THE RELATIONSHIP BETWEEN N-*myc* COPY NUMBER OR EXPRESSION AND RESISTANCE TO THERAPY IN HUMAN NEUROBLASTOMA CELL LINES

By

ANNE LIVINGSTONE

A thesis submitted for the degree of Master of Science
to the Faculty of Medicine, University of Glasgow.

CRC Department of Radiation Oncology, University of Glasgow

May 1993

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT

The copy number of the N-*myc* oncogene provides a prognostic index for neuroblastoma. The mechanism for this is not known, but may be related to cellular resistance to radiation or cytotoxic drugs.

Seven human neuroblastoma cell lines were used to investigate the relationship between N-*myc* copy number or expression and sensitivity to ionising radiation and cisplatin. N-*myc* copy number was assessed by Southern blotting and hybridisation using the p-Nb1 probe. The signal produced from DNA of the cell lines was compared with that of human placental DNA which has single copy N-*myc* (the normal copy number for diploid cells). A range of N-myc copy numbers from 1 - 800 was found. Expression levels of N-*myc* messenger RNA were compared by "dot-blotting" and subsequent hybridising to the p-Nb1 probe. Radiosensitivity was investigated by irradiating multicellular tumour spheroids or cell monolayers using a ^{60}Co source, (dose range 0.5 - 5 Gy). Survival curves were produced using both colony formation and spheroid regrowth delay as end points. The response to radiation was assessed by surviving fraction at 2 Gy (SF_2); values ranged from 0.13 - 0.52. Sensitivity to cisplatin was indicated by comparison of isoeffective concentrations (concentration required to produce 1 log cell kill) from survival curves produced as above. These concentrations ranged from 7.5 - 13 μM.

Cisplatin studies showed a border-line correlation between N-*myc* copy number (though not expression) and resistance to this drug. If this relationship is causal it may explain why treatment fails in those patients with elevated N-*myc* copy number. However, no correlation was found between N-*myc* copy number or expression and sensitivity to radiation. It is possible that N-*myc* amplification confers resistance to some, but not all, treatments used in the therapy of neuroblastoma. Further investigations along these lines may lead to the identification of agents which are most appropriate for the treatment of neuroblastoma with amplified N-*myc* gene.

ACKNOWLEDGEMENTS

Part of the work described in this thesis was supported by a grant from the Cancer Research Campaign.

I should like to thank my supervisors Dr. R.J. Mairs and Dr. T.E. Wheldon for their advice and encouragement. I should also like to thank my colleagues Dr. J. O'Donoghue, Mr. J. Russell (who supplied the data for radiosensitivity of XRNB1-G), Dr. M. Gaze, Dr. W. Millar, Dr. F. Rinaldi and also Miss J. Thomson for her kind assistance in preparing the manuscript.

CONTENTS

		PAGE
ABSTRACT		2
ACKNOWLEDGEMENTS		3
1.	CANCER RELATED GENES	6
2.	NEUROBLASTOMA	12
3.	MOLECULAR GENETICS OF NEUROBLASTOMA	18
4.	CHEMOTHERAPY OF NEUROBLASTOMA	27
5.	RADIOTHERAPY OF NEUROBLASTOMA	32
6.	MATERIALS AND METHODS	38
7.	RESULTS	45
8.	DISCUSSION	72
9.	CONCLUSION	75
REFERENCES		76

CHAPTER 1

CANCER RELATED GENES

	PAGE
Introduction	6
Oncogenes	7
Transformation Assays	7
Proto-oncogenes	8
Activation	8
Tumour Suppressor Genes	10

CHAPTER 1. CANCER RELATED GENES

INTRODUCTION

In the early 1980's, the molecular genetic era of human cancer was ushered in with the discovery of "dominantly acting" activated cellular oncogenes (Krontiris & Cooper, 1981; Shih *et al* 1981). Oncogenes are cellular genes which may, in the activated form, contribute to the process of malignant change. The first activated oncogenes were isolated by transfection of DNA from human tumour cells into mouse NIH 3T3 cells, a process that gave rise to cellular changes thought to be consistent with neoplastic transformation (see page 7). These activated oncogenes were quickly discovered to be homologous to transforming retroviral genes (Der *et al* 1982; Parada *et al* 1982). This finding, which was predicted on the basis of the seminal investigations showing that the avian retroviral src oncogene had evolved from the capture of a cellular proto-oncogene, (Stehelin *et al* 1976) led to the identification of a number of putative cellular oncogenes.

