RelA)) contains variable transactivation domain (TAD) at the C-terminal (Siebenlist, Franzoso et al. 1994).

NF-κB binds to 10 base pair DNA sites (5'-GGGRNYYYCC-3'), also called κB elements or κB sites, as either homo- or heterodimers (Chen and Ghosh 1999). NF-κB generally refers to the p50-p65 (RelA) heterodimer, one of the major Rel complexes in most cells.

1.2.2 Regulation of NF-κB

In unstimulated cells, NF-κB is found in the cytoplasm in a latent, inactive form, bound to its inhibitor (IκB). Several IκB proteins have been identified (IκBα, IκBß, IκBε, IκBγ, IκBζ, and Bcl-3), all of which have different affinities for individual Rel/NF-κB complexes (Karin 1999). The most prevalent and best-studied IκB

protein is IκBα. The IκB proteins bind to NF-κB dimers and block the function of their nuclear localisation sequence (NLS), thereby retaining the complex in the cytoplasm. Stimulation by bacterial lipopolysaccharide (LPS) or proinflammatory cytokines tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) will activate a high molecular weight complex containing a serine specific IκB kinase (IKK). Activated IKK phosphorylates IκB proteins at two serine residues located within the NH_2-terminal region (Ghosh, May et al. 1998). Phosphorylation of the IκB proteins results in rapid ubiquitination and degradation by the 26S proteasome, which subsequently disrupts the NF-κB-IκB complex. This results in the liberation of NF-κB, allowing it to translocate to the nucleus and activate expression of NF-κB target genes, one of which is IκBα. IκBα contains both NLS and nuclear export sequence (NES) and as such, newly synthesised IκBα entering the nucleus can abrogate this signal and remove NF-κB dimers from DNA to the cytoplasm (Figure 1.7) (Sachdev, Hoffmann et al. 1998).

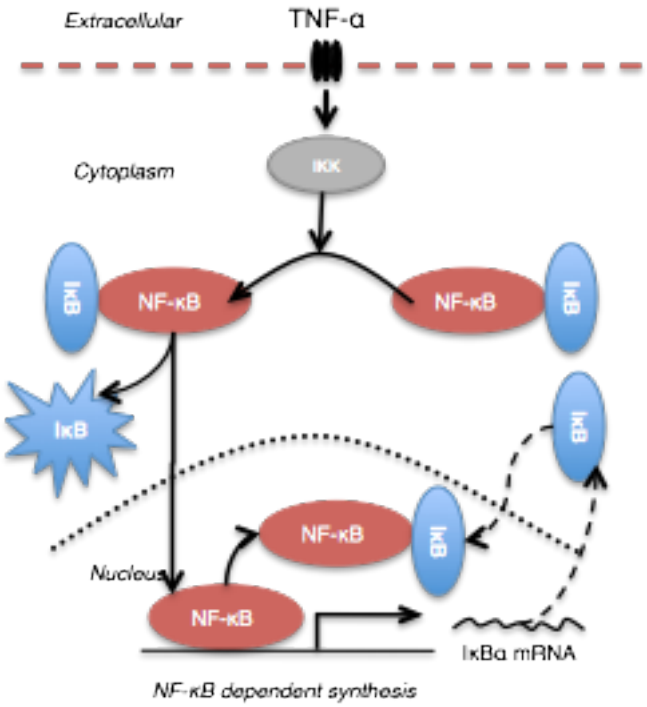


Figure 1.7: Summary of NF-κB signalling pathway

1.2.3 NF-κB activity and oncogenesis

There is compelling evidence regarding NF-κB involvement in oncogenesis. It was first suggested following observation that v-Rel, a viral protein derived from the cellular gene c-Rel, caused aggressive lymphomas in infected young chickens (Gilmore 1999). Chromosomal translocation of the Bcl-3 gene was found in a subset of B-cell chronic lymphocytic leukaemias, this gene is also overexpressed in some B-cell neoplasms (McKeithan, Takimoto et al. 1997). IκBα mutations resulting in constitutively active NF-κB were observed in Hodgkin's lymphoma, suggesting a tumour suppressor role for IκBα (Cabannes, Khan et al. 1999). Constitutively active NF-κB was also noted in other cancer types including liver, skin, breast, renal, and prostate cancers (Sovak, Bellas et al. 1997; Palayoor, Youmell et al. 1999; Tai, Tsai et al. 2000; Oya, Ohtsubo et al. 2001; Dhawan, Singh et al. 2002).

In many cases, NF-κB promotes up-regulation of several anti-apoptotic genes such as Bcl-2 homologues A1/Bfl-1 and Bcl-xL, and inhibition of NF-κB sensitises many tumour cells to death-inducing stimuli, for example by chemotherapeutic agents (Baldwin 2001). Moreover, it has been observed that NF-κB can antagonise p53, a key regulator of apoptosis, possibly through cross-competition for transcriptional co-activators p300/CBP (Webster and Perkins 1999).

In view of the fact that oncogenesis is closely associated with anti-apoptotic mechanisms, the following sections will discuss the mechanism of programmed cell death and how NF-κB might influence this process.

1.2.3.1 Apoptosis

Apoptosis, as opposed to necrosis, is a distinct mechanism of cell death that is tightly regulated with the aim to remove aged, damaged, or unwanted cells. The term *apoptosis* (Greek: *'falling off'* from *apo* – 'from' + *ptosis* - 'falling, a fall') was first coined by Kerr et al. in 1972 to describe a programmed mechanism of cell deletion involving discrete structural changes, observed in various tissues and cell types (Kerr, Wyllie et al. 1972). Regulatory mechanisms controlling apoptosis are fundamental for normal homeostasis and disturbances in signalling cascades which regulate apoptosis can result in a wide variety of diseases (Rudin and Thompson 1997). Failure of apoptosis induction is found in autoimmune diseases, systemic lupus erythematosus, and cancer. In contrast, increased apoptosis is observed in neurodegenerative disorders, acquired immune deficiency syndrome (AIDS), and some blood disorders characterised by low numbers of peripheral cells. In AIDS,

HIV virus proteins may activate CD4 on uninfected T-helper lymphocytes to induce apoptosis, which results in immunodepletion (Goepel 2004).

Two distinguished apoptotic pathways, both of which lead to caspase activation, have been identified. The extrinsic pathway is initiated by ligation of transmembrane death receptors (e.g. CD95) which activates caspase-8 and -10, the activator caspases. These will in turn cleave and activate the effector caspase-3 and -7. In the intrinsic pathway, disruption of mitochondrial membrane releases mitochondrial proteins such as cytochrome c, which leads to the activation of caspase-9, thereby initiating the apoptotic caspase cascade (Green 2000). Cells will undergo stages of morphological changes, starting from nuclear shrinking (pyknosis) and fragmentation (karyorrhexis). The cells shrink and dissociate from surrounding cells, while retaining the organelles and an intact plasma membrane (Span, Pennings et al. 2002). Alteration of plasma membrane rapidly induces phagocytosis, and cells not phagocytosed break into smaller fragments called apoptotic bodies. Another characteristic feature of apoptosis is cytoplasmic ‘boiling’ or blebbing (zeiosis) (Fig 1.8) (Lane, Allan et al. 2005). In contrast, necrosis is a much faster process where swelling of the entire cytoplasm and mitochondrial matrix (oncosis) occurs shortly before the cell membrane ruptures. The differences between apoptosis and necrosis are summarised in Table 1.5 (Goepel 2004).

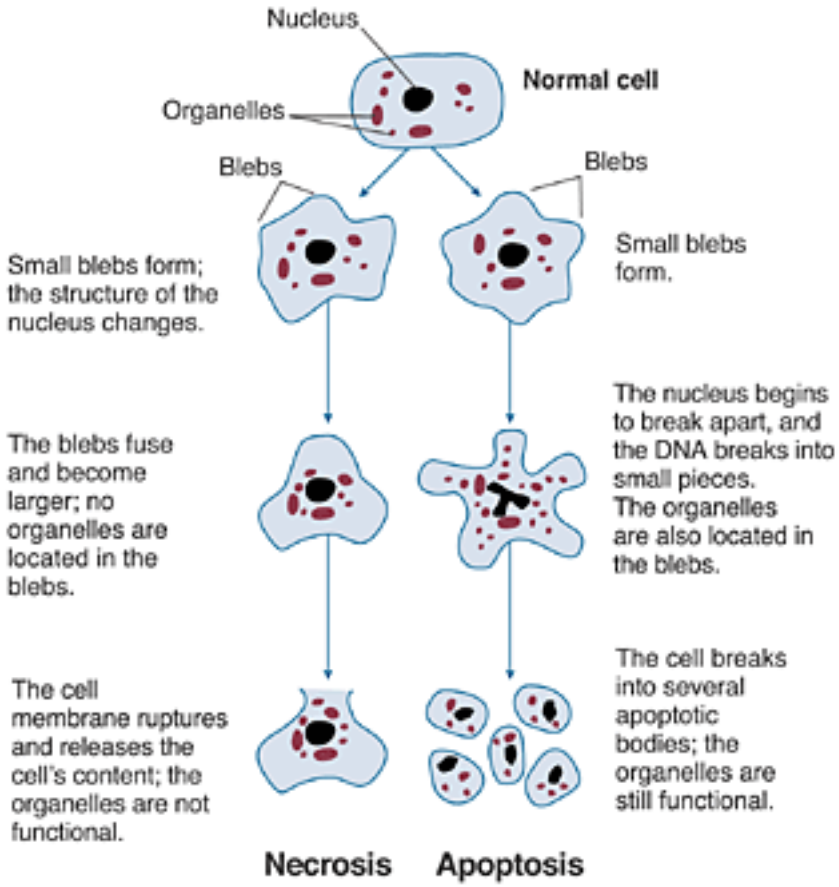


Figure 1.8: Structural changes of cells undergoing apoptosis and necrosis
(Goodlett and Horn 2001)

Table 1.5: Differences between cell death by apoptosis and necrosis (Goepel 2004)

Attribute	Apoptosis	Necrosis
Induction	Physiological or pathological stimuli	Pathological injury
Extent	Single cells	Cell groups
Biochemical process	ATP-dependent DNA fragmentation by endogenous nucleases Intact lysosomes	Ion homeostasis cessation Leaky lysosomes
Integrity of cell membrane	Intact	Damaged
Morphology	Cell shrinkage and fragmentation	Cell swelling and lysis
Inflammatory process	Absent	Present

More recently, it has become apparent that the classic dichotomous model of apoptosis versus necrosis is an oversimplification of a highly complex process to guard an organism against unwanted and potentially harmful cells. Alternative models of programmed cell death (PCD) have been described that occur in the absence of caspases and do not fit conventional definition of apoptosis. Paraptosis occurs in the absence of caspase activation and typical nuclear changes and results in cytoplasmic vacuolation and mitochondrial swelling (Sperandio, de Belle et al.

2000). Another model, autophagy, is characterised by degradation by the cell's own lysosomal system after sequestration of cytoplasm or organelles in autophagic vesicles (Gozuacik and Kimchi 2004). Other proposed mechanisms include mitotic catastrophe (King and Cidlowski 1995) and slow cell death (Blagosklonny 2000). These caspase-independent PCD mechanisms not only occur under physiological conditions but can also be induced by chemotherapeutic agents (Bröker, Kruyt et al. 2005).

Disruption in apoptotic pathways is a common feature of oncogenesis. As a regulator of cellular stress, p53 is a critical mediator of apoptosis and as such, p53 mutation is extremely common in many cancer cells. Moreover, mutation or altered expression in its downstream effectors (*PTEN*, *Bax*, *Bak*, and *Apaf-1*) or upstream regulators (*ATM*, *Chk2*, *Mdm2*) can also suppress apoptosis and accelerate tumour development in transgenic mice (Ryan, Phillips et al. 2001). Although less common, mutations in CD95 and the resulting inactivation of the death-receptor pathway have been linked to tumour growth and metastasis (Rosen, Li et al. 2000). Studies have correlated NF-κB activation and suppression of cell death pathways via transcription of several anti-apoptotic proteins such as TRAF1 and 2 which block caspase-8 activation (Baldwin 2001).

1.2.3.2 NF-κB: pro- or anti-apoptotic?

While in previous sections NF-κB activation has been associated with expression of anti-apoptotic proteins, others have reported that a pro-apoptotic role of NF-κB. In a T-cell hybridoma cell line, glucocorticoid-induced apoptosis was facilitated by

inhibition of NF-κB, whereas NF-κB activation was required for phorbol ester and ionomycin-induced apoptosis through up-regulation of the Fas ligand (FasL) (Lin, Williams-Skipp et al. 1999). This is supported by Kasibhatla et al. who reported that NF-κB induced proapoptotic FasL protein in response to etoposide or T-cell activation signals (Kasibhatla, Genestier et al. 1999).

Moreover, it has been reported that selective inhibition of NF-κB in murine skin through overexpression of IκBα resulted in spontaneous development of squamous cell carcinomas, suggesting an oncogenic role of IκBα (Hogerlinden, Rozell et al. 1999).

Although previously it has been described that NF-κB actively competes against p53 for their co-activator proteins (section 1.2.3) (Webster and Perkins 1999), Ryan et al. stated that induction of p53 via UV radiation caused NF-κB activation which enhanced the ability of p53 to induce apoptosis, and conversely inhibition of NF-κB abrogated p53-induced apoptosis (Ryan, Ernst et al. 2000). Furthermore, they suggested that inhibition of NF-κB in tumour cells which exhibit wild type p53 may diminish, rather than augment, response to chemotherapeutic agent.

Therefore, given the pro- and antiapoptotic role of NF-κB, one might not always expect inhibition of NF-κB to increase the sensitivity of tumour cells to chemotherapy or other apoptosis-inducing stimuli. In fact, whether NF-κB prevents or promotes apoptosis is dependent upon stimulus, cell type, and context.

1.2.3.3 Clinical implementation of NF-κB inhibition

In recent years, NF-κB has become the focus of many cancer researchers, particularly in certain cancer types such as Hodgkin's lymphoma where NF-κB is constitutively active and NF-κB inhibition may have a therapeutic potential (Cabannes, Khan et al. 1999; Baldwin 2001). Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) have been utilised to block the initiation and/or progression of colorectal cancer. Yin et al. have reported that aspirin and sodium salicylate inhibit NF-κB activation by inhibiting ATP-binding to IKK-ß and thereby reducing IKK-ß-dependent phosphorylation of IκBα and subsequent degradation by proteasome (Yin, Yamamoto et al. 1998). More importantly, Pierce et al. have found that indomethacin, a non-salicylate cyclooxygenase (COX) inhibitor, had no effect on NF-κB pathway at concentrations that inhibit COX activity (Pierce, Read et al. 1996). Therefore, it would appear that the ability of aspirin and sodium salicylate to inhibit NF-κB is independent of their COX inhibitory effects.

Another approach to suppress NF-κB is by inhibiting ubiquitin-mediated degradation of IκBα by proteasome. Bortezomib (PS-341, Velcade) is a potent and selective proteasome inhibitor, shown to sensitise human colorectal cancer cell lines to camptothecin analogues CPT-11 and SN-38 (Cusack, Liu et al. 2001). Since then, bortezomib has been approved by the FDA for treatment of multiple myeloma. Nevertheless, since proteasome is also involved in other cellular factors such as cyclins, cyclin-dependent kinase inhibitor p21Waf1 and p27Kip1, and p53, its efficacy may not be solely due to inhibition of NF-κB pathway.

1.2.2 Correlating chemoresistance with NF-κB in neuroblastoma

The mechanism of chemoresistance in neuroblastoma is still poorly understood, and the potential involvement of NF-κB pathway in this process has often resulted in conflicting conclusions. Constitutive activation of NF-κB pathway has been reported in S-type neuroblastoma cells (Bian, Opipari et al. 2002) and high-risk neuroblastoma samples (Brown, Tan et al. 2007) and linked to chemoresistance. Furthermore, inhibition of NF-κB by pyrrolidine dithiocarbamate (PDTC) and *N*-tosyl-$_L$-phenylalanine chloromethyl ketone (TPCK) induced an apoptotic response characterised by caspase-9 activation and apoptotic DNA changes in S-type neuroblastoma cells (Bian, Opipari et al. 2002). However, this view is opposed by Yang et al. who recently reported that NF-κB inhibition appeared to be protective for SH-EP cells (Yang, Wang et al. 2010). In contrast, Armstrong et al. reported that NF-κB activation is required for doxorubicin and etoposide-induced cell death in N-type neuroblastoma cells (Armstrong, Bian et al. 2006).

Commonly used chemotherapeutic agents such as etoposide and doxorubicin have been shown to induce NF-κB-dependent gene transcription in various neuroblastoma cell lines (Nelson, Ihekwaba et al. 2004; Ammann, Haag et al. 2009; Mullassery 2010). Furthermore, inhibition of NF-κB in S-type neuroblastoma cells by either IκBα or a chemical inhibitor BMS-345541 has been shown to enhance apoptosis through mediation by TNF-related apoptosis-inducing ligand (TRAIL) (Ammann, Haag et al. 2009).

Previous work in our laboratory has indicated that etoposide and doxorubicin induced NF-κB activation. Moreover, inhibition of NF-κB, either through over-

expression of IκBα or by chemical inhibitor Bay 11 and BMS-345541, has been shown to induce cell death in both S- and N-type neuroblastoma cells. However, combining NF-κB inhibitor with chemotherapeutic agent did not appear to enhance cell death (Mullassery 2010).

1.3 Aims and Objectives

Recently, the biological properties of many naturally occurring and synthetic compounds have been characterised, and they appear to be able to manipulate the NF-κB pathway (Bratt, Belcher et al. 2000; Yang, Oz et al. 2001; Lo, Liang et al. 2002; Tse, Wan et al. 2007).

Therefore, building on our previous knowledge on NF-κB and development of chemoresistance in neuroblastoma, we proposed to screen a selection of chemical compounds for their effects on NF-κB pathway and induction of cell death in neuroblastoma cells. Furthermore, we aim to investigate a potential synergistic interaction between an NF-κB inhibitor and chemotherapeutic agent.

Moreover, to review the current clinical status of neuroblastoma, a retrospective analysis of the outcomes of patients treated in Liverpool for neuroblastoma between 1985-2005 was conducted. This approach will hopefully highlight the challenges of neuroblastoma management as well as contribute to current understanding of molecular mechanisms of this enigmatic disease.

CHAPTER 2

Materials and Methods

2.1 Neuroblastoma Clinical Outcomes

2.1.1 Patients

All consecutive patients who presented to Alder Hey Children's Hospital, Liverpool between 1985 and 2005 with newly diagnosed neuroblastoma were included in the clinical study. Patient data were obtained from hospital case records, operative notes, and a dedicated oncology database. A cohort of 91 patients was identified. Patients diagnosed in the earlier period of study were staged according to the Evans classification (stage I-IVs) (Evans, D'Angio et al. 1971), those after 1993 were staged in accordance with the new International Neuroblastoma Staging System (INSS, stage 1-4S) (Brodeur, Pritchard et al. 1993). To minimise potential discrepancies between the two neuroblastoma classification systems, patients from the earlier study period were also retrospectively re-classified into the INSS classification.

2.1.2 Treatment

All stage 1 patients (n=3) were diagnosed ante-natally with ultrasound imaging studies. Due to parental anxiety, complete surgical resection was performed in two of these young patients, the other infant was followed up by close observation/serial imaging. Treatment protocol for stage 2 disease included primary tumour resection or delayed resection after induction chemotherapy with alternating OPEC/OJEC (vincristine, etoposide, cyclophosphamide, and cisplatin/carboplatin).

Delayed elective tumour resection was performed for patients with stage 3 disease following induction chemotherapy with alternating OPEC/OJEC (either at standard 3-weekly intervals or rapid 10-day intervals). Radiotherapy to the primary tumour site was given in some cases for microscopic residual disease.

Treatment protocol for patient with advanced staged metastatic neuroblastoma (stage 4) consisted of chemotherapy following tumour biopsy, elective surgery with attempt at resection when possible, radiotherapy to primary site, followed by autologous bone marrow transplant. Since 1998, differentiation therapy with 13-*cis*-retinoic acid has been incorporated into our treatment protocol.

Treatment for stage 4S disease, characterised by spontaneous regression in most instances, varied from observation only, primary tumour resection, to limited cycles of chemotherapy with vincristine and cyclophosphamide.

The aim of surgery in localised disease is complete resection whenever possible, while preserving adjacent vital structures (i.e. visceral arteries supplying the gut, nerves, and kidneys) which are frequently adherent to, or encased by the tumour. Due to the infiltrative nature of the tumour, it is not usually possible to get complete microscopically negative resection margins, therefore the extent of surgical resection was defined by the operating surgeon as "gross macroscopic/complete resection" (CR), partial resection (PR), or biopsy only (BX).

2.1.3 Statistical analysis

Survival time was defined as time from diagnosis to death or the latest follow-up date at the time of writing analysis. Event-free survival (EFS) was defined as the time from diagnosis to relapse, tumour progression, or death from any cause. The probabilities were estimated using the Kaplan-Meier method. The differences between levels within each covariate were tested using the log-rank test. The covariates analysed in Cox regression analysis were INSS stage, age at presentation, time period/eras, surgical treatment, *MYCN* status, site of primary tumour, and gender. Means comparisons were done using either Chi-square test or Fisher's exact test with 95% confidence interval.

2.2 Cell Culture

A number of commercially available neuroblastoma cell lines were used in this study. These cell lines have been derived from patients with metastatic or relapsed disease and selected for the study based on their varying phenotypic characteristics as outlined in Table 2.1.

Table 2.1: Summary of cell lines and culture conditions

Cell Line	Source	Culture Medium	Characteristics
SH-EP	Gift from Professor Manfred Schwab, German Cancer Research Centre, Heidelberg, Germany	RPMI 1640, 10% FCS, 1% L-Glutamine, 1% NEAA	S type (derived from SK-N-SH), bone marrow metastasis
SH-SY5Y	ECACC*	MEM, 10% FCS, 1% NEAA	N type (derived from SK-N-SH), bone marrow metastasis
Kelly	ECACC*	RPMI 1640, 10% FCS	MYCN amplified, brain tissue

*ECACC: European Collection of Cell Cultures. RPMI 1640 and Minimal Essential Medium (MEM) were purchased from Gibco (Invitrogen, UK). Non-essential amino acids (NEAA) and fetal calf serum (FCS) were purchased from Invitrogen.

Apart from commercially available cell lines, a number of primary tumour samples were obtained at elective surgical resections and primary tumour biopsies at diagnosis. Typically, tumour tissue was dissected with scalpel and treated with 0.5% trypsin/EDTA (Gibco, UK) before cultured in complete media (RPMI 1640, 20% FCS) at 37°C in a humidified atmosphere containing 5% CO_2.

2.2.1 Routine cell culture and long-term cryogenic storage of cells protocols

Cells were typically cultured in 75 cm^2 tissue culture flasks (Corning, UK) at 37°C in a humidified atmosphere containing 5% CO_2. They were grown to 70-80% confluence at which point they would be sub-cultured, mostly every two or three days. Firstly, the medium was removed from the flask and cells were washed with serum free medium. Cells were then incubated at 37°C/5% CO_2 for 5 minutes in the presence of 1ml of 0.05% trypsin/EDTA (Gibco, UK) to detach the monolayer from the flask. After 5 minutes, the trypsinisation process was inhibited through the addition of 9 ml growth medium. To remove traces of trypsin, the cells were transferred to a 25 ml universal tube, pelleted by centrifugation at 1,000 rpm for 5 minutes, and the supernatant discarded. The cell pellet was re-suspended in growth medium. The cell count was determined using a particle counter (Beckman Coulter). Between 15-25% of the cells were returned to a new 75 cm^2 tissue culture flask and the remaining cells were distributed to various cell culture vessels (Table 2.2) for use in other experiments.

Table 2.2: Typical plating density for SH-EP cell line

Culture vessel	Cell growth area (cm^2)*	Number of cells plated
96-well	0.34	1.5×10^4
35 mm dish	8	5×10^5
60 mm dish	21	1.5×10^6

*Cell growth area as per manufacturer's information and may be smaller than surface area of the dish

A working stock of cell lines was kept in liquid nitrogen for long-term storage. Cells were pelleted in the same manner as above. However, the final cell pellet was re-suspended in freezing medium containing 90% FCS and 10% dimethylsulfoxide (DMSO). The resultant suspension was stored in cryovials containing 1 ml aliquots. These vials were frozen slowly at a rate of 1°C/min in freezing containers (Nalgene, UK) stored at -80°C overnight, before transferring them to liquid nitrogen.

When needed, a frozen vial was taken from liquid nitrogen and placed in a 37°C water bath for one minute to defrost. The content of the cryovial was slowly mixed with 9 ml growth medium in a 25 ml universal container. The cells were pelleted by centrifugation at 1,000 rpm for 5 minutes and re-suspended in 15 ml growth medium. This suspension was then transferred to a 75 cm^2 culture flask and incubated at 37°C/5% CO_2.

2.2.2 Transfection

Cells were transfected using FuGENE®6 transfection reagent as per manufacturer's instructions. The optimal FuGENE (µl) to DNA (µg) ratios for SH-EP and SH-SY5Y cell lines are listed in Table 2.3. The amount of FuGENE and DNA required was adjusted depending upon the surface area of the culture vessel used (Table 2.2).

Table 2.3: Optimal FuGENE (µl) to DNA (µg) ratios for 35 mm dish

Cell line	FuGENE (µl)	DNA (µg)	Total volume (µl)
SH-EP	2	1	100
SH-SY5Y	1	1	100

2.3 Molecular Biology

2.3.1 Transformation of chemically competent cells

A suitable strain of competent *E coli* (e.g. DH5α) was used for propagation of plasmid DNA. 50 µl of competent cells were thawed on ice, 0.5 µg of plasmid DNA was added and incubated for 30 minutes in a 1.5 ml Eppendorf tube. The cells were then heat-shocked at 42°C for 1 minute and immediately returned to ice for 2 minutes. 900 µl SOC medium was added and the tube was placed in a 37°C orbital incubator for 1 hour. 20 µl and 5 µl of the cell suspension were then plated onto LB-agar plates containing the appropriate antibiotic (50 µg/ml ampicillin or kanamycin) and incubated overnight at 37°C.

2.3.2 Small and large scale amplification of plasmid DNA (Mini and Maxi Prep)

E coli cells containing plasmid DNA were cultured in 5 ml LB broth containing the appropriate antibiotic for a minimum of 8 hours in a 37°C orbital incubator set at 200 rpm. For Mini Prep, 2.5 ml of the culture was harvested by centrifugation at 4,000 x g for 10 minutes. DNA was extracted using the PureLink™ HiPure Plasmid DNA Purification Kit (Invitrogen) according to the manufacturer's instructions. Purified plasmid DNA was confirmed using spectrophotometry and a suitable restriction enzyme digest using gel electrophoresis.

For Maxi Prep, a 1 L conical flask containing 250 ml LB broth with appropriate antibiotic was inoculated with 5 ml overnight culture of transformed *E coli*, and cultured overnight in a 37°C orbital incubator set at 200 rpm. The following day, the culture was harvested by centrifugation at 6,000 x g in a Sorvall GSA rotor for 15 minutes at 4°C. DNA was extracted using the PureLink™ HiPure Plasmid DNA Purification Kit (Invitrogen) according to the manufacturer's instructions. The eluted DNA was concentrated using isopropanol precipitation. This solution was centrifuged at 12,000 x g for 30 minutes at 4°C. The supernatant was discarded and the pellet washed with 1.5 ml 70% ethanol and further centrifuged at 12,000 x g for 5 minutes at 4°C. This step was repeated one more time, before leaving the DNA pellet to air dry for 10-30 minutes and dissolved in 200 µl TE buffer. The DNA plasmid concentration was determined using spectrophotometry and the final concentration was adjusted to 1 µg/µl, and stored as 10 µl aliquots.

2.3.3 Agarose gel electrophoresis and restriction enzyme digestion of plasmid DNA

Appropriate restriction enzymes (New England Biolabs) (Figure 2.1) were used according to manufacturer's instructions. DNA was incubated with restriction enzyme(s) and following digestion, the cleaved DNA fragments were analysed by horizontal agarose gel electrophoresis in 1x TAE buffer. The DNA fragments were loaded into the 1% agarose gel in 1x Orange G loading buffer and run at 100 V for ~15 minutes, after which the fragments were visualised by UV illumination.

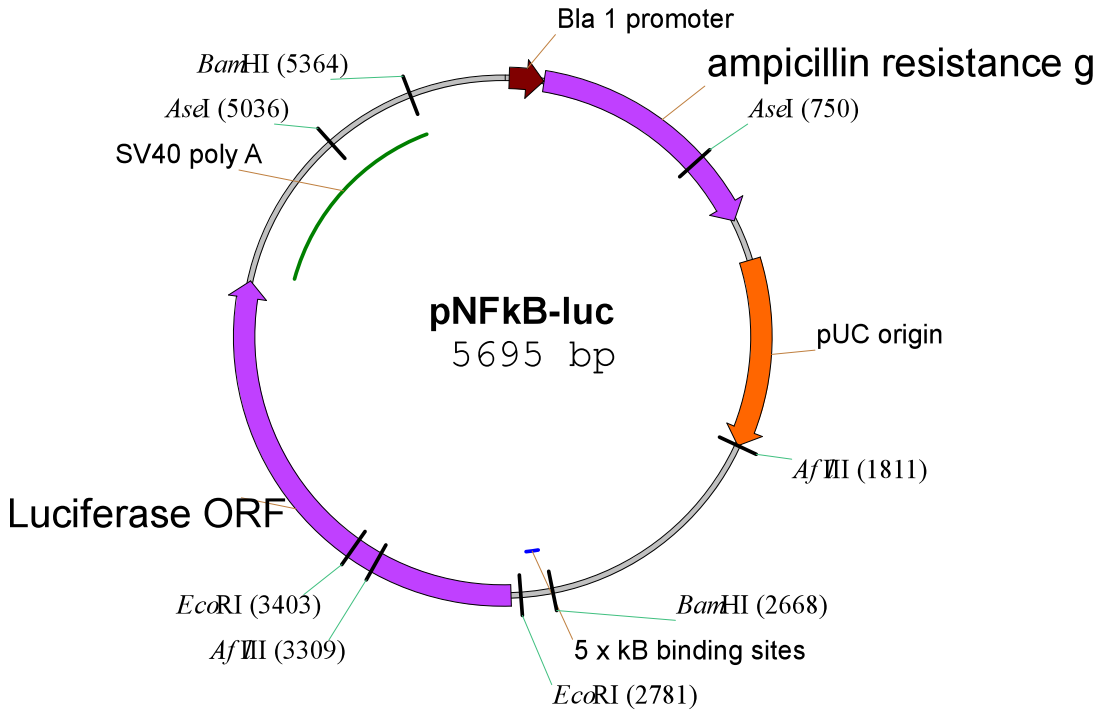


Figure 2.1: Restriction digest map of pNF-κB-Luc

2.3.4 Treatment of cells with chemotherapy agents and NF-κB inhibitors

Cells were plated in 96-well culture plates for 24 hours before treatment with the pharmacological agent of interest, diluted to the desired concentrations in the same culture medium specific to the cell line. In all cases, culture medium was partially removed from the well and replaced with an equal volume of medium containing the pharmacological agent of interest.

2.4 Bulk-cell Analysis

2.4.1 Cell viability assay (MTT)

Cell viability was measured by analysing changes in absorbance of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) after reduction by mitochondrial reductase enzymes. After appropriate duration of treatment (i.e. 24 or 48 hours), culture medium was removed from the plate and replaced with 50 µl of 0.5 mg/ml MTT solution. After 60-90 minutes incubation at 37°C, the MTT solution was removed and the cells lysed with 50 µl of 0.04N hydrochloric acid/isopropanol. The plate was then placed in a shaking table for 10 minutes to mix the precipitate. The absorbance was read at 570 nm using EnVision (PerkinElmer, UK) or Multiskan Ascent (Thermoscientific, UK) plate reader. The results shown were adjusted by subtracting the 'blank' (medium and MTT reagent) from the initial readings. Readings from treated wells were compared to 'control', i.e. untreated cells grown in normal medium. The experiment was performed in replicate of three to six samples and each experiment was repeated at least three to six times.

2.4.2 Luminometry – reporter gene assay using NF-Luc

SH-EP cells stably transfected with NF-Luciferase reporter vector containing five repeats of NF-κB binding sites cloned upstream of the luciferase gene were used for luminometry assays. These cells were plated in white opaque 96-well plates for 24 hours before treated with appropriate drug of interest. After the determined incubation period, the medium was removed and the cells lysed with 80 µl/well luminometry lysis buffer (0.025% $_{(w/v)}$ DTT, 1% $_{(w/v)}$ BSA, 1% Triton X 100, 15% $_{(w/v)}$ glycerol, 25 mM Tris-phosphate, 0.1 mM EDTA, and 8 mM $MgCl_2$) at room temperature with gentle shaking for 20 minutes. Each sample was supplemented with ATP to a final concentration of 1 mM. The plate was then loaded into EnVision or VICTOR (PerkinElmer, UK) plate reader which has been programmed to inject 80 µl of 25 mM luciferin (buffered in 25 mM Tris-phosphate, pH 7.5) to a sample, take photon counts every 0.1 second for 5 seconds, then move to the next well. All measurements were exported and analysed using Microsoft Excel. The experiment was performed in replicate of three to six samples and each experiment was repeated at least three to six times.

2.4.3 Western blotting

Cells were cultured overnight in 60 mm or 90 mm dishes before given the appropriate treatment. Cell lysates were prepared on ice and kept at 4°C everytime. After removing culture medium, cells were washed with PBS before adding lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X 100, 50

mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium ß-glycerophosphate, 0.1 mM PMSF). Cell lysates were collected using a cell scraper and stored in Eppendorf tubes. Cell debris was removed by centrifuging at 10,000 x g for 15 minutes at 4°C. The supernatant was transferred and stored in new Eppendorf tubes at -20°C.

The amount of protein in each sample was determined using bicinchoninic acid assay (BCA assay). Afterwards, Laemmli buffer was added to an aliquot of each sample and the mixture was then boiled for 5 minutes. The same amount of protein from each sample, typically 40 µg, was loaded into each lane of the gel as well as 5 µl of pre-stained molecular weight marker (BioRad). The gel was run at 100 V for 60-90 minutes in a BioRad PROTEAN II xi Cell.

When the run was finished, the gel was transferred to a nitrocellulose membrane in a BioRad Trans-Blot Electrophoresis Transfer Cell run at 100 V for 1 hour with water cooling.

At the end of transfer, the nitrocellulose membrane was blocked in 5% non-fat milk/TBS-Tween 0.1% for 1 hour at room temperature. The membrane was then washed in TBS-Tween 0.1% for 5 minutes before incubated in primary antibody solution overnight at 4°C with gentle rocking (Table 2.4). The membrane was then washed in TBS-Tween 0.1% three times for 5 minutes and incubated in appropriate secondary antibody solution for 1 hour at room temperature. The membrane was washed again in TBS-Tween 0.1% three times for 5 minutes.

Signal was detected by enhanced chemiluminescent (ECL) and manual film development.

Table 2.4: Primary antibodies for Western blotting

Primary antibody	Source	Dilution	Type
Phospho-NF-κB p65 (Ser536)	Cell Signaling	1:1000	Rabbit
IκB-α	Cell Signaling	1:1000	Rabbit
Phospho-IκB-α (Ser32/36)	Cell Signaling	1:1000	Mouse
Phospho-IKKα (Ser180)/IKKß (Ser181)	Cell Signaling	1:1000	Rabbit
Cyclophilin A	Cell Signaling	1:1000	Rabbit

The secondary antibody used was Anti-rabbit IgG HRP-linked 1:1000 (Cell Signaling) and Anti-mouse IgG (whole molecule) Alkaline Phosphatase 1:3000 (Sigma).

2.5 Single-cell Imaging

2.5.1 Confocal microscopy

Confocal microscopy was conducted on cells plated in 35 mm glass bottom dishes (IWAKI, Japan), incubated throughout the experiment in a humidified CO_2 incubator (37°C, 5% CO_2). Either a Plan-Neofluar 63x phase-contrast oil immersion objective (1.3 NA) or a Plan-Neofluar 20x dry objective (0.5 NA) was used. Data capture and extraction were carried out with LSM510 version 3.5 or ZEN software (Zeiss, Germany).

2.5.2 Immunocytochemistry

Cells were plated in 35 mm glass bottom dish (IWAKI, Japan) and incubated overnight to reach 70-80% confluency. The culture medium was removed and the cells were rinsed three times with PBS. Afterwards, the cells were fixed with 4% paraformaldehyde for 10 minutes at 4°C. The cells were rinsed three times for 5 minutes with PBS to remove excess paraformaldehyde. The cells were then blocked for 30 minutes with blocking solution (1% BSA, 0.1% Triton X 100 in PBS). The blocking solution was removed and cells were incubated for one hour with primary antibody diluted as required in the blocking solution (Table 2.5). Afterwards, cells were washed three times for 5 minutes with the blocking solution and incubated for a further 30 minutes with appropriate secondary antibody (Table 2.5). The cells were then washed three times for 5 minutes with the blocking solution again before adding a small volume of PBS and stored at 4°C for microscopy.

Table 2.5: Antibodies used for immunocytochemistry

Primary antibody	Source	Dilution	Type
NB84	Novocastra	1:200	Mouse
CD56	Novocastra	1:50	Mouse
Secondary antibody	**Source**	**Dilution**	
Mouse-Cy3	Sigma	1:500	

Microscopy was carried out with a 63x phase-contrast oil immersion objective (1.3 NA). Excitation of Cy3 was performed using a Helium Neon laser (543 nm). The emitted light was reflected by a 545 nm dichroic mirror through a 560 nm long-pass filter.

2.5.3 Mode of cell death using Annexin V/Propidium Iodide

Analysis of cell death was performed using Annexin V FITC and Propidium Iodide (PI) (Sigma). Cells were plated in four-compartment CellView^{TM} 35 mm glass bottom dish (Greiner, Belgium). Annexin V and PI were added to each compartment immediately prior to imaging with a Plan-Neofluar 20x dry objective, to a final concentration of 1 µl/ml. Excitation of Annexin V FITC was performed using an Argon ion laser at 488 nm. The emitted light was reflected by a 545 nm dichroic mirror through a 505-550 nm band-pass filter. Excitation of PI was performed using a Helium Neon laser (543 nm). The emitted light was reflected by a 545 nm dichroic mirror through a 560 nm long-pass filter. Images were taken from 4 fields of view from each compartment, approximately every 6 minutes.

The analysis was performed by counting the number of cells positive for Annexin V alone, PI alone, and both Annexin V and PI together, expressed as a percentage of the total number of cells at the beginning of each experiment, at different time intervals (Table 2.6) (van Engeland, Nieland et al. 1998).

Table 2.6: Possible outcomes from Annexin V/PI analysis

Cell fate	Annexin V	PI
Early apoptosis	+	-
Late apoptosis	+ (first)	+
Necrosis	-	+
	+	+ (first)
Viable cells	-	-

CHAPTER 3

Clinical Outcomes

3.1 Introduction

Neuroblastoma (NB) is the most common extracranial solid tumour diagnosed in childhood. During the last decades, the introduction of intensive multi-modal therapy for high-risk NB cases has led to improvement in the prognosis for this particular group of patients. This has prompted further debate on the role of aggressive surgery, notably efforts to achieve 'complete tumour resection' for stage 3 and stage 4 NB.

Although complete tumour resection is still widely regarded as the mainstay of treatment for localised tumour (stage 1 and 2 NB), international expert opinion regarding the utility of aggressive surgery for stage 3 and 4 NB is subject to continuing debate. Several studies demonstrated that complete tumour resection may

well be associated with significantly improved clinical outcome (La Quaglia 2001; Tsuchida and Kaneko 2002), however others have shown that the radicality of surgery (i.e. the completeness of resection) did not change the outcome (Castel, Tovar et al. 2002; von Schweinitz, Hero et al. 2002).

Taken against this background of varying international clinical evidence, the present study at Alder Hey was designed to evaluate how the role of surgery may have changed over the last two decades, particularly with the introduction of novel chemotherapy regimes and to seek to correlate these findings with overall survival rate (OS), event-free survival rate (EFS), and local recurrence.

3.2 Results

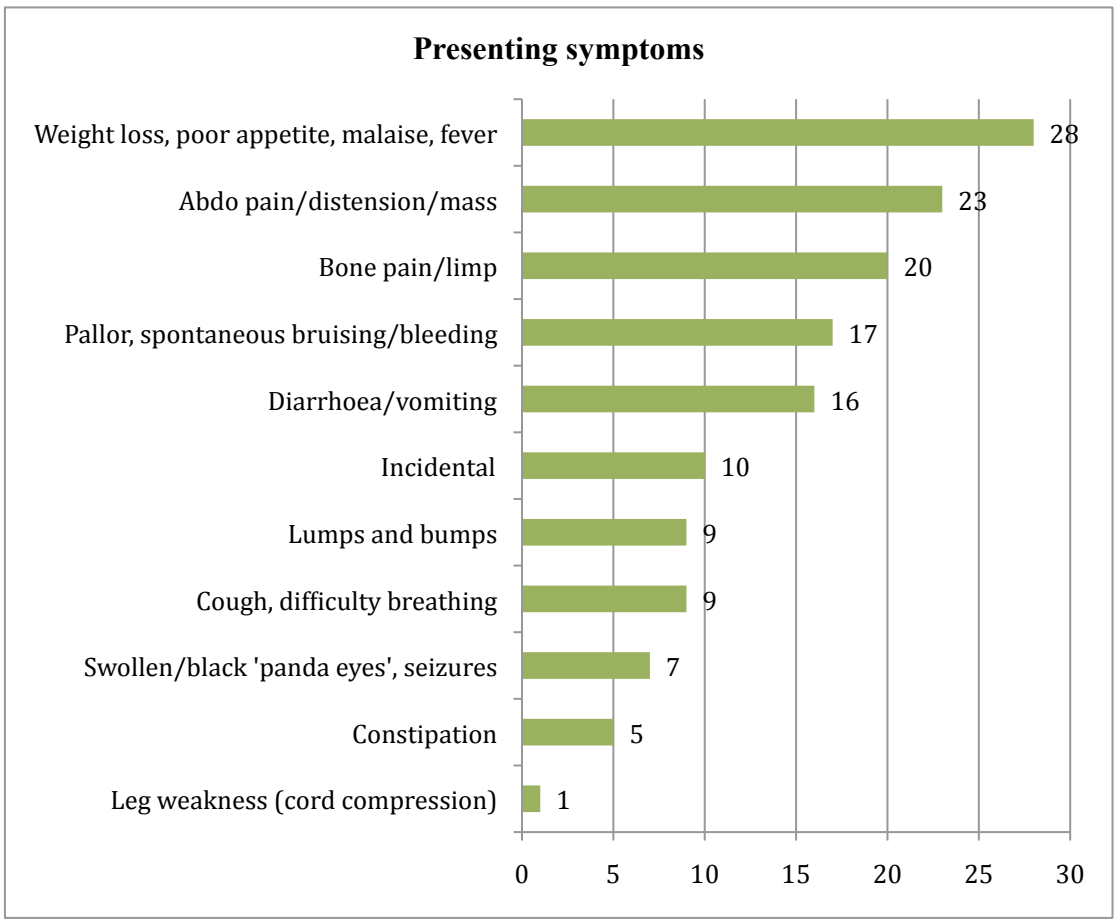
3.2.1 Patient characteristics

The male to female ratio was 0.75:1. Older patients (>2 years) were more likely to present with more advanced disease, the median ages for stage 1-2 and stage 3-4 were 1.32 and 2.68 years respectively (p=0.024). Full information regarding amplification of *MYCN* oncogene was available in a limited number of cases, 6 (16%) stage 3-4 patients were *MYCN* amplified, and 13 (34%) cases were *MYCN* non-amplified (Table 3.1). Abdominal pain in conjunction with palpable abdominal mass, constitutional symptoms, and bone pain were among the commonest presenting clinical features at diagnosis (Figure 3.1).

Table 3.1: Patient characteristics

Characteristic	No (%)
INSS classification:	
•Stage 1	3 (3.3)
•Stage 2	10 (11)
•Stage 3	13 (14.3)
•Stage 4	56 (61.5)
•Stage 4S	9 (9.9)
•Total	91 (100)
Site of primary tumour:	
•Abdomen	
•Adrenal	66 (72.5)
•Paravertebral	9 (9.9)
•Other abdominal	1 (1.1)
•Thorax	12 (13.2)
•Pelvis	1 (1.1)
•Unknown primary	2(2.2)
•Total	91 (100)
MYCN* (1992-2005, stage 3-4S):	
•Non-amplified	13 (34.2)
•Amplified	6 (15.8)
•Undetermined	19 (50)
•Total	38 (100)

** MYCN status was only incorporated into hospital policy in 1992 and sample was analysed at an external site. Unfortunately biopsy procedures at times were performed as emergency, and as such delivery and other logistics problems resulted in undetermined/unequivocal results.*



* Incidental findings include prenatal diagnosis (n=3), or incidental finding at GP/hospital of tumour mass whilst being investigated for another problem.

Figure 3.1: Presenting symptoms at diagnosis

3.2.2 Surgery

In all surgery, attempts were always made to safely remove primary tumour. This was achieved in 53 patients, the majority (74%) being delayed resection after primary tumour biopsy and adjuvant chemotherapy (Table 3.2). In particular, improved tumour resectability was clearly observed in 30 (60%) stage 4 cases following induction chemotherapy, where initially tumour removal was considered unlikely to be feasible. No operations in a group of stage 3-4 cases was due to fully informed family decisions in view of very extensive disease precluding complete

resection (n=10), chemoresistant tumour (n=10), complete remission after chemotherapy (n=2), or early death (n=1).

	Primary resection		Delayed resection		Biopsy only
	CR	PR	CR	PR	
Stage 1	2	-	-	-	-
Stage 2	3	5	1	1	-
Stage 3	1	2	4	3	3
Stage 4	-	-	16	14	20
Stage 4S	1	-	-	-	7
Total	7	7	21	18	30

Table 3.2: Timing and extend of surgical resection

Surgical resection of tumour was either performed as primary resection or delayed resection after induction chemotherapy. The extent of resection was defined by the operating surgeon as: gross macroscopic/complete resection (CR); partial resection (PR); or biopsy only.