The discovery that activated oncogenes could be found in 10 to 30% of human cancers led to theories that activation of single or multiple cooperating cellular oncogenes was sufficient per se for tumourogenesis to occur. These theories were strengthened when it was found that the growing list of oncogene functions included growth factors, growth factor receptors, signal transducers, protein kinases and transcriptional activators - all of which, when behaving aberrantly, might lead to uncontrolled cell proliferation.

Lately another class of cancer related genes has begun to emerge; the so called tumour suppressor genes (Stanbridge 1985). Earlier studies had shown that when malignant cells were fused with normal cells, the resulting hybrids behaved as non-tumourogenic cells. This phenomenon of tumour suppression indicated that a gene (or genes) from a normal cell might replace a defective function in a cancer cell, thus allowing it to be influenced by normal regulators of cell growth. Such genetic elements have been termed tumour suppressor genes.

ONCOGENES

Oncogenes were first described in viruses. In 1911, Rous described a transmissable sarcoma in chickens which was later found to be due to a viral oncogene. Subsequently a large number of such viruses have been isolated from rodents, cats and monkeys and are known to cause tumours in these species. To date RNA viruses have not been implicated in the development of human cancers. Once oncogenes were recognised in RNA tumour viruses, studies were carried out to ascertain the origin of these sequences. cDNA fragments of oncogene regions from the Rous Sarcoma virus were isolated and used to probe DNA from various tissues of the chicken. As a result of these investigations, sequences homologous to oncogenic regions of the virus were found to be present in the genomic DNA of virus-free chickens. Further similar studies revealed DNA sequences homologous to viral oncogenes in yeast, mice and humans. These normal cellular sequences have been referred to as proto-oncogenes (Bishop 1983; Varmus 1984). An oncogene carried by a virus is termed v-*onc*; the proto-oncogene is called c-*onc*. Investigations aimed at determining whether oncogenes firstly arose in viruses or cells of higher organisms led to the conclusion that cellular proto-oncogenes most likely developed first. Proto-oncogenes have both exons and introns, while v-*oncs* possess only the former. Therefore, it seems more likely that the viruses, by infecting host cells, picked up the m-RNA of c-*oncs* (Minden 1987).

TRANSFORMATION ASSAYS

A test of whether a presumed carcinogen - chemical, virus or gene - is in fact a genuine carcinogen is the ability to transform cells or produce tumours in animals. The mouse fibroblast line NIH 3T3, developed by Green *et al* in the early 60's, is a powerful tool in transfection studies. One of the main differences between non-transformed cells and those which have been transformed is in their growth characteristics. Primary cultures of the former grow to a particular density and stop and will not resume growth until diluted. This phenomenon is known as "contact inhibition". Transformed cells grow in a non-ordered way and tend to pile up; i.e.

they appear to have lost contact inhibition. It has been found that DNA isolated from cancer cells, when taken up by 3T3 cells, leads to the transformed phenotype. The most advanced assay for *in vivo* transformation studies currently available involves introducing oncogenes into the germ lines of mice or other species. These so called transgenic animals are then observed for the development of tumours (Burck *et al* 1988).

PROTO-ONCOGENES

Proto-oncogenes are normal cellular genes which are thought to be involved in control of proliferation or differentiation and which, when activated, may contribute to tumourogenesis. They can be activated by a number of mechanisms including point mutation, amplification and translocation. There are now known to be about forty or so cellular proto-oncogenes.