3.2.3 Morbidity

Post-operative morbidity was noted in 13 patients (15.7%). There was no surgery-related death. Nephrectomies were performed in 4 patients to aid complete tumour resection or as part of *en-bloc* excision, one of whom developed renal failure, currently managed by the nephrology team (latest GFR 50). Horner's syndrome (miosis, ptosis, anhidrosis) was observed in 5 patients (41.7%) following resection of thoracic neuroblastomas, one of whom also suffered from phrenic nerve palsy. One

patient experienced post-operative diarrhoea which resolved within 24 hours. Damage to the right renal vein during a difficult resection led to a massive intra-operative haemorrhage in one patient, requiring 5-litre blood transfusion during the operation. One patient returned to theatre due to a wound dehiscence, while another patient suffered injury to the sympathetic nerve supply to the left leg, which led to a unilateral 'sympathectomised' peripheral extremity.

3.2.4 Survival analysis

The probability of survival at 5 years for the entire series is 0.37 and the EFS is 0.35. Overall survival and EFS according to tumour stage is shown in Figure 3.2. Survival for stage 3 disease had improved significantly from 25% to 80% (p=0.04) between the eras 1985-1994 and 1995-2005, similar trends were also observed in stage 4 disease (18% to 22%, p=0.098) and stage 4S disease (40% to 75%, p=0.381) (Figure 3.3). Across tumour stages 1-4S, there was a clear trend towards improved survival (OS and EFS) by achieving complete resection, although the difference in survival rates between complete vs partial resection was not statistically significant (OS 60% vs 44%, p = 0.216; EFS 56% vs 40%, p = 0.121). Significant difference was only observed when compared to the 'biopsy only' group (5-year OS and EFS 16.7%, p <0.001). Median survival time for stage 4 patients who had biopsy only was 6.1 months (95% CI 4.3-7.9) vs 35.4 months (95% CI 14.6-56.3) for those who had complete/partial surgical resection.

Relapse of tumour occurred in 26 stage 3-4 patients: 4 local, 7 local and distant, and 15 distant relapses. Relapse pattern was similar across the different resection groups

(Table 3.3). Younger age at presentation (<18 months) was associated with improved survival (Table 3.4) particularly in the groups who had tumour resection (complete or partial). Significant difference was observed when patients were analysed by their *MYCN* status, 5-year EFS for non-amplified patients was 58.8% vs 0 for amplified cases ($p = 0.008$). Advanced stage disease, older age at presentation, patients treated in the earlier era (1985-1994), unresected primary tumour, and *MYCN* amplification were all associated with poorer prognosis (Table 3.5).

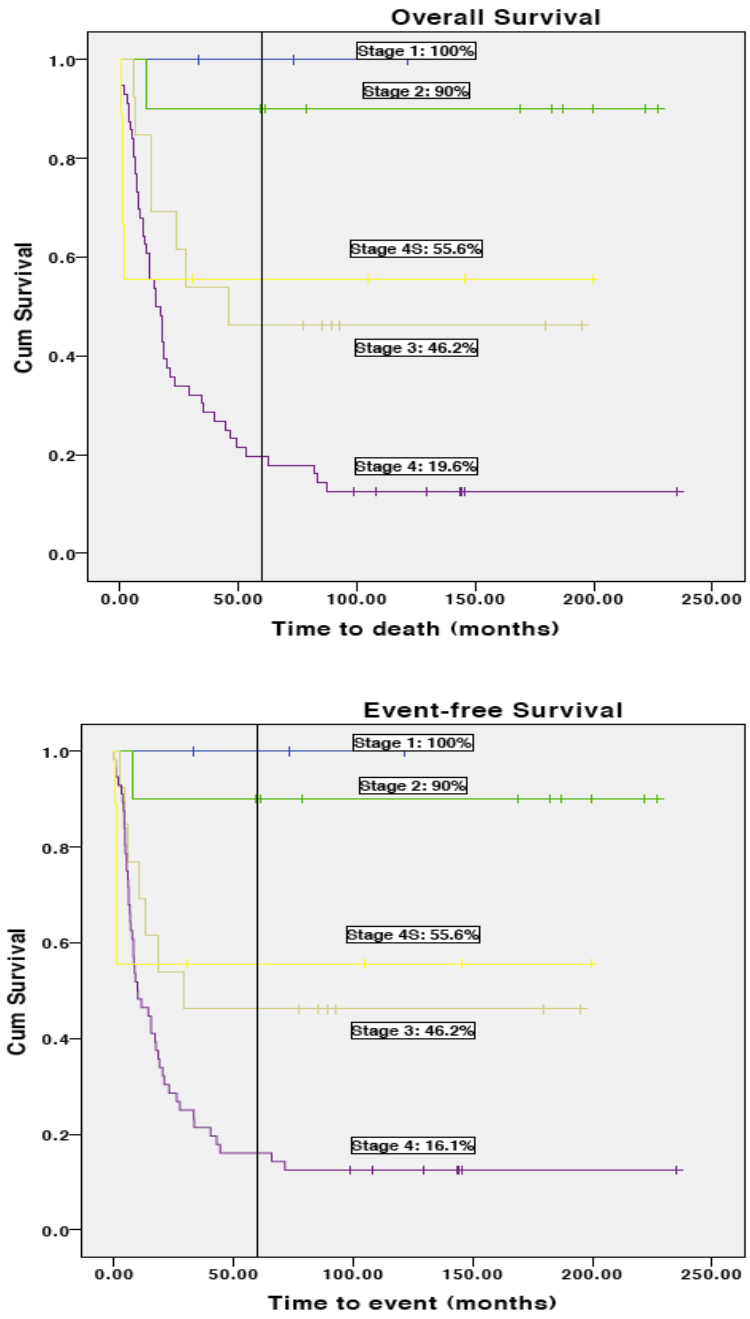


Figure 3.2: OS and EFS curves by stage

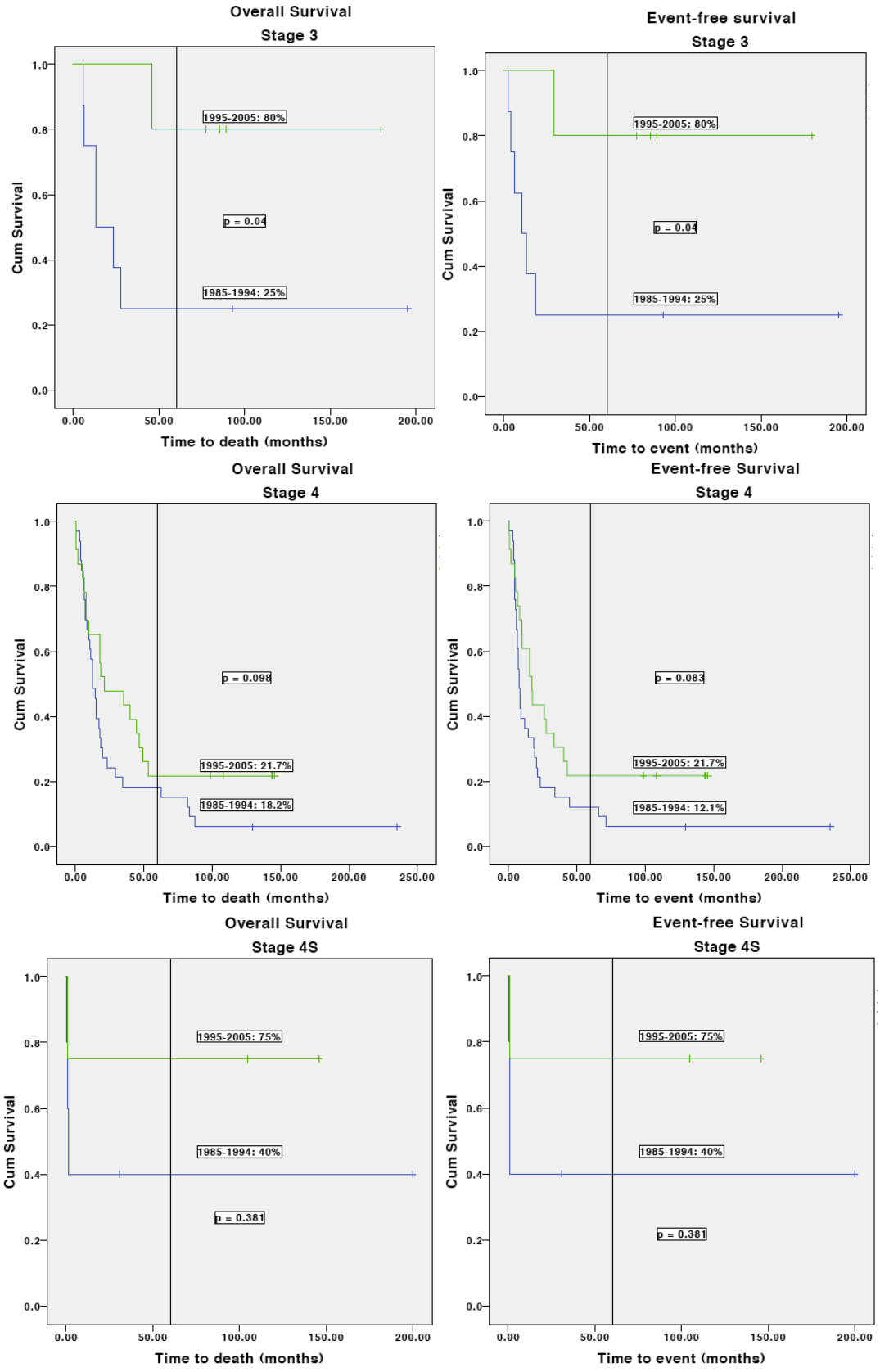


Figure 3.3: OS and EFS curves comparing the two treatment eras

Table 3.3: 5-year survival rates, patterns of relapse, and extend of surgical resection of stage 3 and 4 patients

Extend of resection	5-Year		N	Relapse			
	OS (%)	EFS (%)		Local only	Distant only	Both	Total
Complete resection	47.6	42.9	21	2	6	3	11
Partial resection	31.6	26.3	19	1	8	3	12
Biopsy only	4.3	4.3	23	1	1	1	3

* p = 0.245 ✯ p = 0.126 ** p < 0.001

Table 3.4: 5-year survival rates according to age at presentation

Age	5-Year Overall Survival (%)			5-Year Event-free-survival (%)		
	Complete resection	Partial resection	Biopsy only	Complete resection	Partial resection	Biopsy only
<18m	87.5	63.6	33.3	87.5	63.6	33.3
>18m	50.0	28.6	-	45.0	21.4	-
P value	0.060	0.034	0.346	0.047	0.029	0.183

Table 3.5: Univariate Cox regression analysis with overall survival as dependent variable

Variable	P value
INSS stage	0.012
Age at presentation	0.017
Time period (85-94, 95-05)	0.014
Tumour resection vs biopsy only	<0.001
MYCN amplification	0.011
Site of primary tumour	0.087
Gender	0.839

Table 3.6: Overview of tumour resectability over the last two decades

Extend of resection	1985-1994		1995-2005	
	No (%)	% Alive	No (%)	% Alive
Complete resection	10 (21.3)	40.0	18 (50.0)	61.1
Partial resection	17 (36.2)	35.3	8 (22.2)	37.5
Biopsy only	20 (42.5)	10.0	10 (27.8)	30.0
Total	47 (100.0)	25.5	36 (100.0)	47.2

3.3 Discussion

Treatment modalities for neuroblastoma are constantly evolving. Whilst surgery remains the mainstay treatment for localised tumours with favourable biology, the past two decades have observed dramatic intensification of therapy for moderate to high-risk neuroblastoma (Maris 2010). Accordingly, clinical outcomes of neuroblastoma patients have arguably improved, the biggest gain observed in the Alder Hey study was amongst stage 3 patients where 5-year survival rate has increased from 25% to 80% (Figure 3.3). In contrast, outcomes of stage 4 and 4S patients have shown only modest improvement.

Following initial tumour biopsy, surgery for moderate to high-risk neuroblastoma was performed after six to ten courses of intensive induction chemotherapy. As a result, tumour resectability increased from 23.1% to 53.8% in stage 3 patients and from zero to 53.6% in stage 4 patients (Table 3.2). This observation concurred with findings from other comprehensive imaging studies (Cecchetto, Mosseri et al. 2005; Davidoff, Corey et al. 2005; Simon, Hero et al. 2008). Improvements in chemotherapy protocols and surgical techniques over the last two decades are also associated with an increased proportion of patients having successful tumour resection, from 57.4% to 72.7%. In addition, the number of patients achieving complete macroscopic tumour resection has also doubled between the eras 1985-1994 and 1995-2005 respectively (Table 3.6). Despite these advances, however, we have only observed modest improvement in cure rates.

Current opinions are divided regarding the exact role of surgery in high-risk neuroblastoma. La Quaglia and colleagues at Memorial Sloan-Kettering Cancer Center have observed significant survival benefit associated with gross total resection (GTR) (La Quaglia, Kushner et al. 2004), but other neuroblastoma studies have failed to demonstrate a significant advantage for radical surgery in high-risk cases (Losty, Quinn et al. 1993; Castel, Tovar et al. 2002; von Schweinitz, Hero et al. 2002; Adkins, Sawin et al. 2004). Castel had observed no significant association or advantage between the extent of surgical resection and clinical outcome in a multi-centre Spanish study of stage 4 patients treated under a single protocol (Castel, Tovar et al. 2002). A recent long-term outcome study reported by Von Schweinitz, et al (von Schweinitz, Hero et al. 2002) noted that the correlation between surgical radicality and improved outcome was observed only in the earlier study periods but not in the later periods following the introduction of intensified chemotherapy regimens. Our findings strongly correlate with a published study by Adkins et al. (Adkins, Sawin et al. 2004) which reported a trend towards improved outcomes by achieving complete resection but the benefits in terms of overall survival were marginal. These findings hold true for both study eras in the Alder Hey series (1985-1994 and 1995-2005).

Surgery proved effective in local control of tumour progression and relapses were mainly metastatic. However, unlike findings reported by von Allmen, et al. (von Allmen, Grupp et al. 2005) and La Quaglia, et al. (La Quaglia, Kushner et al. 2004) which demonstrated good local control only in the GTR group, we observed similar clinical patterns in both complete and partial resection groups.

Being a highly infiltrative tumour, neuroblastoma resection presents a significant challenge to paediatric oncology surgeons. Kiely has proposed a novel subadventitial approach for resection, however this demanding technique has been associated with aortic injury and post-operative troublesome diarrhoea secondary to intestinal sympathetic denervation (Kiely 2007). Kubota, et al. from Japan have reported up to 15% incidence of renal infarction and atrophy as a result of aggressive surgery devitalising renal blood flow (Kubota, Yagi et al. 2004). Our operative technique was associated with a low complication rate of 15.7% or 10.8% if one excludes the nephrectomies that were removed *en bloc* to achieve complete resection.

In the current era of intensive chemotherapy, radiotherapy, stem cell transplantation, differentiation therapy, and now immunotherapy, the real benefits of surgery in achieving long-term cure rates in high-risk neuroblastoma remain uncertain. New induction chemotherapy regimens have clearly permitted better tumour response rates and in turn likely to improve tumour resectability. Further improvements in supportive care protocols and infection prophylaxis following stem cell transplant have also reduced treatment-related deaths (Fish and Grupp 2008). Correspondingly, can aggressive surgery, with the risk of damaging adjacent neurovascular structures, be truly justified? Further characterisation of tumour biology is being increasingly used to predict neuroblastoma behaviour and disease progression. New multi-disciplinary approaches in the future will probably focus on targeted biological treatments based on personalised tumour profiles, perhaps obviating the need for aggressive surgery in advanced staged disease.

CHAPTER 4

The effects of chemotherapy and NF-κB inhibitors on neuroblastoma cell fate

4.1 Introduction

Except in low risk cases where surgery alone is potentially curative, chemotherapy remains the most common treatment modality for neuroblastoma. Induction chemotherapy normally uses a combination of cisplatin or carboplatin, etoposide, cyclophosphamide, vincristine, and doxorubicin (Mullassery, Dominici et al. 2009). These anti-proliferative chemotherapy agents induce DNA damage and consequently initiate apoptosis.

While low and intermediate-risk cases usually respond well to treatment, the majority of high-risk cases ultimately progress or recur despite initial positive response to treatment. Tumour recurrences most commonly appear within 2 years after bone marrow transplant (Matthay, Villablanca et al. 1999).

Etoposide (Figure 4.1), derived from epipodophyllotoxin, delivers its cytotoxic activities through its action on topoisomerase II. Topoisomerase II enzyme is able to cleave DNA by generating transient double-stranded breaks in the DNA backbone. This process results in the formation of short-lived intermediary topoisomerase II-cleaved DNA complex (the cleavage complex), which is tolerated by the cell. Etoposide acts primarily by inhibiting the ability of this enzyme to religate cleaved DNA molecules. A cell-cycle specific compound, etoposide is mainly active in the G2 phase, and to a lesser extent in the S phase (Pommier, Fesen et al. 1996). Rapidly proliferating cells have a high concentration of topoisomerase II and as such are prime targets for etoposide.

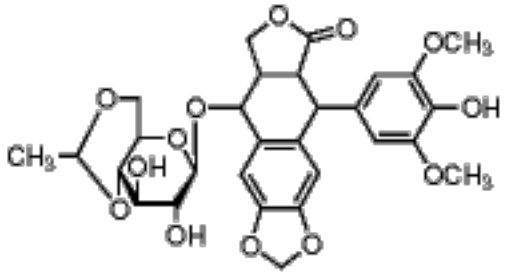


Figure 4.1: Chemical structure of etoposide

However, a number of neuroblastoma cell lines obtained from patients after relapse indicated significant resistance to etoposide, although cell lines obtained at diagnosis from the same patients were sensitive (Matthay and Kushner 2005). Several ideas have been postulated for the underlying mechanisms of tumour resistance. The cancer stem cell hypothesis suggests the presence of stem cells which may escape cytotoxic agents through slower proliferation rate (Dick 2008). The presence of stem cells in neuroblastoma tumours is associated with worse prognosis (Ross and Spengler 2007). Acquired drug resistance may result from increased expression of cell surface drug transport proteins, most notably the p-glycoprotein-mediated multi-drug resistance, altered DNA repair, or mutations in the p53 gene resulting in decreased apoptotic activity (Norris, Bordow et al. 1996; Haber, Bordow et al. 1999; Blanc, Goldschneider et al. 2003).

This chapter will investigate the sensitivity of a number of neuroblastoma cell lines to etoposide treatment and whether this can be improved through manipulating the signalling pathway, NF-κB.

4.2 Effect of etoposide on neuroblastoma cells

Two neuroblastoma cell lines were selected for this study to represent the phenotypic variation of the tumour: SH-EP cell line as a representative of the stromal type (S-type) cells and SH-SY5Y cell line as a representative of the neuronal type (N-type) cells. These cell lines were derived from post-treatment bone marrow aspiration of a 4-year old female patient who presented with an aggressive thoracic tumour (Biedler,

Helson et al. 1973).

4.2.1 Cell viability after etoposide treatment

As one of the commonest chemotherapy agents for neuroblastoma, etoposide has been incorporated into a number of chemotherapy schedules with varying doses and intervals. Pharmacokinetic studies have demonstrated greater anti-tumour activity at plasma concentration 1-5 μg/ml (~1.7-8.5 μM) (Splinter, van der Gaast et al. 1992).

The maximum tolerated dose (MTD), defined as the quantity that produced grade 4 haematological toxicity, grade 3 mucositis, diarrhoea, or skin toxicity, and grade 2 hepatic, renal, pulmonary, cardiac, or neurological toxicity in at least half of the patient population (National Cancer Institute 1999), was observed to be 8.59 μg/ml (~14.59 μM) (Gregianin, Brunetto et al. 2002).

This information was taken into consideration when choosing the doses of etoposide for our cell viability assays. Cell viability of SH-EP and SH-SY5Y cells, measured after 24-hour exposure to etoposide, are shown in Figure 4.2. Moderate reduction in cell viability was observed in both cell lines following 24 hours of treatment with etoposide, suggesting a degree of resistance of the cell lines to etoposide-induced cell death.

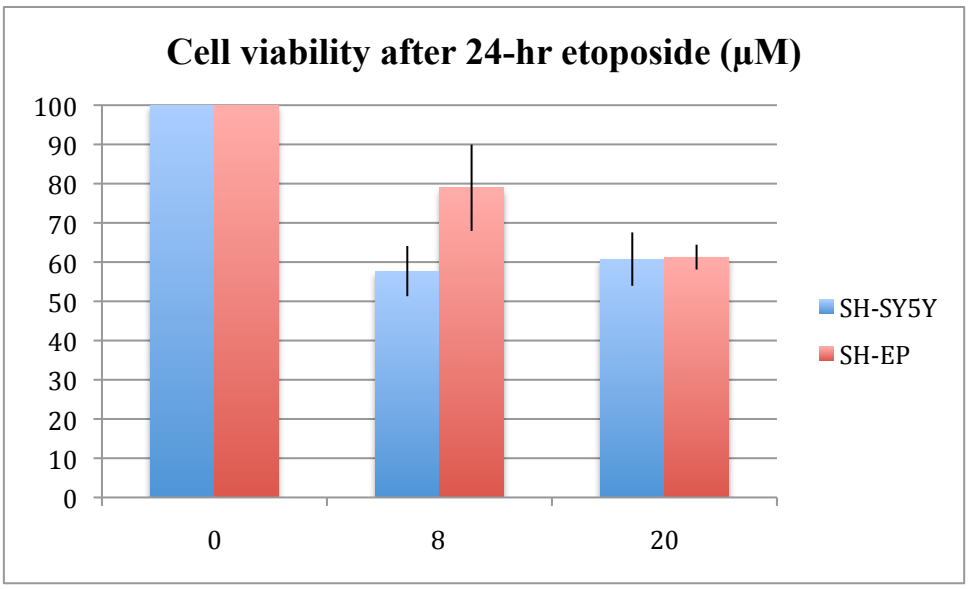


Figure 4.2: Neuroblastoma cell viability after etoposide treatment

Neuroblastoma cell lines (SH-EP and SH-SY5Y) plated in 96-well plate with cell density of 15,000 cells/well, treated with 8 µM and 20 µM etoposide. Mean cell viability after 24 hours was calculated using MTT assay from 3 or more experiments, standard deviation was represented by the error bars.

4.2.2 Effect of etoposide on NF-κB pathway

The role of NF- κB as a significant signalling pathway involved in chemotherapy resistance has been reviewed extensively (Baldwin 2001; Johnstone, Ruefli et al. 2002). The activation of NF-κB dependent gene transcription in several cell lines is associated with decreased apoptosis through the expression of antiapoptotic genes such as Bcl-xL, a member of the Bcl-2 family (Lee, Dadgostar et al. 1999; Chen, Edelstein et al. 2000). Moreover, it has been observed that exposure to some chemotherapy agents (including etoposide) leads to NF-κB activation in some cancer cells (Nelson, Ihekwaba et al. 2004; Nakanishi and Toi 2005).

In view of this, we investigated whether resistance to etoposide as observed in the previous section is possibly linked to activation of the NF-κB pathway in these neuroblastoma cell lines.

NF-κB dependent gene transcription was investigated in luciferase reporter gene assay. To eliminate variability in transfection efficiency between experiments, all experiments were carried out using SH-EP cells stably transfected with NF-Luc (Figure 4.3).

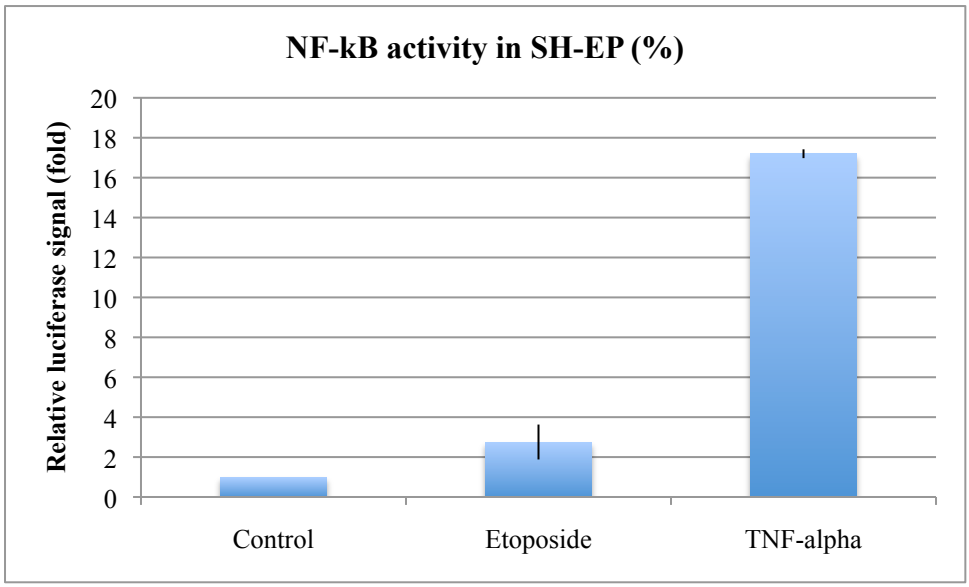


Figure 4.3: Etoposide-induced NF-κB gene transcription in SH-EP cells

SH-EP cells stably transfected with NF-Luc were treated with 20 μM etoposide and 10 ng/ml TNF-alpha. The relative luminescence was determined 16 hours after treatment as indicated in the y-axis as number of folds relative to untreated control samples. Data shown are the mean of 3-6 experiments, error bars indicate the standard deviation.

Treatment with etoposide caused an increase in NF-Luc activity by 2.75 ± 0.87 times in SH-EP cells when compared to untreated control samples. However, a much higher activity is normally observed in typical NF-κB activators, in this case represented by TNF-alpha.

4.2.3 Discussion

Chemotherapy agents exert their cytotoxic activity through induction of apoptosis. However, it has also been demonstrated that these agents, in this case etoposide, also activated the transcription factor NF-κB, which potentially dampened the apoptotic effect of chemotherapy. Furthermore, previous work in our laboratory has contributed more evidence of the link between NF-κB activation and chemotherapy resistance as it was demonstrated that NB cell lines which showed more resistance to etoposide showed higher NF-κB activation (Mullassery 2010).

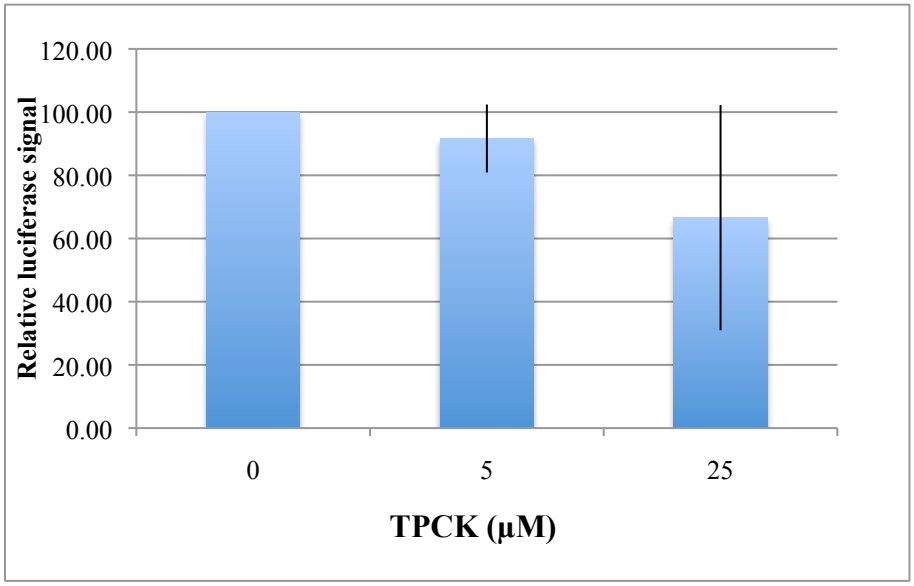
4.3 Screening of NF-κB inhibitors

Data from the previous section has suggested a possible mechanism by which NF-κB can contribute to chemotherapy resistance. By extension, it also raised the possibility that certain types of NF-κB inhibition may synergistically improve response to chemotherapy.

Therefore, working alongside an ongoing project in our laboratory which screened compounds for changes in NF-κB dynamics (i.e. nuclear-cytoplasmic translocation of NF-κB), we investigated these compounds for their effect on neuroblastoma cells. Each compound was assayed for its effects on NF-κB activation and cell viability.

4.3.1 *N*-Tosyl-$_L$-phenylalanine chloromethyl ketone (TPCK) and related compounds

N-Tosyl-$_L$-phenylalanine chloromethyl ketone (TPCK) and *N*-α-p-tosyl-L-lysine chloromethyl ketone (TLCK) are serine protease inhibitors which have been reported in several studies to inhibit the activation of NF-κB by suppressing the degradation of the inhibitor of NF-κB, IκBα (Wu, Lee et al. 1996; Jeong, Kim et al. 1997; Ha, Byun et al. 2009).



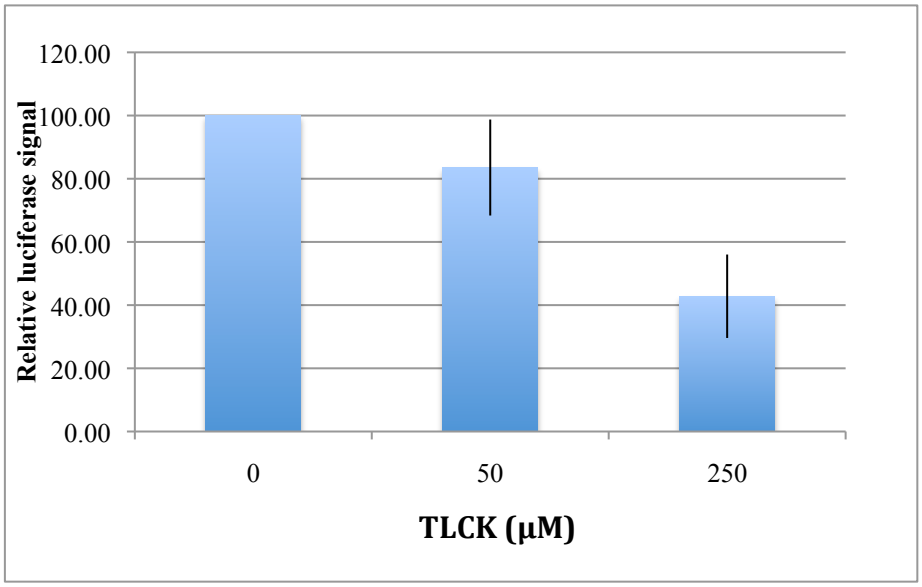


Figure 4.4: TPCK and TLCK inhibit NF-κB dependent gene transcription

SH-EP cells were treated with TPCK and TLCK at the indicated doses (µM). The relative luminescence was determined 16 hours after treatment as indicated in the y-axis as a percentage relative to untreated control samples. The experiment was repeated 3 times and standard deviation was indicated by the error bars.

Both TPCK and TLCK were able to inhibit NF-κB dependent gene transcription in a dose-dependent manner (Figure 4.4). Cells treated with 25 μM TPCK demonstrated a reduced level of NF-Luc transcription to 66% of the basal transcription observed in untreated cells. Similarly, treatment with 250 μM TLCK decreased NF-Luc transcription to 43%.

Furthermore, we investigated whether this inhibition of NF-κB had any effect in S-type neuroblastoma cells. Cell viability results shown in Figure 4.5 indicated that treatment with the NF-κB inhibitors TPCK or TLCK was associated with cell death response in S-type neuroblastoma cells (SH-EP).

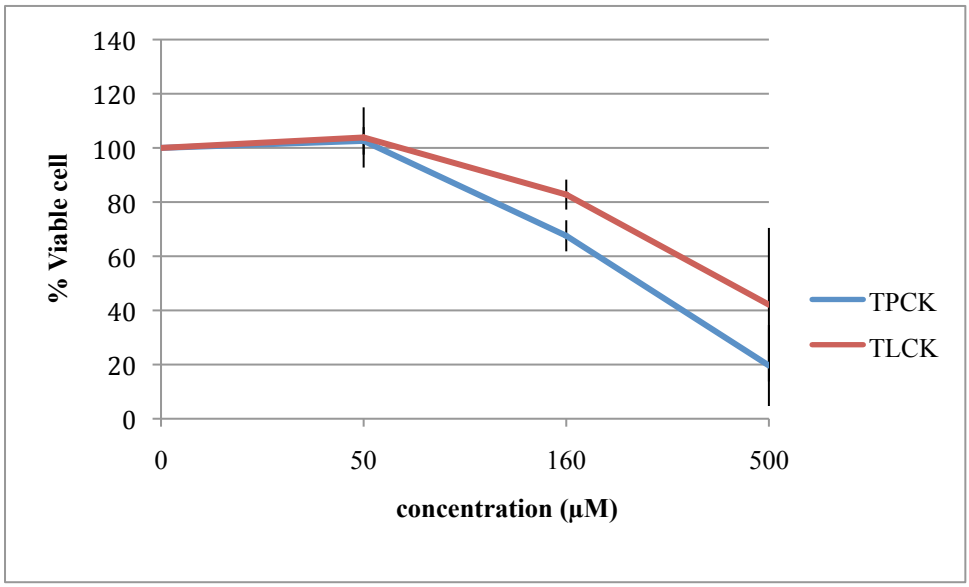


Figure 4.5: TPCK and TLCK killed S-type neuroblastoma cells (SH-EP)

Cell viability determined by MTT assay 24 hours after treatment with TPCK and TLCK in the indicated doses (μM). Values represented as relative to untreated samples. The experiment was repeated 3-6 times and standard deviation was represented by the error bars.

4.3.2 Penta-Galloyl-Glucose (PGG) and related compounds

1, 2, 3, 4, 6-penta-O-galloyl-ß-$_D$-glucose (PGG), a natural polyphenolic compound found in many traditional prescriptions, exhibited a number of biological properties which is of interest to cancer research, one of which is its ability to suppress the activation of NF-κB through inhibition of IKK activity (Pan, Lin-Shiau et al. 2000; Oh, Pae et al. 2001).

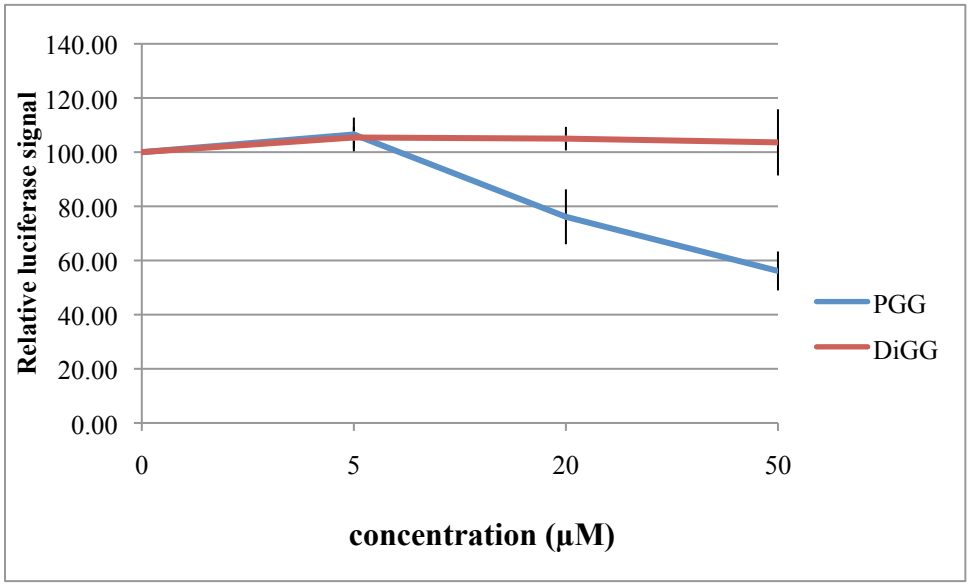


Figure 4.6: PGG suppressed TNF-induced NF-κB activation

SH-EP cells were treated with PGG and DiGG at the indicated doses (µM) and incubated for 90 minutes before stimulated with TNF-alpha (10 ng/ml). Cells were lysed 8 hours afterwards and luciferase activity recorded. Values in the y-axis represented the mean luciferase signal relative to untreated control samples taken from 3 experiments. Error bars indicated standard deviation.

PGG decreased TNF-alpha induced NF-κB activity in a dose-dependent manner (Figure 4.6) and caused a decrease in cell viability (Figure 4.7). A 50% decrease in cell viability was observed in SH-EP cells treated with 25 µM PGG. In addition to affecting IKK activity, PGG may also affect reactive oxygen species (ROS) in the

cell, which in turn alter NF-κB activity. To separate these two effects, a structurally related compound that should have less impact on ROS, 1, 2-di-galloyl-glucose (DiGG) was synthesised. DiGG, however, did not affect NF-κB gene transcription (Figure 4.6). Nevertheless, treatment with 200 μM DiGG showed 52% reduction in cell viability (Figure 4.7), suggesting the effect of PGG on cell viability is not primarily due to its action on NF-κB, and other pathways must be involved.

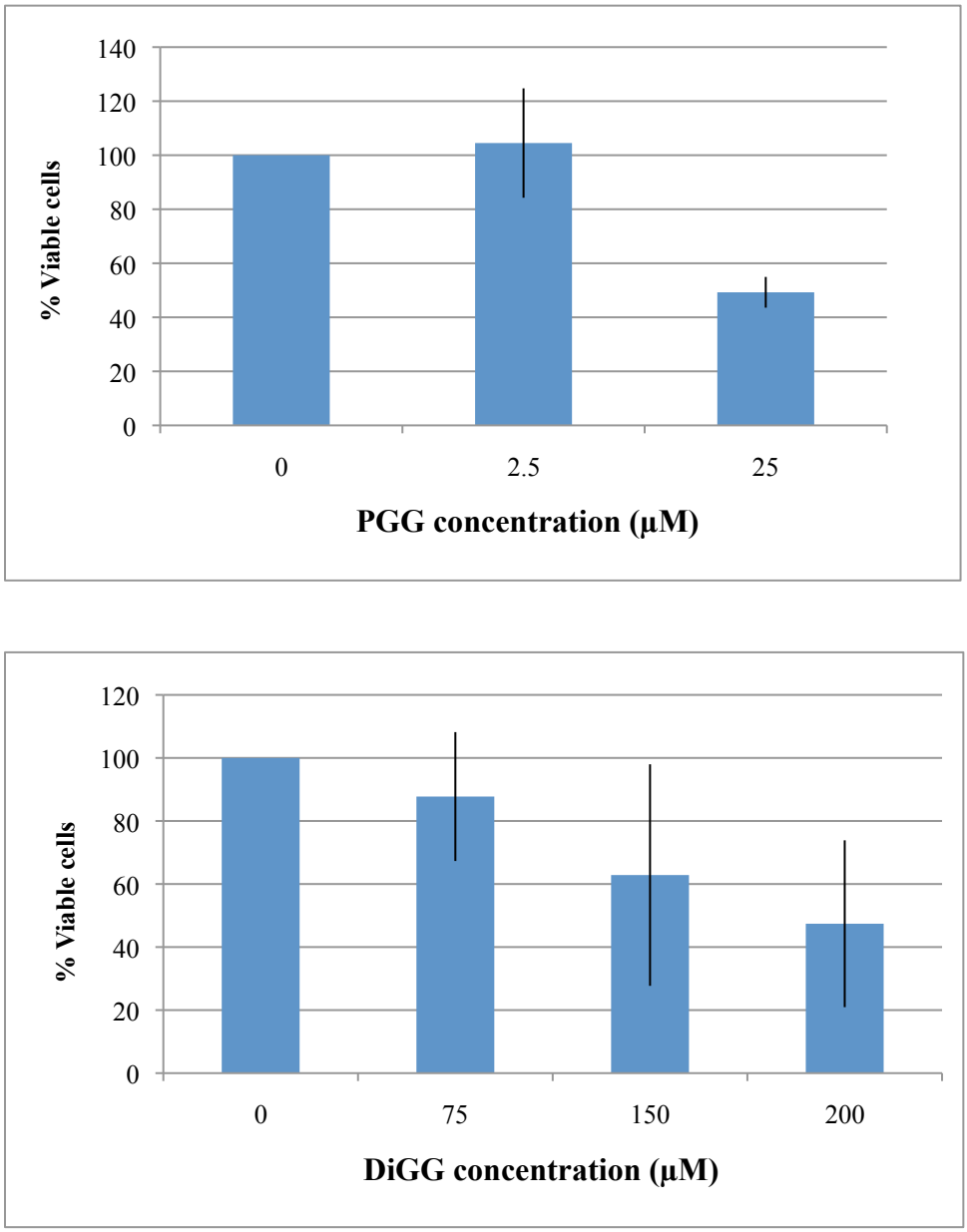


Figure 4.7: PGG and DiGG killed S-type neuroblastoma cells

Mean cell viability recorded 24 hours after treatment with PGG and DiGG at the indicated doses (µM), normalised to untreated samples. Error bars indicated standard deviation from 3-6 experiments.

4.3.3 Auranofin and related compounds

Several studies have described anti-rheumatic gold containing compounds, auranofin (AF) and sodium aurothiomalate hydrate (SAH) to have inhibitory effects on NF-κB activity by suppressing IKK activity (Bratt, Belcher et al. 2000; Jeon, Byun et al. 2003).

Auranofin demonstrated a potent inhibitory effect on TNF-alpha-induced NF-κB gene transcription, a 50% decrease in luciferase signal was observed at a concentration of 1.8 μM. However, similar effect was not observed with SAH (Figure 4.8).

Auranofin also indicated potent cytotoxicity on neuroblastoma cells, a 50% decrease in cell viability was observed at a concentration between 1-1.5 μM (Figure 4.9). Again, similar effect was not observed with SAH.

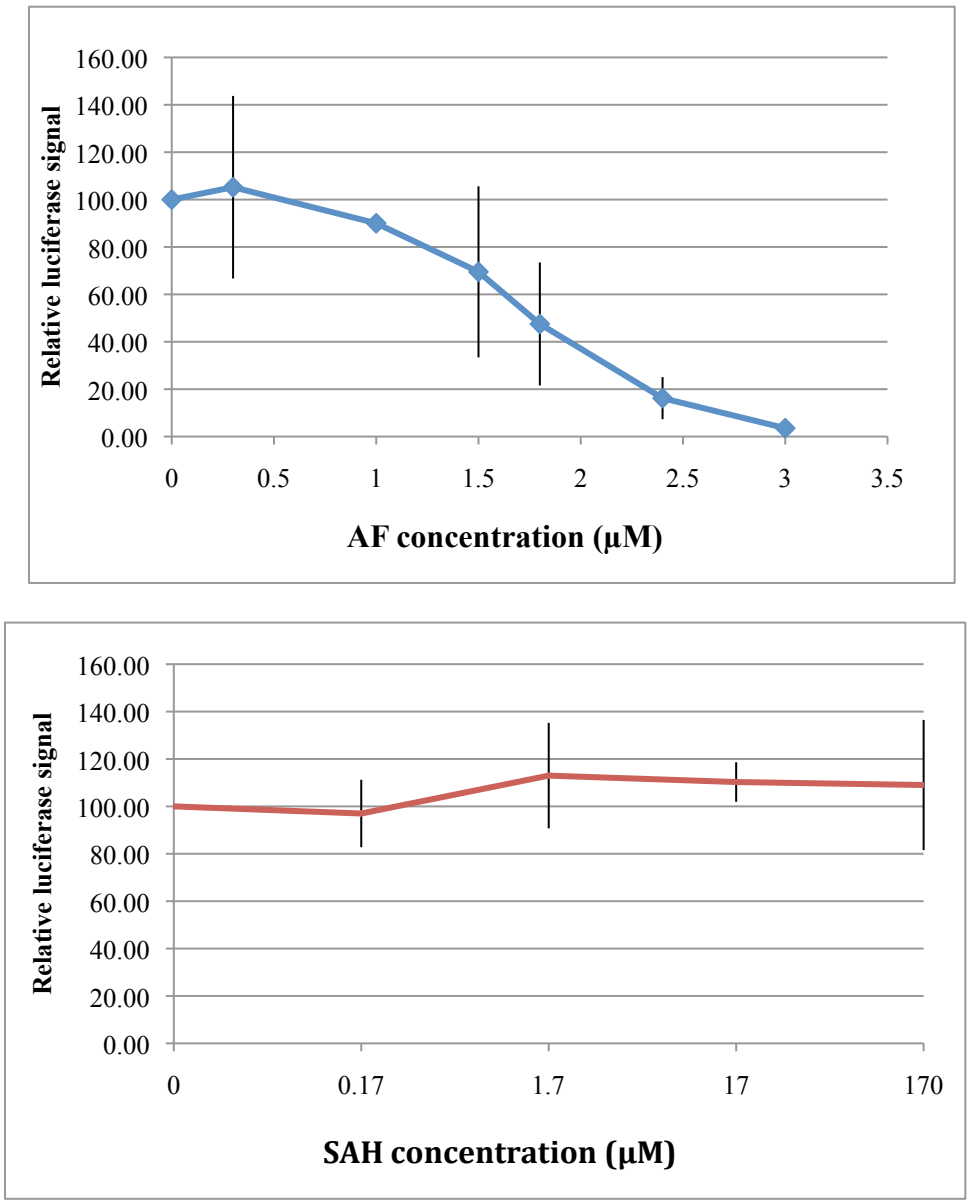


Figure 4.8: TNF-induced NF-κB activity was suppressed by AF but not SAH

SH-EP cells were treated with AF and SAH for 90 minutes before stimulated with TNF-alpha (10 ng/ml). Cells were lysed after 7 hours. Values in the y-axis represented the mean luciferase signal relative to untreated control samples taken from 5 experiments with standard deviation indicated by the error bars.