In order for non malignant cells to enter a proliferative state they require an external signal which is received by a membrane receptor and is transmitted via the cytoplasm to the nucleus (Minden 1987). Proto-oncogenes have been found which function at each step of the signal transduction process. For example, the erb-B proto-oncogene appears to be homologous to the gene encoding Epidermal Growth Factor. The products of the src, abl, and *ras* proto-oncogenes act at the level of secondary messengers on the cytoplasm whereas the *myc* family of proto-oncogenes encode nuclear proteins.

ACTIVATION

Point Mutation

A point mutation in a gene is the substitution of one nucleotide base for another. This in turn may result in a single amino acid alteration in the resulting protein, sometimes with dire consequences. For example, in the activated H-*ras* gene associated with bladder cancer, a single base substitution in the 12th codon causes

the substitution of valine for glycine giving rise to a different protein product (Tabin *et al* 1982; Reddy *et al* 1982). This altered product has been implicated in the initiation of tumourogenesis, and has been found in a bladder tumour cell line. However, activated H-*ras* is only found in a proportion of patients with bladder cancer and the significance of this finding to the majority of patients is unknown (Thomas and Waxman 1989). Mutations involving the family of *ras* genes are associated with a number of neoplasms including human leukaemias and colon carcinomas.

Translocations

These occur frequently in some types of tumours and therefore they may play a role in the development of neoplasia. The best known example occurs in chronic myeloid leukaemia where there is a reciprocal translocation between chromosome 9 and chromosome 22 giving rise to a novel gene product and the possible onset of tumour development (Rowley 1984). In 1981 Klein proposed that chromosome breaks might give rise to activation of oncogenes situated proximal to break points (Klein 1981). Recently several genes situated at the sites of common translocations have been identified and indeed many of these have turned out to be known oncogenes (Minden 1987).

Amplification

Multiple copies of proto-oncogenes are often found in tumour cells; for example c-*myc* amplification in acute promyelocytic leukaemia and N-*myc* amplification in neuroblastoma cells. Amplifications of H-*ras*, N-*ras* and K-*ras* have been reported in various tumours (Minden 1987). Amplification of N-*myc* in neuroblastoma and the erb-2/neu proto-oncogenes in human breast cancer have been correlated with advanced stage disease and a poor prognosis (Burck *et al* 1988). However, the role of these amplified oncogenes in the development of tumours is not yet known. It is possible that amplified oncogenes maintain the aggressiveness of disease rather than initiate tumourogenesis.

TUMOUR SUPPRESSOR GENES

Certain experimental findings suggest that cancer is a recessive disease at the cellular level. For example, experiments have shown that the malignant phenotype can be suppressed by hybridisation of normal cells with tumour cells (Burck *et al* 1988). In recent years we have witnessed the discovery of a class of cancer-related genes which unlike cellular oncogenes act not to promote malignant change, but to suppress it. Accordingly, these tumour suppressor genes or anti-oncogenes are manifest not when they are activated (as are cellular oncogenes) but rather when they are absent or inactivated. Examples of these include the retinoblastoma susceptibility gene (Rb) on chromosome 13 and the deletion on chromosome 11 associated with Wilms' tumour. Both these types of tumour require the loss of two alleles for onset of tumourogenesis, and in the case of retinoblastoma the loss of the first allele is often inherited. Deletion of the short arm of chromosome 1 is the most consistent cytogenetic abnormality in primary neuroblastomas. This will be described in greater detail later. Some familial cases of neuroblastoma have been documented (Hayes and Smith 1989; Brodeur and Fong 1989). Identification and cloning of this putative suppressor gene on chromosome 1 may lead to pre-natal diagnosis in affected individuals. The importance of these tumour suppressor genes in the development of neoplasms is beginning to emerge and it may eventually be possible to restore the lost gene as a means of cancer therapy. It is worth mentioning the p53 gene at this stage which seems to function both as a proto-oncogene and a tumour suppressor gene depending on the type of mutation it has undergone. The p53 gene is found in normal cells in all tissues. High intracellular levels of its mutant product (a nucleophosphoprotein) is associated with various tumours. In fact, about half the common cancers contain mutations of the p53 gene, located on the long arm of chromosome 17, and this gene plays a central role in cellular growth regulation (Burck *et al* 1988). Examples of these cancers include colon, lung, oesophagus, breast, liver, brain and haemopoietic tissues (Hollstein *et al* 1991). Analyses of these mutations may provide clues to the cause of this variey of tumours and to the function of specific regions of the p53 gene (Hollstein *et al* 1991).