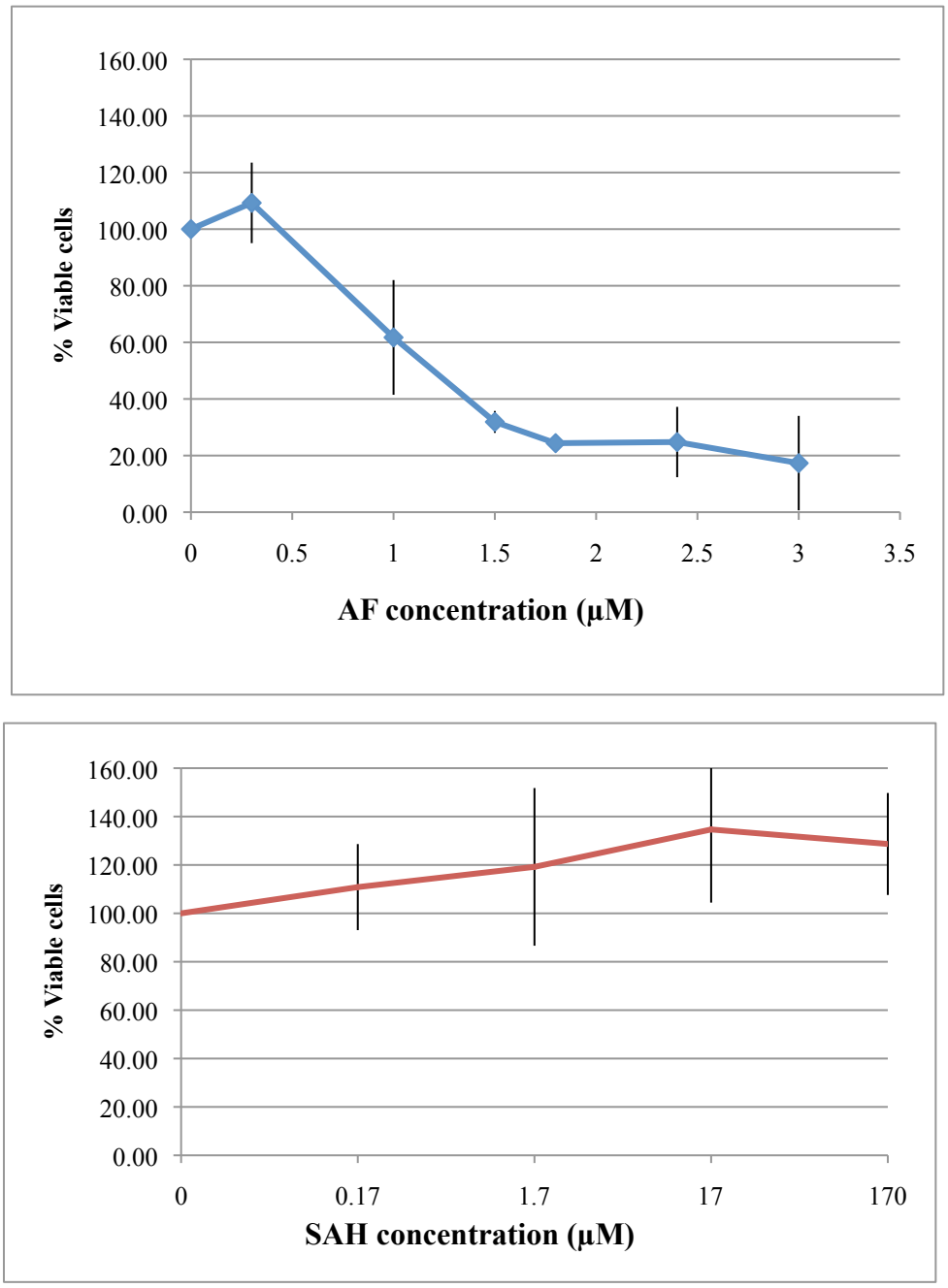


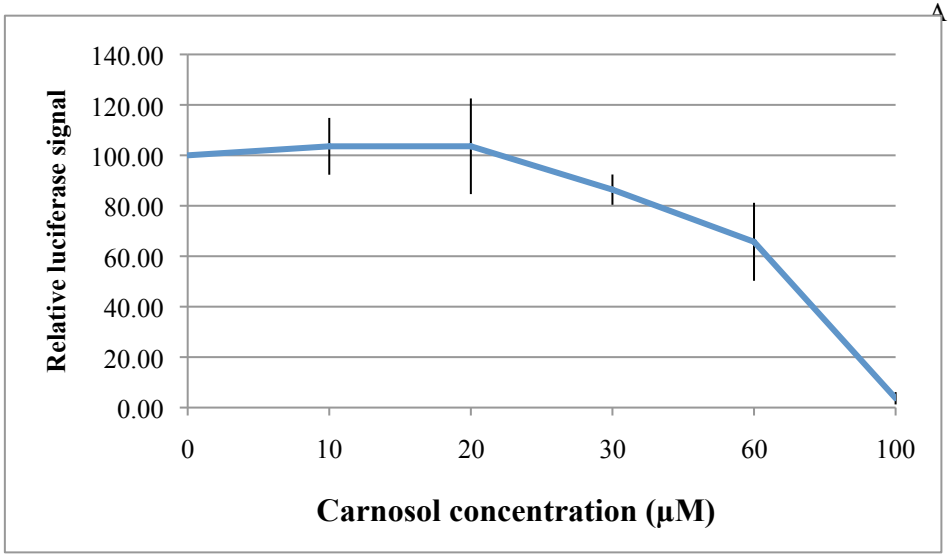
Figure 4.9: AF, but not SAH, demonstrated potent cytotoxicity in neuroblastoma cells

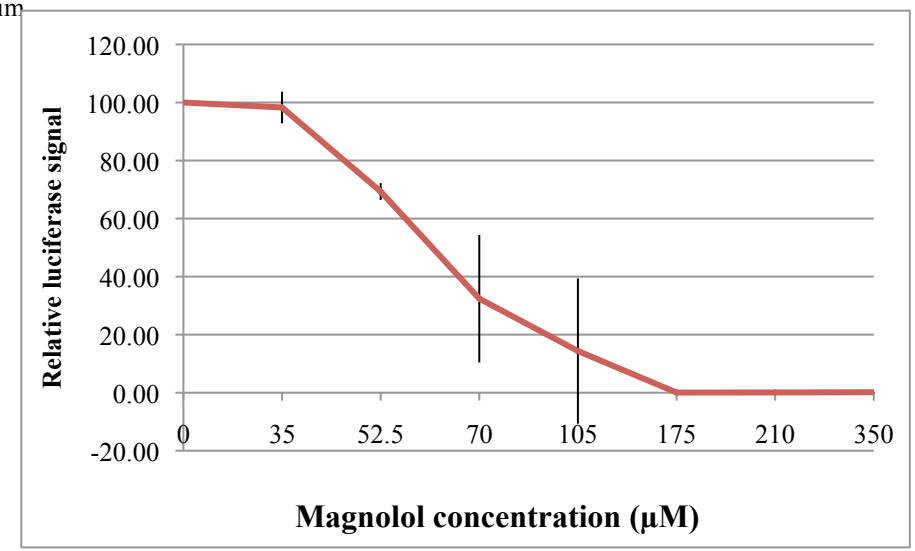
Mean cell viability recorded 24 hours after treatment with AF and SAH at the indicated doses (µM), normalised to untreated samples. Error bars indicated standard deviation from 3-6 experiments.

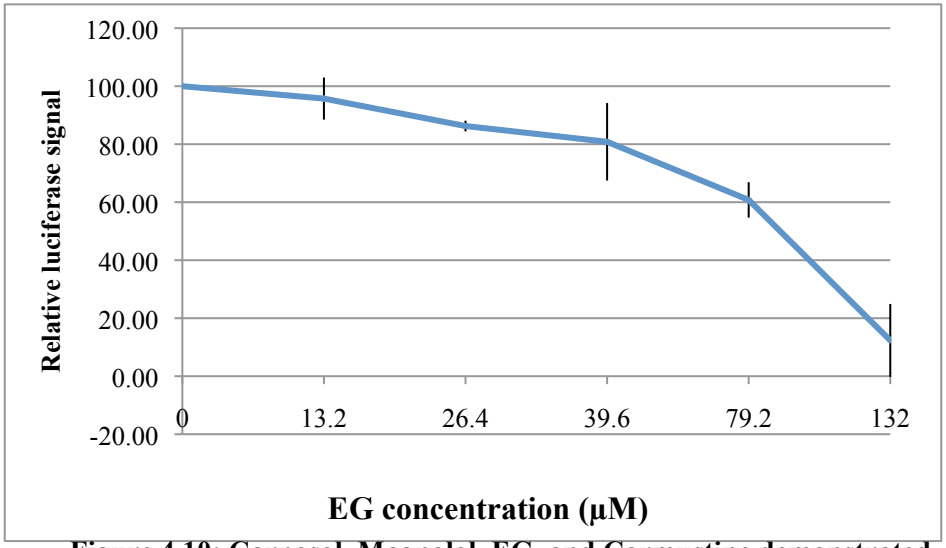
4.3.4 Carnosol, magnolol, epigallocatechin gallate, and carmustine

Carnosol, a natural antioxidant derived from rosemary; magnolol, a lignan isolated from *Magnolia officinalis* plant; and epigallocatechin gallate (EG), a major green tea polyphenol, have been described in various studies to modulate NF-κB activity (Yang, Oz et al. 2001; Lo, Liang et al. 2002; Tse, Wan et al. 2007). Carmustine is a DNA alkylating agent widely used in treatment of gliomas (Weaver, Yeyeodu et al. 2003).

The ability of these compounds to affect NF-κB pathway in neuroblastoma cells was investigated (Figure 4.10) and their effects on cell viability was assessed correspondingly (Figure 4.11). All four compounds demonstrated inhibitory effects on NF-κB activity and cytotoxicity against S-type neuroblastoma cells.







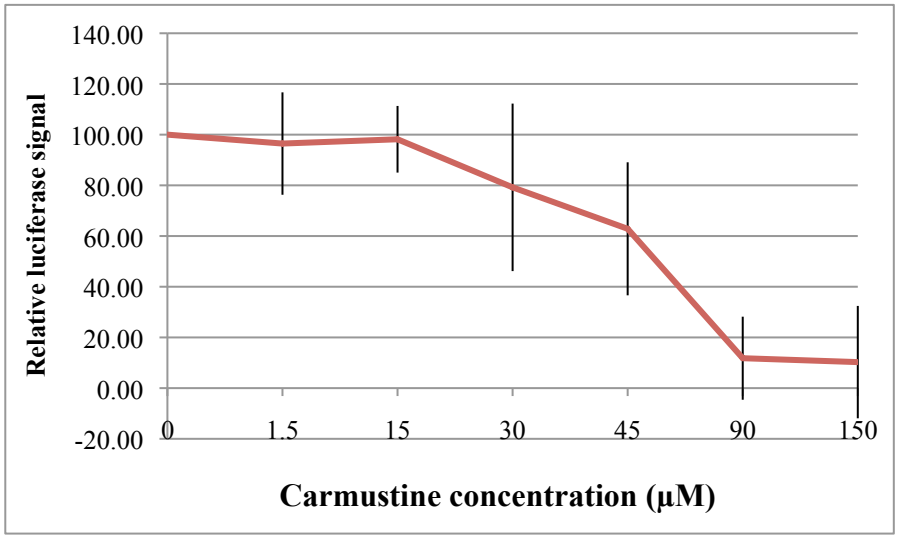


Figure 4.10: Carnosol, Magnolol, EG, and Carmustine demonstrated inhibitory effect on TNF-alpha induced NF-κB activity

SH-EP cells were treated with these 4 compounds for 90 minutes before stimulated with TNF-alpha (10 ng/ml). Cells were lysed after 7 hours. Values in the y-axis represented the mean luciferase signal relative to untreated control samples taken from 6 experiments with standard deviation indicated by the error bars.

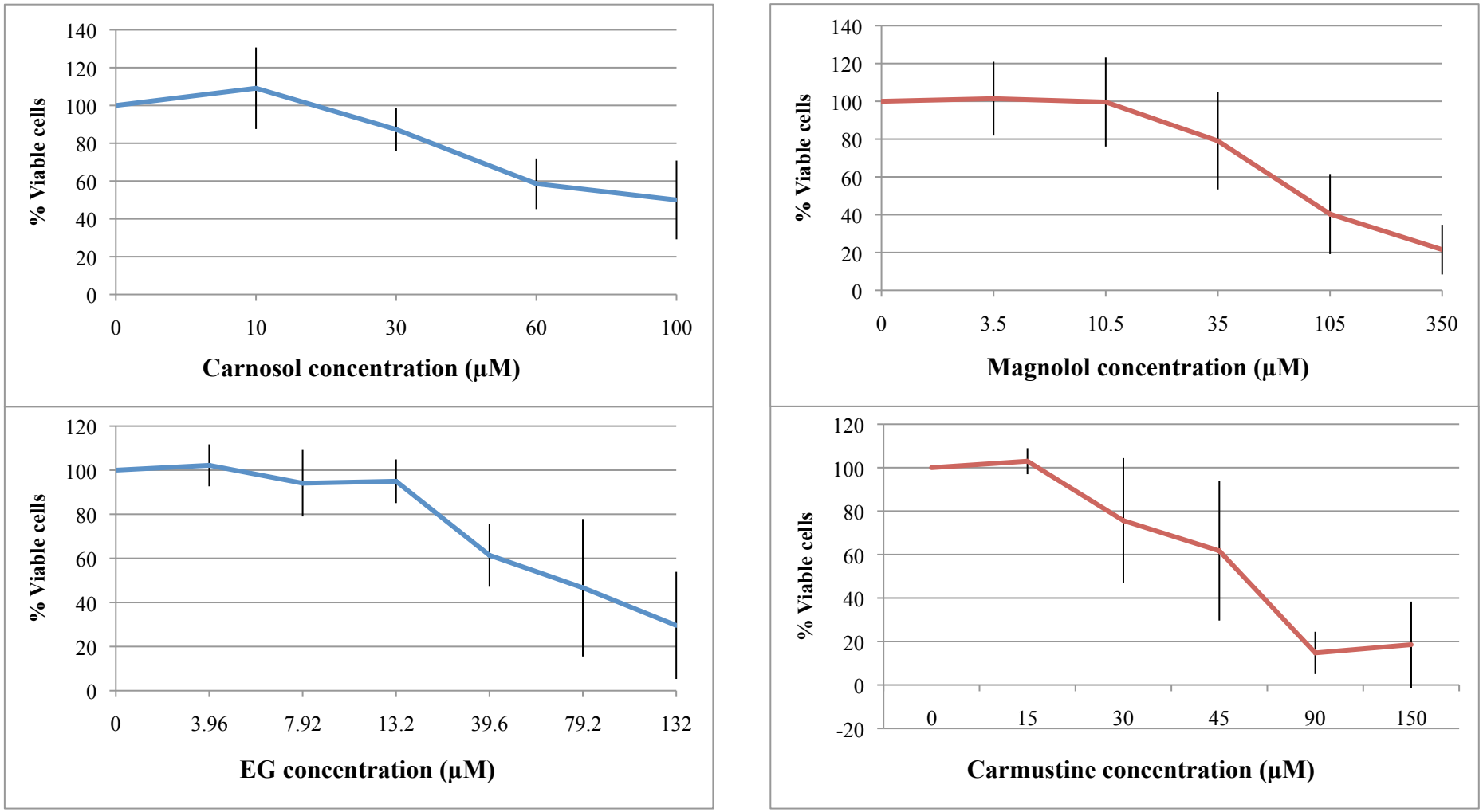


Figure 4.11: Carnosol, Magnolol, EG, and Carmustine decreased cell viability of neuroblastoma cells

Mean cell viability recorded 24 hours after SH-EP cells were treated with these 4 compounds at the indicated doses demonstrated a dose-dependent decrease. Values in the y-axis were normalised to untreated samples and error bars indicated standard deviation from 6 experiments.

4.3.5 Deferoxamine mesylate and gabexate mesylate

In a previous study by Li (Li and Frei 2006), iron chelator deferoxamine mesylate (DFOM) inhibited LPS-induced NF-κB activation *in vivo*. DFOM has been shown to have anti-proliferative properties by causing G1 arrest. Gabexate mesylate, a synthetic protease inhibitor, has been shown to inhibit phosphorylation of IκBα in several studies (Uchiba, Okajima et al. 2003; Yuksel, Okajima et al. 2003).

However, neither of these compounds demonstrated inhibitory effect on TNF-induced NF-κB activation in SH-EP cell line (Figure 4.12) nor cytotoxicity (Figure 4.13).

4.3.6 1α, 25-dihydroxyvitamin D_3 and CP-55940

1α, 25-dihydroxyvitamin D_3, the active form of vitamin D3 has effects on cellular differentiation and proliferation. The ability of this compound as well as the cannabinoid receptor 1 agonist, CP-55940, to inhibit the NF-κB pathway and induce cell death was investigated (Figure 4.14 and Figure 4.15).

1α, 25-dihydroxyvitamin D_3 did not demonstrate either inhibitory effects on the NF-κB pathway nor cytotoxicity. CP-55940, however, was found to inhibit NF-κB activation at 24 and 48 μM, which also corresponded to a reduction of cell viability in SH-EP cells (Figure 4.15).

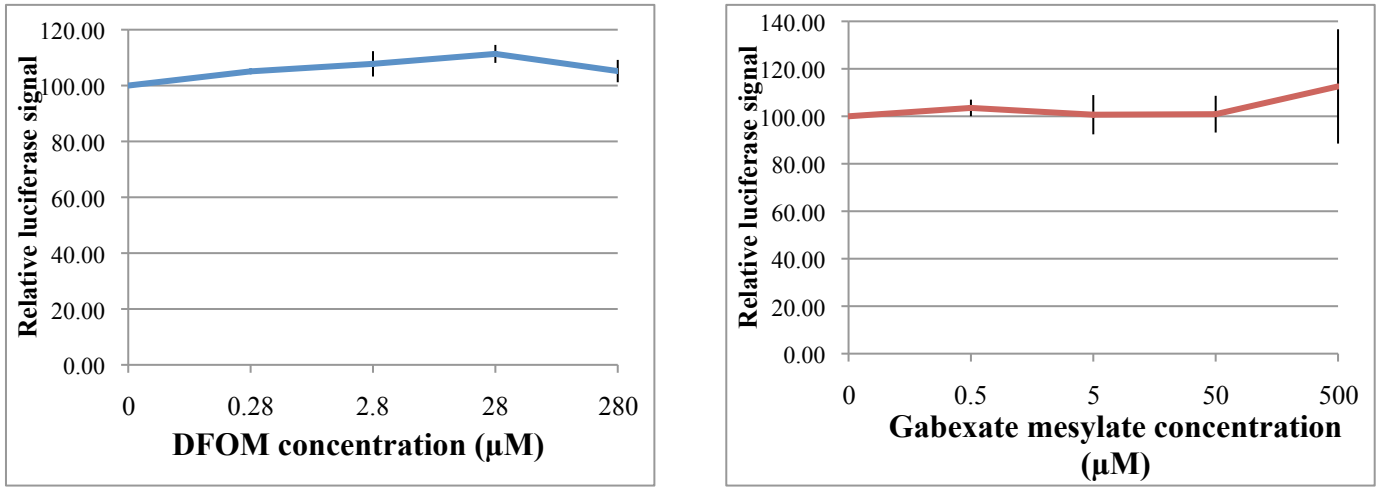


Figure 4.12: Neither DFOM nor gabexate mesylate demonstrated inhibitory effects on TNF-induced NF-κB activation

SH-EP cells were treated with these compounds for 90 minutes before stimulated with TNF-alpha (10 ng/ml). Cells were lysed after 7 hours. Values in the y-axis represented the mean luciferase signal relative to untreated control samples taken from 3 experiments with standard deviation indicated by the error bars.

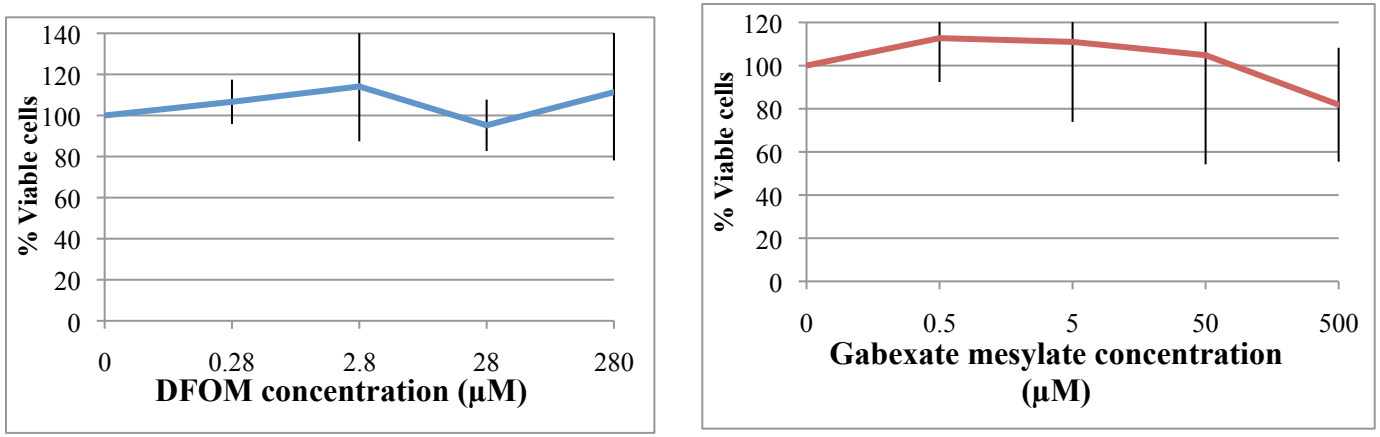
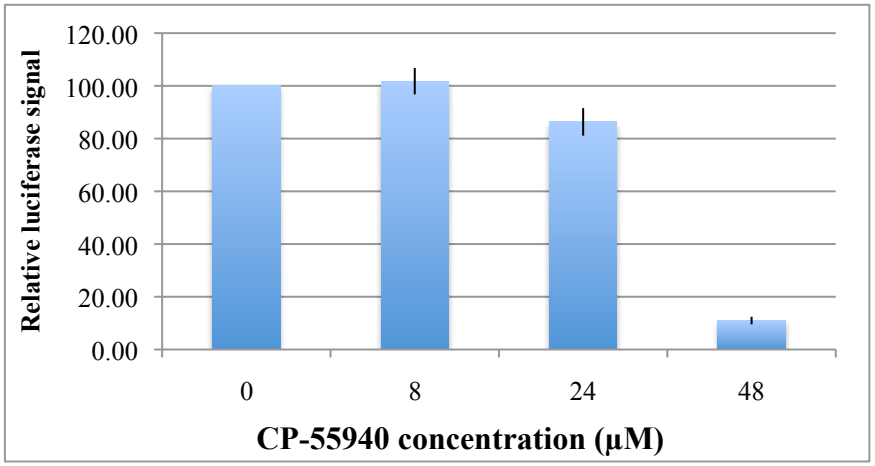


Figure 4.13: Neither DFOM nor gabexate mesylate showed cytotoxicity towards S-type neuroblastoma cells

Mean cell viability recorded 24 hours after SH-EP cells were treated with these compounds at the indicated doses demonstrated a dose-dependent decrease. Values in the y-axis were normalised to untreated samples and error bars indicated standard deviation from 6 experiments.