CHAPTER 2

NEUROBLASTOMA

	PAGE
Introduction	12
Staging	13
Current Treatment and Survival	14
Chemotherapy	16
Radiotherapy	16

CHAPTER 2. NEUROBLASTOMA

INTRODUCTION

Neuroblastoma is a tumour of post-ganglionic sympathetic neurones and is neuro-ectodermal in origin. It is the most common extra-cranial solid tumour in children and comprises 25-50% of all malignant tumours in neonates. It occurs at a rate of 1 per 10,000 live births with the male:female ratio 1.2:1 and the white to black ratio 3:2. On presentation, 45% of neuroblastoma patients already have distant skeletal metastases (O'Brien *et al* 1989). Of all patients with neuroblastoma 50% are less than two years old and 75% are less than four years old. It is extremely rare for any patient to exceed fourteen years of age (Voute 1988). Genetic pre-disposition to the disease is a possibility the presence of neuroblastoma in siblings and identical twins has been well documented (Hayes and Smith 1989).

Primary tumours can arise in numerous sites within the peripheral nervous system from the base of the skull to locations deep in the pelvis and adrenals. 70% of cases originate in the retroperitoneal region and 30% in the adrenal glands. The site of the primary tumour is an important prognostic factor; neuroblastomas arising in the cervical, thoracic and pelvic regions are more favourable than those of adrenal origin (Voute *et al* 1986). Liver, bone and lymph nodes are common metastatic sites, whereas brain, spinal cord, heart and lungs are rarely affected (Voute *et al* 1986: Voute 1988). Biological markers for neuroblastoma include urinary excretion of Vanillylmandelic and Homovanillic acids and the ratio of one to the other predicts outcome: the higher the value, the better the outcome (Hayes and Smith 1989). Some patients also excrete increased levels of cystathionine and homocysteine, both of these amino acids occuring in nervous tissue and liver (Voute 1988).

STAGING

The current system is the International Staging System for Neuroblastoma (Brodeur *et al* 1988a). Table 1 shows this system and the older Paediatric Oncology Group (POG) system of staging for comparison (Kretschmar 1991)

International Staging System for Neuroblastoma	**Paediatric Oncology Group**
Stage 1: Localised tumour confined to the area of origin; complete gross excision, with or without microscopic residual disease; identifiable ipsilateral and contralateral lymph nodes negative microscopically. Stage 2A: Unilateral tumour with incomplete gross excision; identifiable ipsilateral and contralateral lymph nodes negative microscopically. Stage 2B: Unilateral tumour with complete or incomplete gross excision; with positive ipsilateral regional lymph nodes; identifiable contralateral lymph nodes negative microscopically. Stage 3: Tumour infiltrating across the midline with or without regional lymph node involvement; or unilateral tumour with contralateral regional lymph node involvement; or, midline tumour with bilateral regional lymph node involvement. Stage 4: Dissemination of tumour to distant lymph nodes, bone marrow, liver, and/or other organs (except as defined in stage 4S). Stage 4S: Localised primary tumour as defined for stage 1 or 2 with dissemination limited to liver, skin, and/or bone marrow.	Stage A: Complete gross resection of primary tumour, with or without microscopic residual. Intracavitary lymph nodes, not adherent to but removed with the primary, must be histologically free of tumour. Nodes adherent to or within the tumour resection may be positive for tumour without restaging patient to Stage C. If primary is in abdomen or pelvis, liver must be histologically free of tumour. Stage B: Grossly unresected primary tumour. Nodes and liver as in Stage A. Stage C: Complete or incomplete resection of primary. Intracavitary nodes that are not adherent to the primary are histologically positive for tumour. Liver as in Stage A. Stage D: Any dissemination of disease beyond intracavitary nodes, that is, extracavitary nodes, liver, skin, bone marrow, or bone.