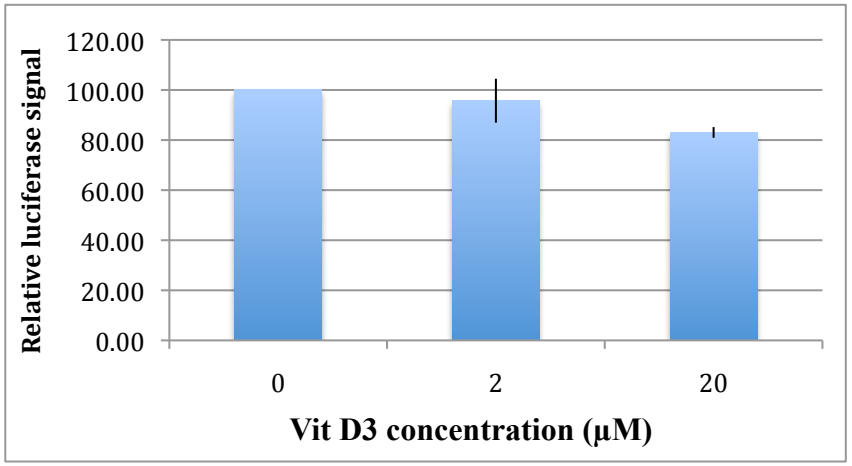
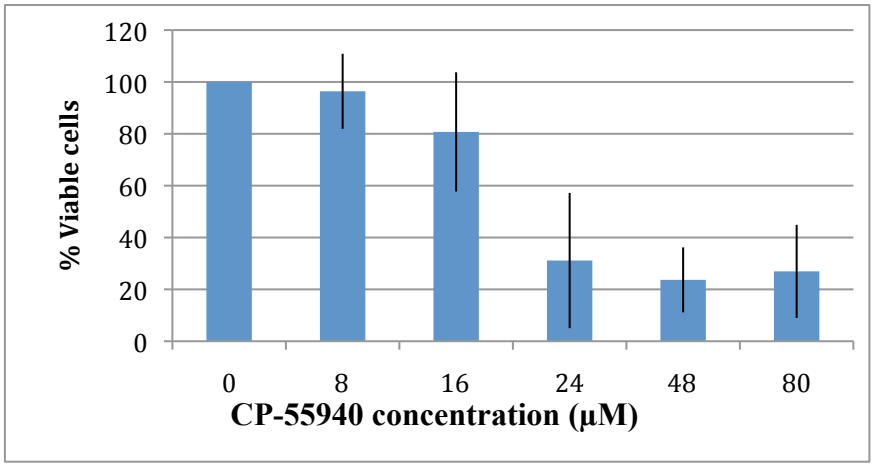


Figure 4.14: CP-55940 caused prominent NF-κB inhibition, but only modest decrease in luciferase signal was observed with vitamin D_3

SH-EP cells were treated with these compounds for 90 minutes before stimulated with TNF-alpha (10 ng/ml). Cells were lysed after 7 hours. Values in the y-axis represented the mean luciferase signal relative to untreated control samples taken from 3-6 experiments with standard deviation indicated by the error bars.



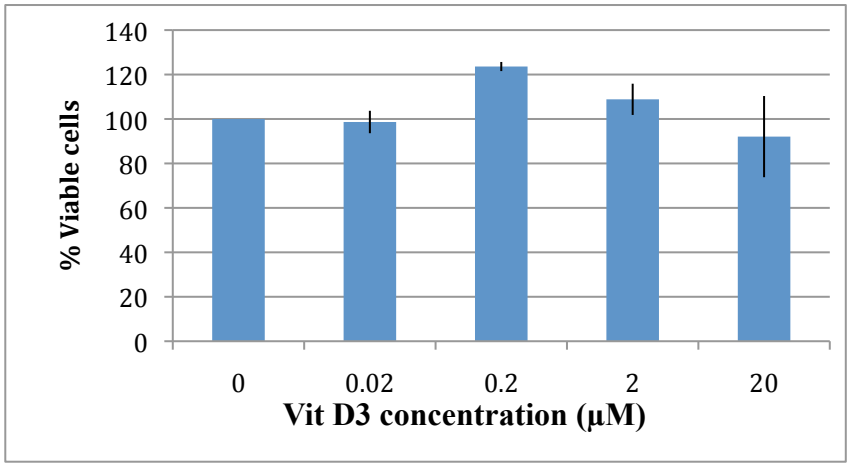


Figure 4.15: CP-55940 reduced neuroblastoma cell viability, however vitamin D3 was not found to be cytotoxic

Mean cell viability recorded 24 hours after SH-EP cells were treated with these compounds at the indicated doses demonstrated a dose-dependent decrease. Values in the y-axis were normalised to untreated samples and error bars indicated standard deviation from 3-6 experiments.

4.3.7 Tacrolimus, cyclosporin D, and rapamycin

The immunosuppressants tacrolimus (FK506) and cyclosporine D (CsD) inhibit calcineurin by binding to FKBP12 and cyclophilin respectively. Published studies investigating NF-κB inhibition have usually focused on FK506 and cyclosporine A (CsA) and little is known about CsD (Meyer, Kohler et al. 1997; Du, Hiramatsu et al. 2009). Rapamycin binds with FKBP12 to inhibit mammalian target of rapamycin (mTOR) which results in G1 cell cycle arrest. It has been found to inhibit NF-κB activation by suppressing IKK activity (Romano, Avellino et al. 2004).

In SH-EP cells, FK506 and CsD decreased TNF-alpha-induced NF-κB activation by 40% at concentrations 40 and 36 μM respectively (Figure 4.16). At these concentrations, 51% and 36% cells were killed respectively (Figure 4.17). At 20 μM rapamycin caused 16% NF-κB inhibition and 30% cell death.

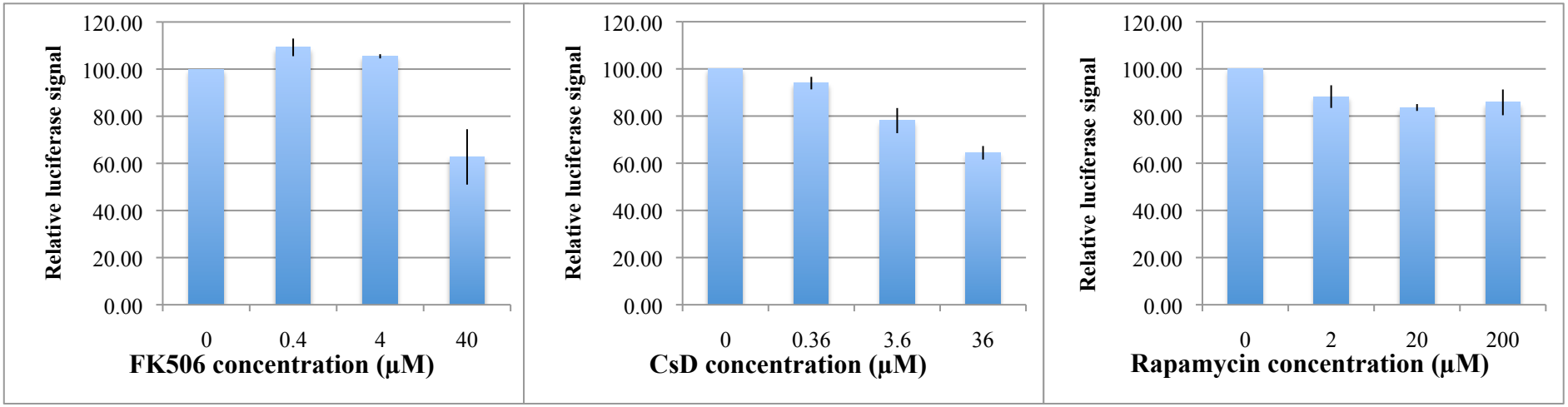


Figure 4.16: NF-κB gene transcription after treatment with FK506, CsD, and rapamycin

SH-EP cells were treated with these compounds for 90 minutes before stimulated with TNF-alpha (10 ng/ml). Cells were lysed after 7 hours. Values in the y-axis represented the mean luciferase signal relative to untreated control samples taken from 3 experiments with standard deviation indicated by the error bars.

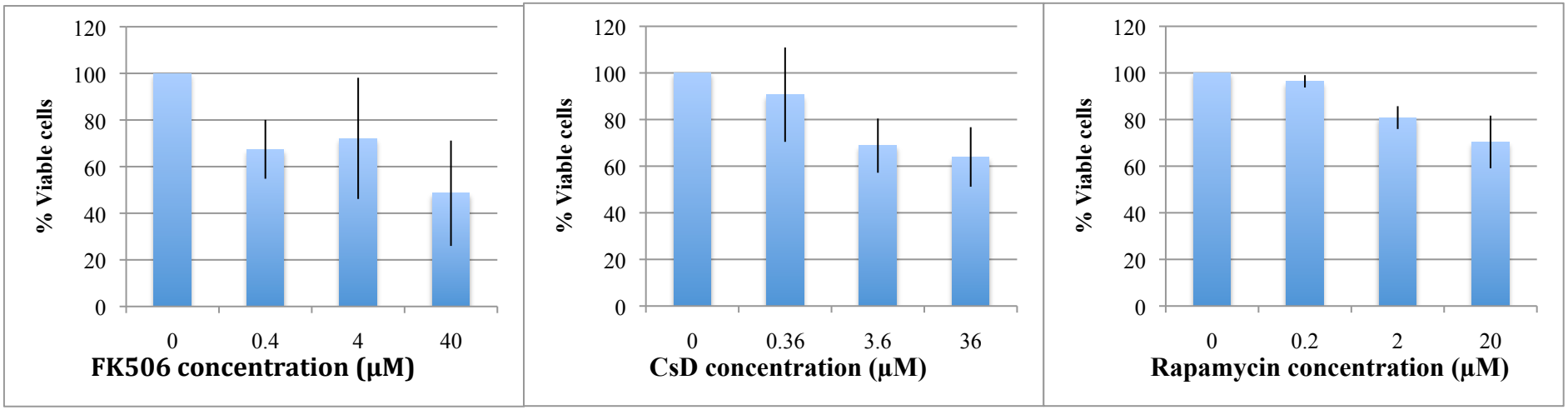


Figure 4.17: Cell viability after treatment with FK506, CsD, and rapamycin

Mean cell viability recorded 24 hours after SH-EP cells were treated with these compounds at the indicated doses demonstrated a dose-dependent decrease. Values in the y-axis were normalised to untreated samples and error bars indicated standard deviation from 3-6 experiments.

4.3.8 RO 106-9920

RO 106-9920 has been described as a small molecule that selectively inhibits essential ubiquitination activity associated with TNF-induced IκBα degradation and subsequent NF-κB activation (Swinney, Xu et al. 2002).

This compound was observed to reduce NF-κB gene expression by 68% at a concentration of 2.4 μM (Figure 4.18). At this concentration, 40% cytotoxicity was demonstrated (Figure 4.19).

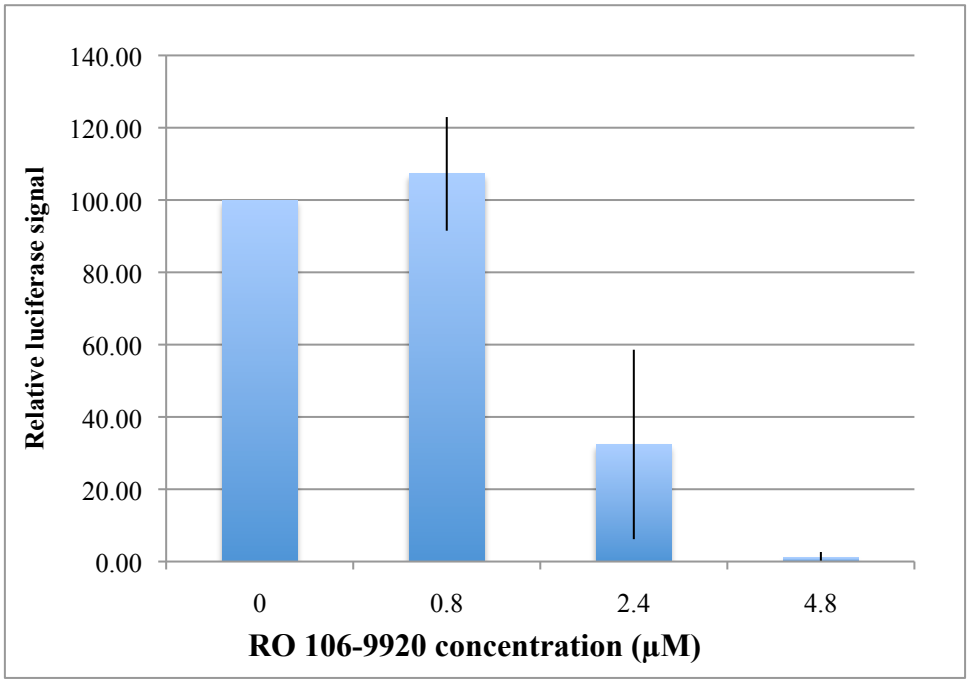


Figure 4.18: Low doses of RO 106-9920 decreased NF-κB dependent gene expression

SH-EP cells were treated with RO 106-9920 for 90 minutes before stimulated with TNF-alpha (10 ng/ml). Cells were lysed after 7 hours. Values in the y-axis represented the mean luciferase signal relative to untreated control samples taken from 6 experiments with standard deviation indicated by the error bars.

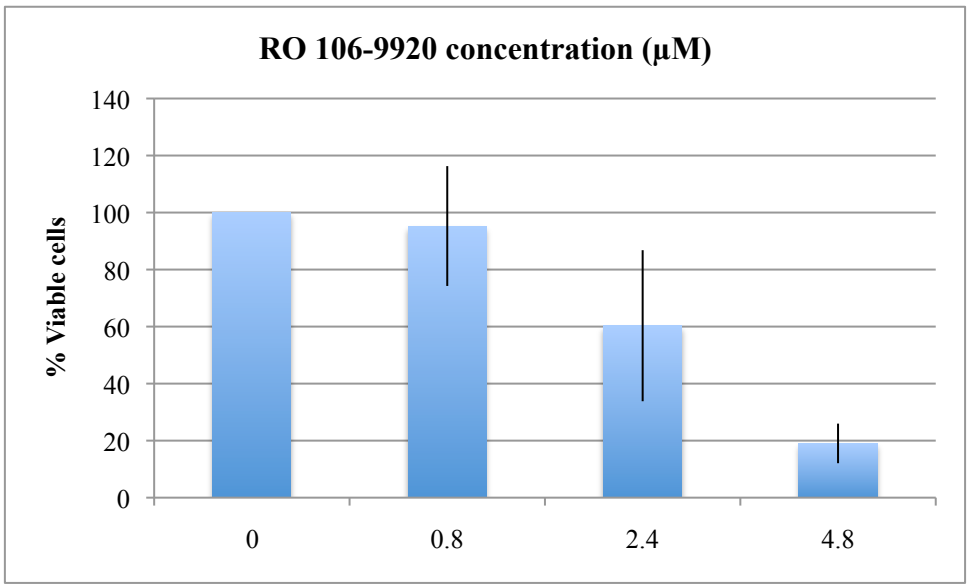


Figure 4.19: RO 106-9920 demonstrated cytotoxicity towards neuroblastoma cells

Mean cell viability recorded 24 hours after SH-EP cells were treated with RO 106-9920 at the indicated doses demonstrated a dose-dependent decrease. Values in the y-axis were normalised to untreated samples and error bars indicated standard deviation from 6 experiments.

4.3.9 H25(R) and H26(S)

The 2-amino-3-cyano-4, 6-diarylpyridine analogues, H25(R) and H26(S) (Figure 4.20) have been described in a previous study to have an inhibitory effect on IκB kinase ß (IKK-ß) (Murata, Shimada et al. 2003).

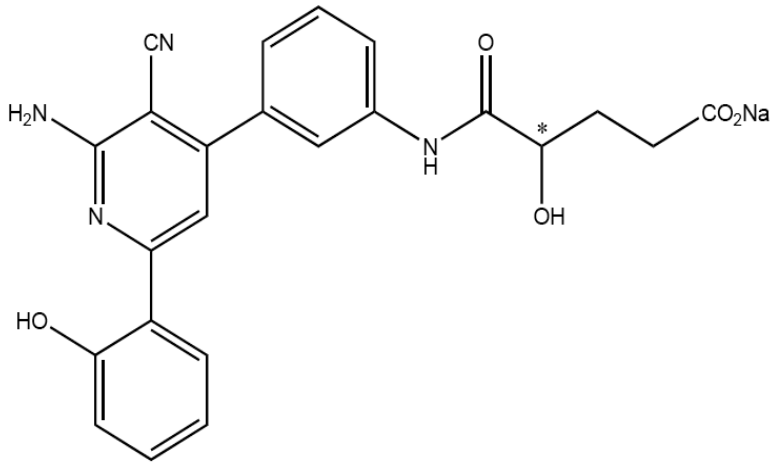


Figure 4.20: Chemical structure of H25 and H26
The asterisk * marked the location of the right (H25) and left (H26) isomers

Treatment with H26(S) at 320 µM caused 60% reduction of NF-κB gene expression (Figure 4.21) and 40% cell death (Figure 4.22). Interestingly, the same dose-dependent response was not observed in H25(R) treated SH-EP cells.

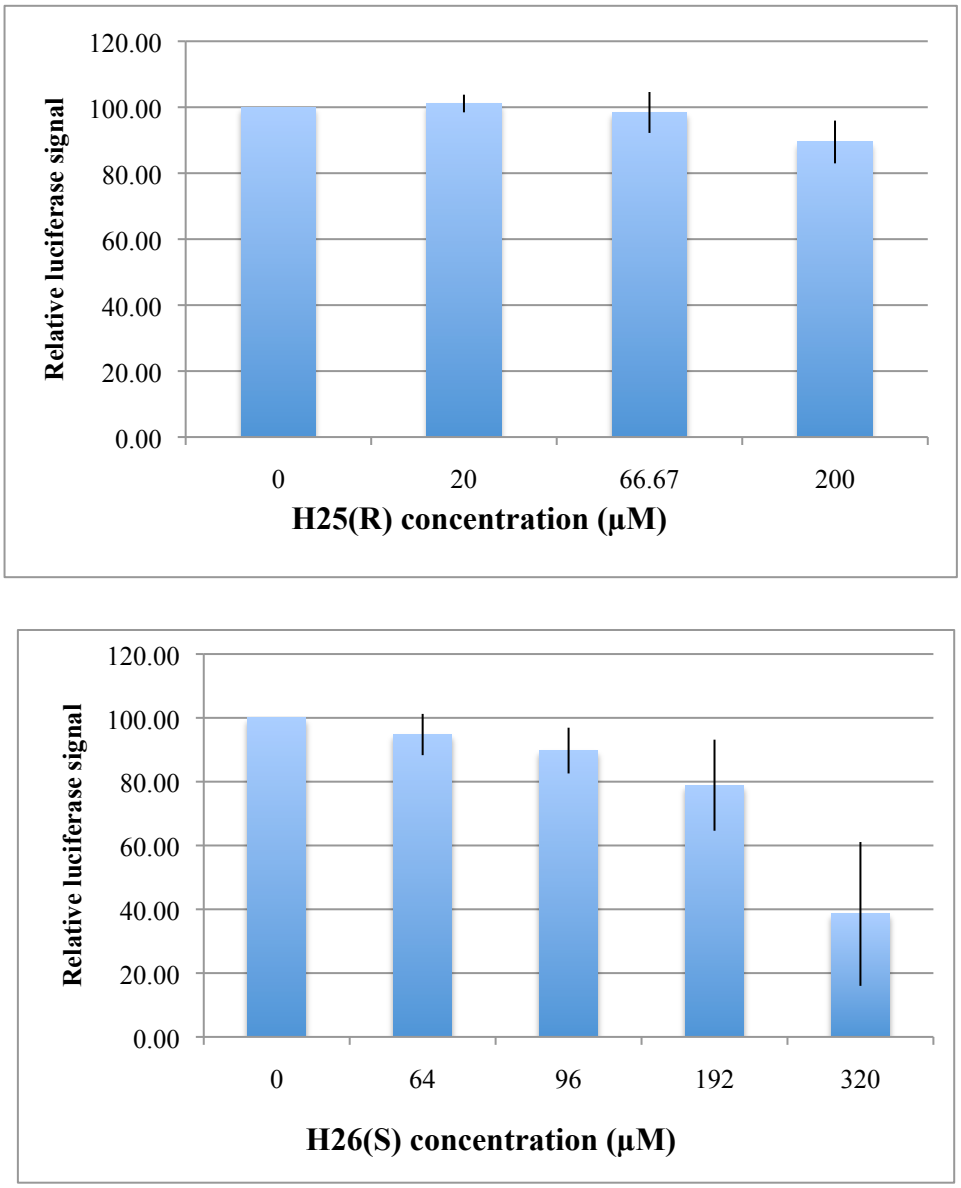


Figure 4.21: H26(S), but not H25(R), demonstrated inhibitory effects on NF-κB
SH-EP cells were treated with H25(R) and H26(S) for 90 minutes before stimulated with TNF-alpha (10 ng/ml). Cells were lysed after 7 hours. Values in the y-axis represented the mean luciferase signal relative to untreated control samples taken from 6 experiments with standard deviation indicated by the error bars.

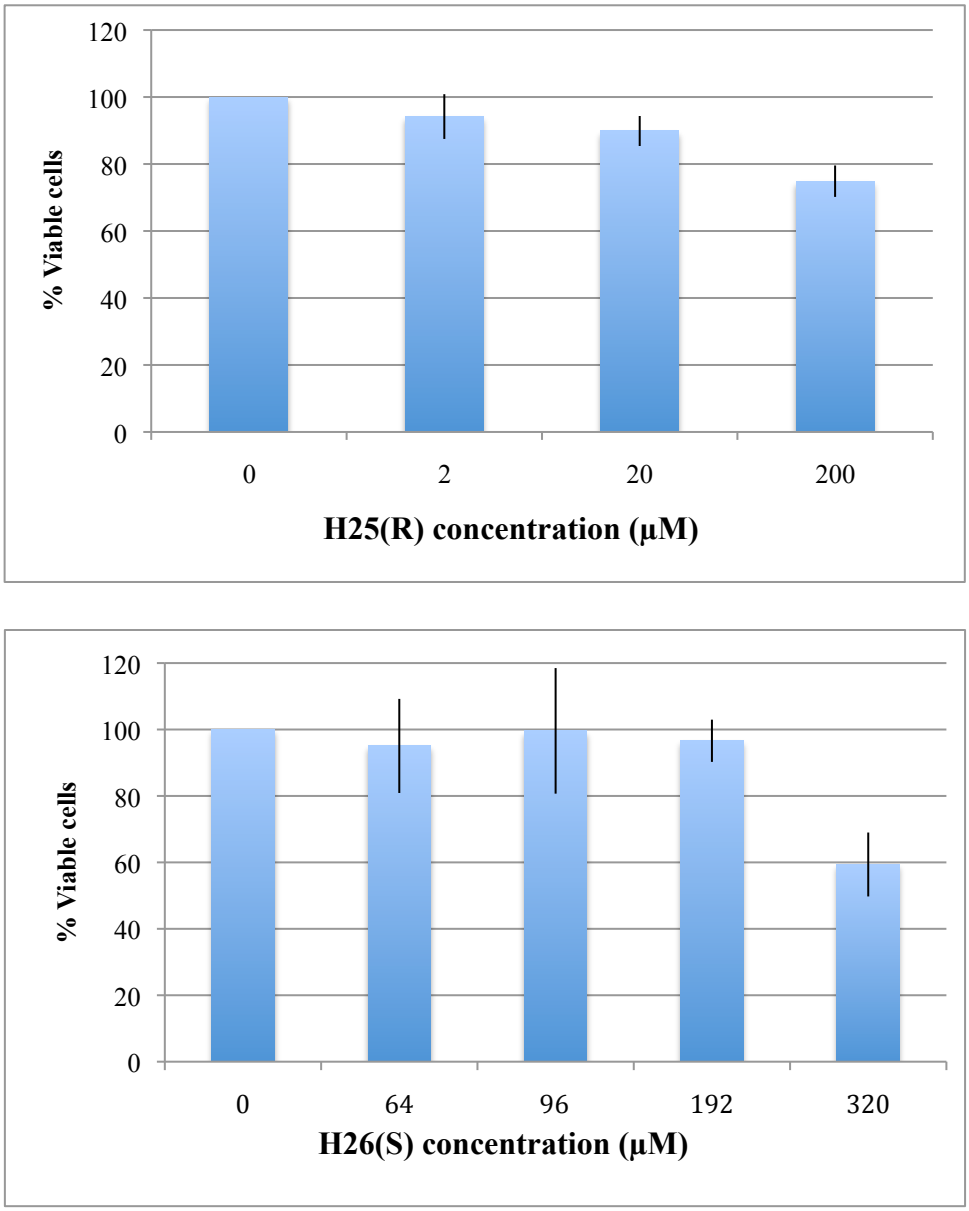


Figure 4.22: Cell viability after 24 hour treatment with H25(R) and H26(S)
Mean cell viability recorded 24 hours after SH-EP cells were treated with H25(R) and H26(S) at the indicated doses. Values in the y-axis were normalised to untreated samples and error bars indicated standard deviation from 6 experiments.