Table 1 Comparison of the new International Staging System for Neuroblastoma with the older POG System.

CURRENT TREATMENT AND SURVIVAL

Although some patients, especially infants, seem to be curable with little or no therapy, conventional treatment has little effect on the long term outcome notably for older children with disseminated disease (Brodeur and Fong 1989). Since the majority of patients present with stage 4 and are over 1 year of age, current therapy is far from satisfactory. Spontaneous regression occurs in about 1% of children with neuroblastoma, largely among infants with stage 4S (Hayes and Smith 1989). By virtue of its neural crest origin, neuroblastoma has the potential to mature into either ganglioneuroblastomas or pheochromocytomas (Voute 1988). Survival depends on age (the younger the patient the better the outcome) and, as mentioned previously, the site of the primary. Survival also depends on the stage at the time of presentation and a study of 100 children showed an 80% two year survival for stage 1 disease compared to 7% for stage 4 (Voute *et al* 1986). Figure 1 shows survival of children older than 1 year of age with POG staged disease (Hayes and Smith 1989). No historical date has been collected for the International Staging System. Current treatment for neuroblastoma is multimodal, typically involving surgery, chemotherapy and possibly radiotherapy.

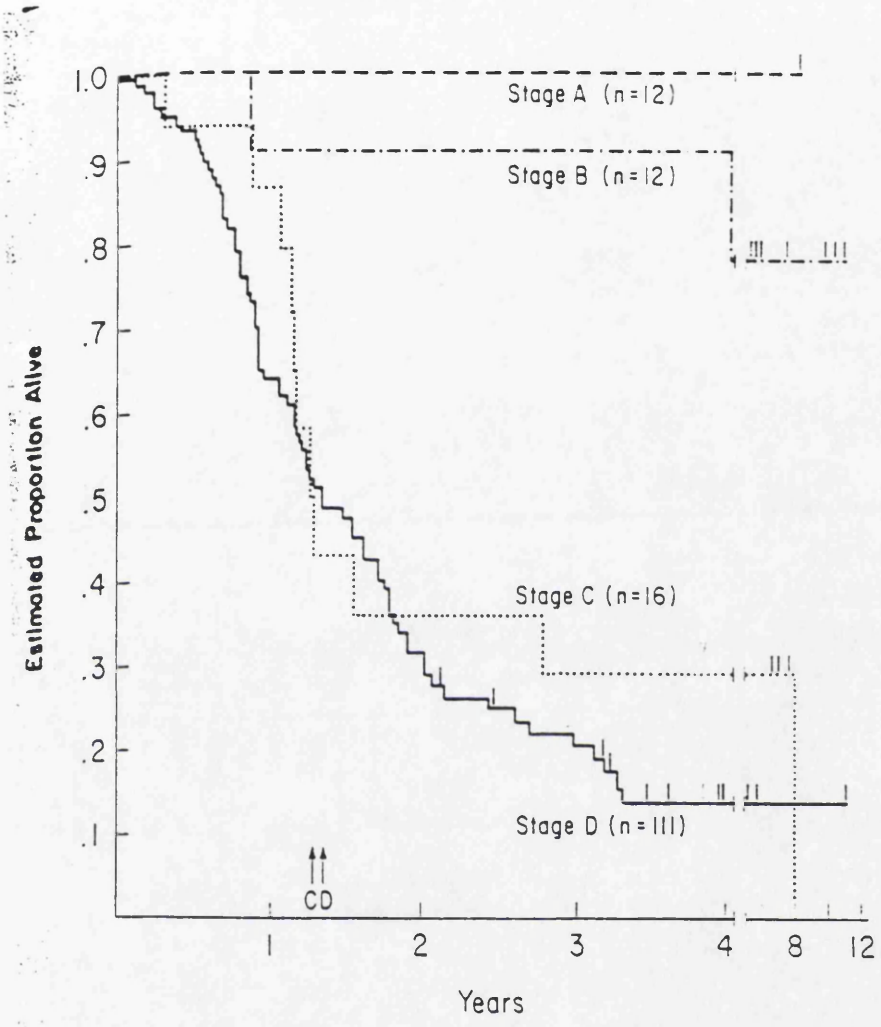

Figure 1. Survival of children older than 1 year of age with POG stage disease treated at St. Judes Childrens Research Hospital, 1974-1984.