4.4 Drug combination

Each compound screened in the previous section had been selected as representative of structurally distinct NF-κB inhibitors. It has been observed that some of these NF-κB inhibitors caused cell death in SH-EP cells. Several published studies have suggested the potential of utilising chemical NF-κB inhibition to sensitise certain types of cancer cells to chemotherapy (Nakanishi and Toi 2005; Li and Sethi 2010). To investigate this hypothesis, we combined several NF-κB inhibitors with etoposide and assessed whether this increased the extent of cell death.

4.4.1 Principles of drug combination

When two drugs, A and B, are combined, a number of outcomes can be observed: each constituent might contribute to the combined effect in accord to its individual potency (additive); or in some cases the combination may exaggerate (synergistic) or diminish (antagonistic) the individual potency of each drug (Tallarida 2001).

A commonly used method for quantifying the effect of drug combination is by measuring the interaction index (γ) (Tallarida 2002). Doses of drug A (alone), drug B (alone), and doses of drug A and B in combination that produce the same intended effect level were determined experimentally. These doses are called *isoboles* and this method is also called the isobolar method.

The interaction index (γ) is defined as follows:

$$\gamma = \frac{a}{A} + \frac{b}{B} \qquad (1)$$

Where *A* is the concentration of drug A alone, *B* is the concentration of drug B alone, *a* is the concentration of drug A in combination, and *b* is the concentration of drug B in combination that produces the chosen effect level. The interaction is additive if $\gamma = 1$, synergistic if $\gamma < 1$, and antagonistic if $\gamma > 1$.

Another useful way to determine γ from equation (1) above is to use fixed proportions of drug A and B in experiments, so that the total dose (Z_t) is:

$$Z_t = a + b$$

The proportions of drug A and B respectively are:

$$p_A = \frac{a}{Z_t} \quad p_B = \frac{b}{Z_t}$$

Therefore, a = pA . Zt and b = pB . Zt and equation (1) can be rewritten as follows:

$$\gamma = \frac{p_A \cdot Z_t}{A} + \frac{p_B \cdot Z_t}{B}$$

$$\gamma = \frac{B \cdot p_A \cdot Z_t}{AB} + \frac{A \cdot p_B \cdot Z_t}{AB}$$

$$\gamma = \frac{Z_t(B \cdot p_A + A \cdot p_B)}{AB}$$

$$Z_t = \frac{\gamma \cdot AB}{B \cdot p_A + A \cdot p_B} \qquad (2)$$

And if R is the ratio A/B, therefore $A = B.R$ and equation (2) can be substituted as follows:

$$Z_t = \frac{\gamma \cdot A \cdot B}{(p_A + R \cdot p_B)B}$$
$$Z_t = \frac{\gamma A}{p_A + R \cdot p_B} \quad (3)$$

If the interaction is additive and $\gamma = 1$, then the total dose (Z_{add}) will be:

$$Z_{add} = \frac{A}{p_A + R \cdot p_B} \quad (4)$$

Combining equation (3) and (4) will be:

$$Z_t = \gamma \cdot Z_{add}$$
$$\gamma = \frac{Z_t}{Z_{add}}$$

In summary, this calculation demonstrates that the interaction index can be measured as the ratio between the total dose needed to give the desired effect in combination and the calculated total additive dose (Tallarida 2002).

Another way of calculating drug synergism is by comparing means between treatments (single and combination therapy) using one-way ANOVA (calculated using statistical programme PASW 18.0).

4.4.2 Combining etoposide with NF-κB inhibitors

Cell viability was investigated 24 hours after treatment with a combination of etoposide with either H26(S) or carnosol. Doses which corresponded to 40% cell death (IC_{40}) in previous experiments were selected (Table 4.1).

Table 4.1: Compounds selected for combination treatment

Compound	Dose (μM)	% Viable cells
Etoposide	20	61.27
H26(S)	320	59.37
Carnosol	60	58.57

Combining etoposide with H26(S) resulted in increased cell death (Figure 4.23). Extrapolating from the graph, the combination dose that caused 40% cell death was 6 and 95 μM for etoposide and H26(S) respectively, much lower than the individual doses of 20 and 320 μM. One-way ANOVA was significant ($p = 0.002$) and multiple comparison test using Bonferroni method found significant difference between single treatment groups (either etoposide or H26(S)) and combination treatment group ($p = 0.003$). Increased cell death was also noted at a lower concentration, however no statistical test was performed because the doses investigated were not identical. The interaction index (γ) is calculated as follows:

$$\gamma = \frac{Z_t}{Z_{add}}$$

$$\gamma = \frac{94.9 + 5.93}{160 + 10}$$

$$\gamma = 0.593$$

The interaction index (γ) is <1, indicating this combination is synergistic.

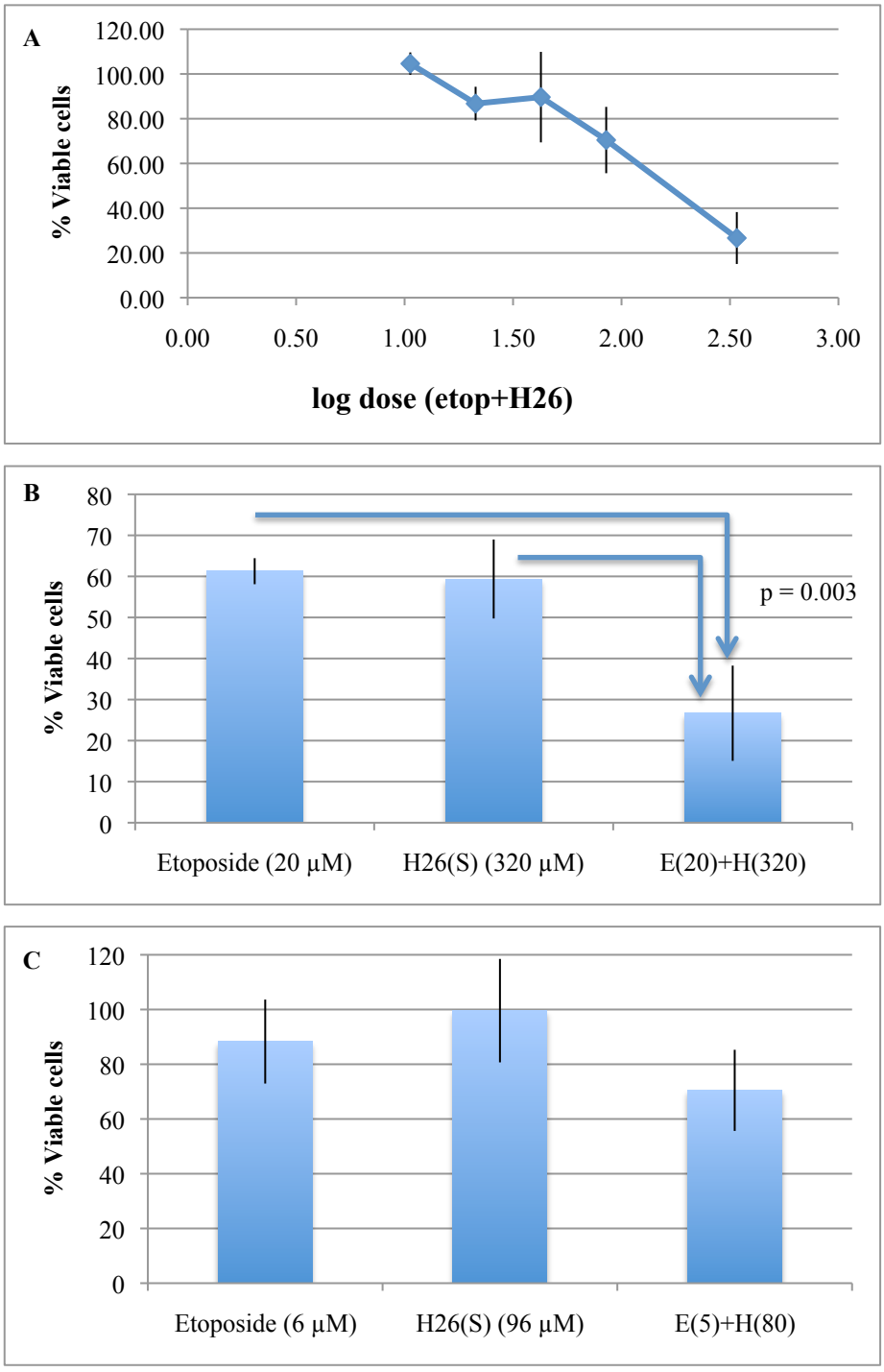


Figure 4.23: Significant increase in cell death when etoposide and H26(S) treatments are combined

Mean cell viability assessed 24 hours after SH-EP cells were treated with etoposide, H26(S), or both. A: Cell viability decreased in a dose-dependent manner. Values in the x axis represent the log total dose of etoposide and H26(S). B: Bonferroni test confirmed that increased cell death was statistically significant ($p = 0.003$). C: Increased cell death was also observed at lower doses.

The same synergistic interaction was not observed when etoposide was combined with carnosol (Figure 4.24). One-way ANOVA and multiple comparison test found the difference in mean cell viability between treatments not significant ($p > 0.05$) and the interaction index (γ) was 2.03, indicating no synergy for this combination.

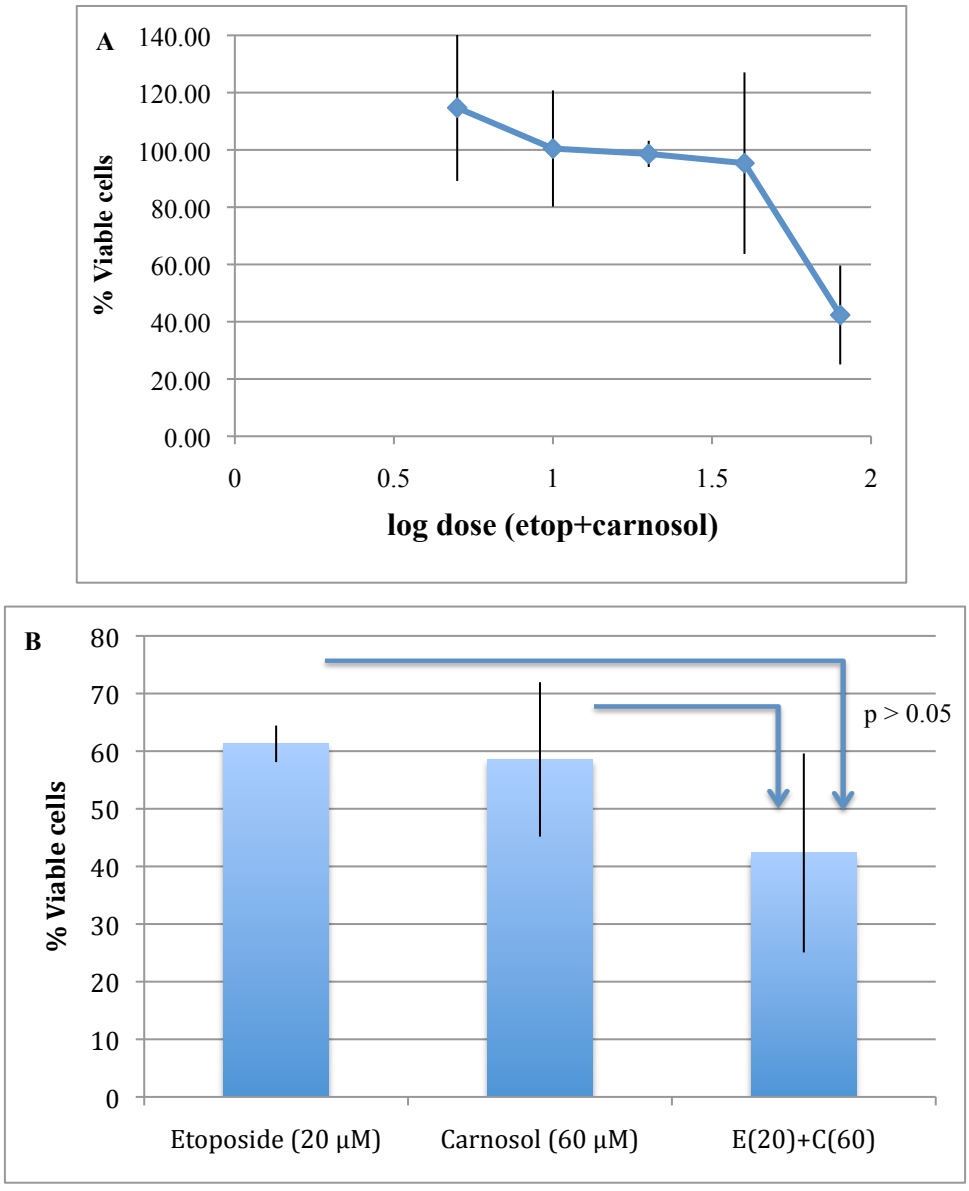


Figure 4.24: No significant difference observed when etoposide was combined with carnosol

Mean cell viability assessed 24 hours after SH-EP cells were treated with etoposide, carnosol, or both. A: Cell viability decreased in a dose-dependent manner. Values in the x axis represent the log total dose of etoposide and H26(S). B: No significant difference in cell viability between treatments ($p > 0.05$).

4.5 Discussion

Results from this chapter indicate that neuroblastoma cell lines conferred a degree of resistance towards one of the commonest chemotherapy agents, etoposide (Section 4.2). Etoposide also induced NF-κB activation in our cell lines, this is in agreement with other studies which suggested that chemotherapy induced NF-κB activation mediates chemoresistance through the transcription of many anti-apoptotic genes (Baldwin 2001; Nakanishi and Toi 2005; Li and Sethi 2010).

This project, therefore, focused on manipulating the NF-κB pathway to assess its role in determining neuroblastoma cell fate. Previous work in our laboratory has confirmed that NF-κB inhibition achieved by over-expression of IκBα resulted in significant cell death (Mullassery 2010), prompting the question whether the same effect could be achieved chemically. A number of chemical compounds which have been described in literature as NF-κB inhibitors had been identified and it is hoped that this can lead to the identification of new compounds which can enhance cell death in neuroblastoma.

Inhibition of TNF-α induced NF-κB activity in SH-EP cell line was achieved by several compounds (Table 4.2). However, for the majority of these compounds, the dose required to achieve a significant inhibition of NF-κB activity also caused a significant degree of cell death. Such similar dose-response curves for cell viability and NF-κB inhibition meant that some of the effect on cell viability was not due to

effects on NF-κB, and it would be difficult to assess synergy if these compounds were combined with etoposide. Moreover, many of the compounds are proteasome inhibitors and since proteasome is also involved in the degradation of other cellular factors (Kucharczak, Simmons et al.), it was decided that further investigations should focus on more specific NF-κB inhibitors.

Table 4.2: Summary of NF-κB inhibitors

Compounds which inhibit NF-κB	Compounds which do not inhibit NF-κB
TPCK	DiGG
TLCK	Sodium aurothiomalate hydrate
PGG	Deferoxamine mesylate
Auranofin	Gabexate mesylate
Carnosol	1α, 25-dihydroxyvitamin D_3
Magnolol	Rapamycin
Epigallocatechin gallate	H25(R)
Carmustine	
CP-55940	
Tacrolimus	
Cyclosporin D	
RO 106-9920	
H26(S)	

Consequently, potential synergy with etoposide was investigated in only five compounds: H26(S), carnosol, magnolol, EG, and RO 106-9920 (data not shown for the last three compounds). In previous experiments, these compounds were able to strongly inhibit NF-κB whilst only causing moderate cell death.

Enhanced cell death was only observed in the H26(S) and etoposide combination group (Figure 4.23). The interaction index for this combination was 0.593, indicating a synergistic interaction.

The next step approach of investigation would be to assess the mechanism of action of H26(S) and how the compound induces cell death in neuroblastoma. In cancer therapy, compounds which are able to activate apoptotic pathways will be more relevant than those which induce cell death through necrosis, as this means the toxic effect is non-specific and these compounds might be toxic to normal cells too.

The mechanism of action of H26(S) was assessed by Western blotting (data not shown). Initial results indicated that H26(S) treated samples showed a reduced degradation of IκBα compared to control samples, which would support Murata, et al. who described the compound as specifically inhibit IKK-ß (Murata, Shimada et al. 2003). Further experiments are required to confirm this, however it was not completed as new stock of H26(S) had to be synthesised and was not available before the end of the project.

The mode of cell death induced by H26(S) was investigated by two methods: firstly by assessing the activation of caspase 3/7 pathway (Promega), and secondly by

investigating Annexin V/PI binding using confocal microscopy. Preliminary results suggested that H26(S) induced apoptosis in a non-caspase 3/7 dependent manner (data not shown). However, as these experiments were only run once, we must not reach a conclusion solely based on this.

In summary, this work has identified the inhibition of IKK by H26(S) as a potential mechanism for increasing the effectiveness of etoposide-induced cell death. However, further investigation into the mechanism of induced cell death is warranted before such a method could be considered for use in a clinical setting.

CHAPTER 5

Overview and concluding remarks

A devastating childhood tumour, neuroblastoma continues to account for a significant proportion of paediatric oncology deaths. While recent advances have seen survival rates rise to >90% in other paediatric malignancies, most notably Wilms' tumour, survival for high-risk neuroblastoma, which accounts for the majority of patients presenting with advanced disease, remains dismally low at 20-30%.

This work herein described firstly a clinical review of contemporary management of neuroblastoma in a leading UK cancer centre, linking evolved therapies over a 20-year period and how they correlated with outcomes. Additionally, the study permitted comparison of institutional data with other international centres.

With deeper understanding of tumour biology, a number of molecular targets implicated in the development of chemoresistance in neuroblastoma have been identified. The second part of the research project explored the role of a key signalling pathway, NF-κB, in neuroblastoma cells. Building upon previous work in the Centre for Cell Imaging, this project screened a selection of pharmacological compounds for their effects on NF-κB pathway and induction of cell death in neuroblastoma cells. Furthermore, a potential synergistic interaction between an NF-κB inhibitor and chemotherapeutic agent was also investigated.

5.1 Key points

The clinical study highlighted several important findings. We observed that in correlation with dramatic intensification of therapy over the past twenty years for moderate to high-risk neuroblastoma, clinical outcomes of patients have arguably improved. The biggest gains were achieved in locally advanced stage 3 patients where 5-year survival rate has improved from 25% to 80% (Chapter 3). Similar improvements were also noted in stage 4 and 4S patients. Refined chemotherapy protocols together with surgical techniques to achieve "total" macroscopic tumour resection where possible were also associated with increased proportion of children having successful tumour resection. It is noteworthy that a doubling of the total number of patients achieving complete macroscopic tumour resection was achieved from the era 1985-1994 to 1995-2005. Current international expert opinions regarding the defining role of 'aggressive' surgery in high-risk neuroblastoma are conflicting. Findings from our study concurred with published works by Adkins et al.

and Castel et al. where we observed a trend towards improved outcomes for children having complete resection while the benefits in terms of overall survival were marginal (Castel, Tovar et al. 2002; Adkins, Sawin et al. 2004). In the current era of intensive multi-modal therapies, the evidence for surgery achieving long-term cure in high-risk neuroblastoma remain uncertain. These results cannot be taken in isolation from other significant advances in treatment modalities (e.g. stem cell transplantation, differentiation therapies, and possibly immunotherapy). Deeper understanding of tumour biology and molecular signalling pathways may guide future directions of therapy, holding the potential for "personalised" treatments based on unique tumour profiles, obviating the need for aggressive surgery in high-risk neuroblastoma cases.

One emerging candidate for molecular biology based study is the transcription factor NF-κB. Previous work in the laboratory reported that treatment with chemotherapeutic agents induced activation of NF-κB pathway in several neuroblastoma cell lines (Nelson, Ihekwaba et al. 2004; Mullassery 2010). Furthermore, inhibition of NF-κB through over-expression of its inhibitor IκBα resulted in increased cell death, thereby indicating that NF-κB is required for neuroblastoma cell survival. To explore whether NF-κB pathway is involved in the mechanism of drug resistance in neuroblastoma, a number of pharmacological compounds were screened for their effects on NF-κB pathway and induction of cell death in neuroblastoma cells.

Findings from my work highlighted a number of key observations. Many of the NF-κB inhibitors screened induced neuroblastoma cell death, yet combining an NF-κB inhibitor with a chemotherapy agent (etoposide was used throughout this study) did not always result in additive or synergistic interaction for four of the five combinations tested. This finding suggests that there may be other pathways involved that led to the reduced overall effect on cell death. For instance, a few of the compounds screened were inhibitors of the proteasome. However, the proteasome is also involved in the degradation of many other cellular pathways such as cyclins, cyclin-dependent kinase inhibitors p21Waf1 and p27Kip1, and tumour suppressor p53 (Kucharczak, Simmons et al. 2003). Nevertheless, towards the end of this research project, a potentially synergistic interaction between etoposide and an NF-κB inhibitor, H26(S) was noted. Only preliminary mechanistic investigations were conducted and therefore we must view this result with a grain of salt.

Neuroblastoma tumours exhibit a variety of morphological properties (i.e. stromal or S-type, neuroblastic or N-type, and intermediate or I-type) and most commercial neuroblastoma cell lines were cloned to express only certain characteristics. This biological heterogeneity must be taken into account during experimental works and for this reason, a growing stock of primary neuroblastoma cells, harvested at elective surgical resections and primary tumour biopsies at Alder Hey Children's Hospital are being characterised. It is hoped that these primary NB cells will resemble clinical behaviour of the tumour more closely. The potential contribution of these unique primary cultures to future research work cannot be underestimated.

5.2 Limitations of study

The clinical study relied on retrospective analysis of hospital case notes with inherent limitations on the availability of data recorded. It was fortunate to have a dedicated Alder Hey oncology database to retrieve information. Future prospective studies will be beneficial to observe ongoing trends, whilst long-term outcome data on these vulnerable patients (e.g. tumour relapse and secondary malignancies) may be gathered for additional studies. It may be debated that reporting from a single UK paediatric centre limits the size of the neuroblastoma study population. Whilst recent international groups have included multi-centre data with large patients populations, it is noteworthy that key findings from our UK centre largely support observations from several groups worldwide (Castel, Tovar et al. 2002; Adkins, Sawin et al. 2004; La Quaglia, Kushner et al. 2004).

As can be expected from an experimental work, screening pharmacological compounds for effect on NF-κB was not without its limitation. Optimisation of drug concentrations was time- and resource-consuming. Due to the large number of compounds available to our group, it was not always possible to test them in all the phenotypical variants of neuroblastoma cell lines or indeed in combination with a number of chemotherapeutic agents. My work focused on the SH-EP cells, an S-type neuroblastoma tumour and the chemotherapy agent etoposide, a topoisomerase-II. To eliminate variability in transfection efficiency, stable transfection was preferred over transient transfection, such that there were limitations in the choice of NBL cell lines. Utilising additional cell lines would undoubtedly add useful key findings to the conclusions. Time constraints within the twelve month research project precluded further work.

The techniques used in the experimental works all have their advantages and disadvantages. For this project, quantitative analysis was preferred whenever possible, e.g. cell death was assessed by MTT assay over a semi-qualitative manner utilising confocal microscopy, as this method was more time- and resource-efficient. However, this approach also required a larger sample amount, which proved restrictive at a number of times when compounds tested were not commercially available.

Furthermore, luminometry assay measures photons of light produced when injected luciferin is oxidised in the presence of the enzyme luciferase. This process requires the cells to be viable, and as such there is a risk of confounding when the compounds used are highly toxic.

5.3 Future directions and conclusions

The therapeutic potential of NF-κB inhibition as an adjunct to neuroblastoma therapy is an exciting prospect. A variety of anti-inflammatory agents widely used in clinical practice exhibit NF-κB inhibition and some NF-κB inhibitors have recently been introduced in cancer treatment, e.g. colorectal cancer and multiple myeloma. However, questions have been raised regarding the specificity of these compounds, e.g. proteasome inhibitor bortezomib. The potential effects of prolonged NF-κB inhibition in humans has not been fully studied. Since NF-κB plays an important role

in a number of key cellular responses, pharmaceutical companies developing these agents and clinicians prescribing them should reflect on potential adverse outcomes. Nevertheless, it is envisaged that specific IKK inhibitors will likely play a future role in cancer treatment to enhance the efficacy of conventional chemotherapy agents.

Neuroblastoma is a complex yet fascinating tumour which exhibits a spectrum of clinical behaviour. Cellular responses to chemotherapy are varied and likely to involve a number of key molecular/signalling pathways which interact as a network rather than individually. Manipulating NF-κB pathway represents an exciting therapeutic opportunity and further comprehensive studies are crucially required. Combining such observations with additional *in vitro* cell line work will provide key data for future laboratory-based studies on xenograft NBL models and the limited pool of primary neuroblastoma cultures harvested from these vulnerable models.

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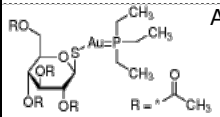
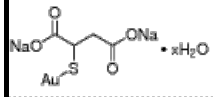
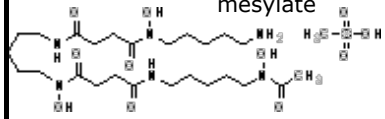
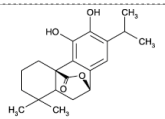
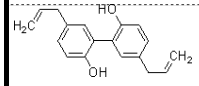
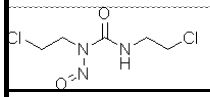
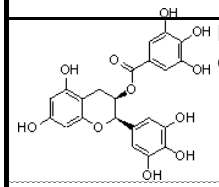
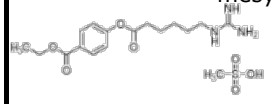
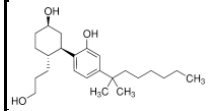
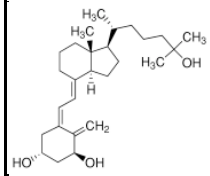
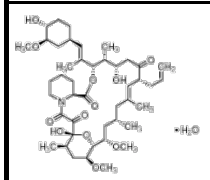
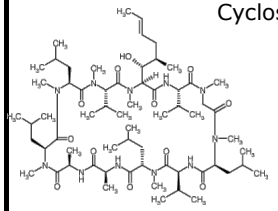
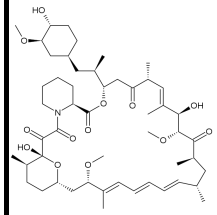
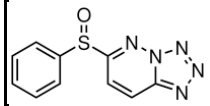
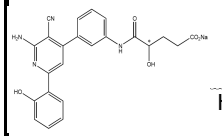
APPENDIX A

Summary of NF-κB inhibitors

	Compound	Description	M. Wt	Amount sent (mg)	mmol	Stock concentration	Active concentration	Solubility, Incubation time	Target	Ref
	Auranofin	Inhibits IKB kinase (IKK) by modifying Cys-179 of the IKKβ subunit 5.	678.48	10	0.014738828	100 mM	10-30 μM	1/500 → 1/1000	IKK-ß inhibitor	Clin.Exp.Immunol, 120, p79
	Sodium aurothiomalate hydrate		390.08 (anhydrous)	250		34.5 mg/ml		1/250 → 1/500		Clin.Exp.Immunol, 120, p79
	Deferoxamine mesylate	Iron chelator, cell arrest in G1 phase, anti-proliferative effects on vascular smooth muscle cells in vitro and in vivo, induce p53. Antioxidant properties in some studies.	656.79	1000	1.522556677	140 mM (in H_2O)	100 μM 70% inhibition	1/250 → 1/500		J.Exp.Med.1992, 175, p1181
	Carnosol	A phenolic diterpene with antioxidant and anticarcinogenic activities.	330.42	5	0.015132256	200 mM	10 μM	1/1000 → 1/2000	Inhibits IκBα phosphorylation	Carcinogensis 2002, 23, p983
	Magnolol	Bioactive plant component with antifungal, antibacterial, and	266.33	10	0.037547404	700 mM	15 μM, 44.8% inhibition	1/1000 → 1/2000	Downstream of MEKK-1	Planta Med. 2005 Apr; 71(4):338-43.
	Carmustine	DNA alkylating agent causing DNA interstrand crosslinks. In solid tumours e.g. glioma.	214.05	25	0.116795141	75 mg/ml		1/250 → 1/500		
	Epigallocatechin gallate	Antioxidant polyphenol flavonoid that inhibits telomerase and DNA methyltransferase.Blocks the activation of EGF receptors and HER-2 receptors.	458.37	50	0.109082183	66 mM	100 mM, 30% inhibition NF-kB, MTT 25% killing 500 mM	1/250 → 1/500		J.Nutr.1998, 128, p2334
	Gabexate mesylate	Serine protease inhibitor.	417.48	5	0.011976622	500 mM (only 14 μl!)	0.1 mM	1/250 → 1/500 but use only 0.5 μl hence 1/500 → 1/1000	Inhibits phosphorylation of IκBα	Crit.Car.Med, 2003, 31, p1147
	CP-55940	Selective cannabinoid receptor agonist.	376.57	10	0.026555488	40 mM	2 μM	1/250 → 1/500		Biochem.Pharma 64, p487
	1a, 25-dihydroxyvitamin D3	Biologically active form of vitamin D_3 in calcium absorption and deposition. Chemopreventive against prostate and colon ca, shows synergy with other anticancer compounds.	416.64	1	0.002400154	10 mM	0.2 μM	1/250 → 1/500	Stimulates phosphorylation of serine residues of IκBα	Exper.Cell.Res.2002, 272, p176

	Tacrolimus (FK-506 monohydrate)	Potent immuno-suppressant. Binds with FKBP12 to inhibit calcineurin and thus inhibit T lymphocyte signal transduction and IL-2 transcription.	822.02	1.7	0.002068076	20 mM	10-50 µM?	1/250 → 1/500		
	Cyclosporin D	Weak immuno-suppressant. Binds to cyclophilin to inhibit calcineurin. Prevents mitochondrial permeability transition pore from opening, inhibiting cytochrome c release (apoptotic stimulation factor).	1216.67	8.5	0.006986282	18 mM	CsA IC_{50} 50 µg/ml	1/200 → 1/400	Inhibits IKK activation	FEBS Letters 413 (1997) 354-358 (CsA)
	Rapamycin (Sirolimus)	Potent immuno-suppressant (inhibits response to IL-2, so blocking activation of T and B cells) and anticancer activity. Binds with FKBP12 to inhibit mammalian target of rapamycin (mTOR) which results in cell cycle arrest at G1.	914.172	12.5	0.013673576	100 mg/ml	100 ng/ml	1/250 → 1/500	Inhibits IKK activation	Eur.J.Cancer. 40, (2004), 2829-2836
	RO 106-9920	Inhibits NF-κB via selective inhibition of LPS and TNF-α-induced IκBα ubiquitination (IC_{50} = 3 µM). Blocks expression of NF-κB dependent cytokines: TNFα, IL-1β, and IL-6 in both cell culture (IC_{50} 600-700 nM) and in vivo in animal models	245.2605	14.2	0.057897623	12 mg/ml	IC_{50} = 3-10 µM	1/250 → 1/500	IKBα ubiquitination inhibitor	J.Biol.Chem. (2002), vol 277, 26, 23573-23581
	HHA025 ®		454.1434	22.4	0.049323628	100 mM	IC_{50} = 8 µM	1/250 → 1/500, 1 hr before TNFα stimulation	IKK-ß inhibitor	Bioorg.Med.Chem.Lett 13, (2003), 913-918
	HHA026 (S)		454.1434	20	0.044038953	160 mM	IC_{50} = 8 µM	1/250 → 1/500, 1 hr before TNFα stimulation	IKK-ß inhibitor	Bioorg.Med.Chem.Lett 13, (2003), 913-918

APPENDIX B

Poster submitted to Postgraduate Science and Medicine (PRISM) Conference, October 2009, Manchester

Winner of Best Poster award

Screening NF-κB Inhibitors for Effects on Neuroblastoma Cell Fate

Adeline Salim,[1,2] Catherine A Heyward,[1] Dhanya Mullassery,[1,2] Heather P McDowell,[3] Edwin C Jesudason,[2] Violaine Sée,[1] Mike R H White,[1] Paul D Losty.[1,2]
1 Centre for Cell Imaging, School of Biological Sciences, University of Liverpool. 2 Institute of Child Health, Royal Liverpool Children's Hospital NHS Trust, Alder Hey. 3 Department of Oncology, Royal Liverpool Children's Hospital NHS Trust, Alder Hey.

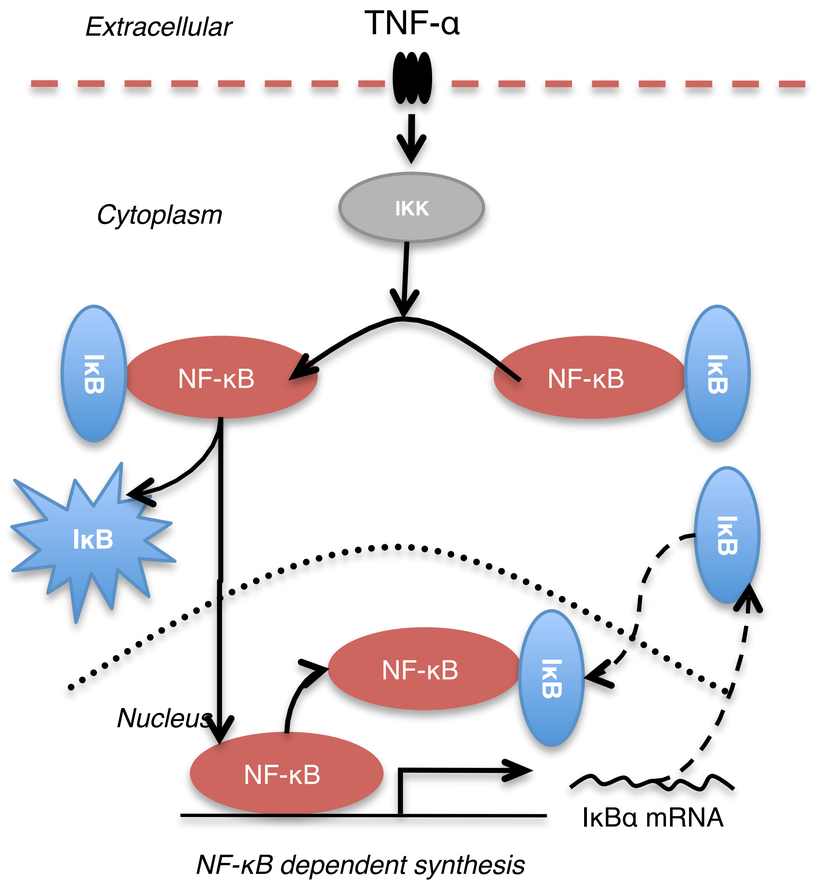
Background

Neuroblastoma

- A neuroendocrine tumour rising from the neural crest.
- The commonest childhood solid tumour, approximately 1500 new cases in Europe per annum.[1]
- Known for its heterogeneity:
  - The majority is highly resistant despite intensive multi-modal therapy.
  - Long term survival for these cases remains poor, currently less than 40%.[2]
  - However, a subset of tumours will spontaneously regress.
  - This has lead scientists to focus research on "switching on" apoptosis in neuroblastoma cells.

Nuclear factor kappa B proteins (NF-κB)

- A family of dimeric transcription factors found in virtually all cell types.
- Regulates cell survival, proliferation, as well as immune and inflammatory responses.
- NF-κB is activated by numerous different stimuli, causing oscillations in NF-κB localisation (Fig 1) required for neuroblastoma cell survival, as observed in live single cell microscopy (Fig 2).



- The frequency of oscillations has been shown to regulate gene expression.[3]
- Previous work has shown that NF-κB inhibition by endogenous inhibitor IκB kills neuroblastoma cells, and similarly treatment with a well-known NF-κB inhibitor Bay-11 also achieves significant cell death (Fig 3).

Figure 1: The NF-κB pathway

- NF-κB is activated by numerous different stimuli, a common one, for example, is TNF-α.
- This leads to activation of IκB kinase (IKK) complex, and its substrate IκB, an inhibitor of NF-κB.
- Degradation of IκB releases NF-κB.
- Activation of NF-κB causes increased synthesis of IκB, which then binds nuclear NF-κB causing its return to the cytoplasm (oscillation in NF-κB localisation).
- Activation of NF-κB has various consequences such as upregulation of genes involved in cell proliferation, cell invasion, and cell death (anti-apoptotic genes).

Current techniques

Cell viability

- MTT assays are used to assess whether chemicals from the LOPAC1280 library can increase the extent of etoposide-induced cell death in neuroblastoma cell lines.

NF-κB activity

- The ability of these compounds to regulate NF-κB activity is measured using an NF-Luc reporter in a luminometry assay.

Mode of cell death

- Compounds demonstrating synergistic effects will be further studied by caspase assay and qPCR to determine the NF-κB –regulated genes involved and the mode of cell death.

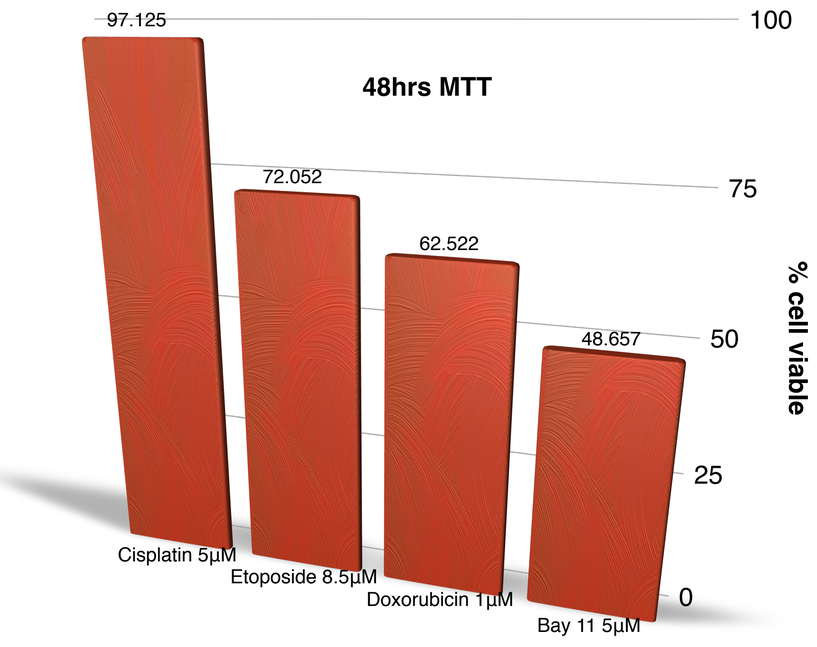


Figure 3: Comparison of cell viability following 48 hours treatment with chemotherapy agents and NF-κB inhibitor Bay 11 as assessed by MTT assay.

- The extent of cell death after treatment with Bay 11 was significantly more than that achieved by all three chemotherapy agents (means comparison by Bonferroni test, $p<0.05$) in S-type neuroblastoma cell line.

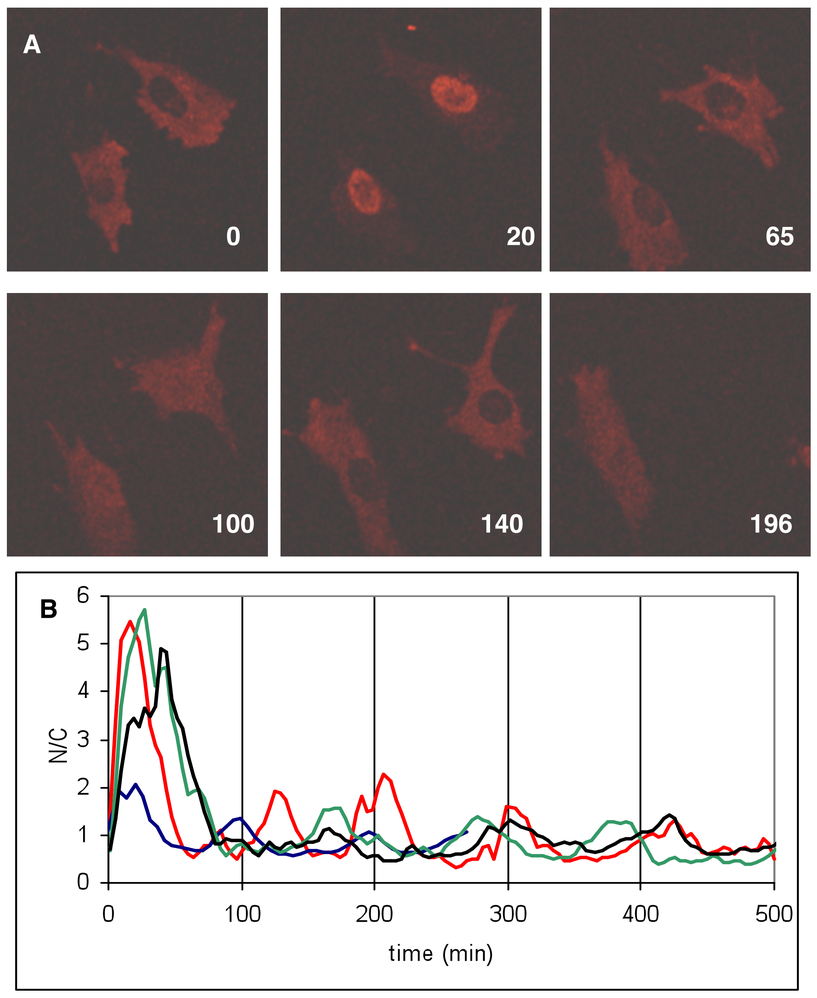


Figure 2: SHEP cells stably transfected with p65dsRed show 100 minute oscillations in p65 localisation.

A. Time-lapse confocal images of SHEP cells stably transfected with p65dsRed showing nuclear:cytoplasmic oscillations in p65dsRed localisation following stimulation with 10ng/ml TNFα. Scalebar 50μm, time in minutes.
B. Time-course of nuclear:cytoplasmic localisation of p65dsRed. Each line shows data for an individual cell.

Aim

- To develop a new therapy for neuroblastoma based on NF-κB manipulation, we are screening compounds from a chemical library for NF-κB inhibitors capable of synergistically increasing cell death in combination with etoposide treatment.

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C Heyward and D Mullassery contributed data that have been used in this poster.

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APPENDIX C

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Neuroblastoma: a 20-year experience in a UK regional centre.

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NEUROBLASTOMA: A 20-YEAR EXPERIENCE IN A UK REGIONAL CENTRE

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Purpose: The role of surgery in the management of neuroblastoma yields conflicting reports. We report a 20-year experience from a UK centre that analyses trends in survival in the context of evolving cancer therapies for neuroblastoma.

Method: Hospital records of 91 neuroblastoma patients from 1985-2005 were studied. Patient demographics, data from operating notes and tumour biology (MYCN status) where available were analysed.

Results: Male:female ratio=0.75:1, median age at presentation 1.9years (newborn-14.9years). Primary tumours were in the adrenal gland (71%), thorax (13%), paravertebral region (9%), pelvis (1%), and other sites (6%). Surgery consisted of primary resection or delayed operation following tumour biopsy/chemotherapy. Overall survival (INSS classification) was 100% for stage 1(n=3),90% for stage 2(n=10),46% for stage 3(n=13),13% for stage 4(n=55)and 56% for stage 4s disease(n=9). During the eras 1985-1994 vs 1995-2005, survival for stage 3 lesions was 25% and 80%(p=0.04) with marginal benefits observed in stage 4 disease (6% vs 22%, p=0.156). Delayed tumour resection was not performed in **20 (36%)** stage 4 patients due to progressive disease. Their median survival was 8.2months vs 44.5months for those who had surgery (p<0.001). Complete tumour resection was achieved in **62%** of stage 3 and 4 patients during 1995-2005 compared to **38%** in 1985-1994. The extent of surgical resection (complete vs partial) showed no significant differences in overall survival or relapse rates. Postoperative morbidity occurred in 22.4% of cases highlighting technical challenges in resection of neuroblastoma. No child with MYCN amplification survived vs 59% survival in non- amplified cases (p=0.012).

Conclusion:
Whilst complete tumour resection may be desirable in advanced neuroblastoma (stage 3 and 4), our findings suggest that the degree of resection is not significantly associated with better overall survival/relapse. Improved outcomes in the 1995-2005 era with stage 3 and 4 tumours complements the introduction of new high dose-intensive chemotherapy regimens and other adjuvant therapies for this enigmatic disease.