CHEMOTHERAPY

Although chemotherapy has not altered long term survival it has been shown to induce complete or partial response in some patients (O'Brien *et al* 1989). Examples include cis-Diamminedichloroplatinum (11) (cisplatin) with a 46% complete or partial response rate, and cyclophosphamide, 59% (Hayes and Smith 1989). Currently these agents are not used singly but in combination with others (see page 27 also).

RADIOTHERAPY

Radiotherapy mostly improves local control and may provide palliation in painful boney metastases and spinal cord compression (O'Brien *et al* 1989). Therapeutic doses range from 15-35 Gy depending on age and the site being irradiated. Total body irradiation is used in conjunction with marrow transplantation in some programmes (Hayes and Smith 1989).

CHAPTER 3

MOLECULAR GENETICS OF NEUROBLASTOMA

	PAGE
Introduction	18
N-myc Amplification	18
Chromosome 1p deletion and Ploidy	19
Genetic Predisposition	20
Possible role of N-myc	21
N-myc Gene Product	22
N-myc Expression	22
Structure and size of amplified N-myc	24

CHAPTER 3. MOLECULAR GENETICS OF NEUROBLASTOMA

INTRODUCTION

There are two genetic events known to be associated with neuroblastomas. These are the deletion of the distal portion of the short arm of chromosome one and N-*myc* amplification. The two events are thought to be related and loss of heterozygosity of chromosome 1p (LOH) may precede amplification (Brodeur 1990).

N-*myc* AMPLIFICATION

A specific oncogene (N-*myc*) has been found to be amplified in certain neuroblastoma cell lines and tumours (Schwab *et al* 1983; Kohl *et al* 1983). Amplification or increased expression of N-*myc* would so far appear to be restricted to a subset of tumours of neural origin, including small cell lung cancer and retinoblastoma (Lee *et al* 1984), although an exception to this is rhabdomyosarcoma which sometimes contains amplified copies of the N-*myc* gene (Dias *et al* 1990). The N-*myc* gene has been sequenced and was found to be related to but only partially homologous to v-*myc* and c-*myc* (Schwab *et al* 1983; Kohl *et al* 1983).

One of the first studies of N-*myc* amplification in a number of primary untreated tumours indicated that 38% of these had gene amplification and that this phenomenon correlated with poor prognosis. Those patients with stage 3 and 4 disease were found to have N-*myc* amplification far more frequently than stage 1, 2A, 2B or 4S (Brodeur *et al* 1984). In a later study 2/16 patients described as stage 2A, 2B had amplification and rapid tumour progression whereas only 1 of the remaining patients without amplification progressed (Seeger *et al* 1985; Brodeur *et al* 1986).

The studies were later extended to over 600 patients and the results confirmed earlier findings: 5-10% of patients with 1, 2A, 2B and 4S stage disease had amplification and poor outcome similar to advanced stage disease and 30% of children with advanced stage neuroblastoma had amplification and poor prognosis (Brodeur *et al* 1988b; Brodeur 1990). These observations still seem to be generally true as seen from investigations in other laboratories (Tsuda *et al* 1988; Nakagawara *et al* 1987; Bartram and Bertold 1987; Combaret *et al* 1989; Hiyama *et al* 1991; Look *et al* 1991; McQuaid and O-Meara 1990). Therefore N-*myc* amplification may be a valuable prognostic indicator.

CHROMOSOME 1p DELETION AND PLOIDY

Flow cytometry studies on neuroblastoma patients have shown that $^1/_3$ of tumours have a near diploid karyotype, with the remaining $^2/_3$ showing hyperdiploidy (Delattre *et al* 1991; Look *et al* 1991). A recent study showed that for children less than 12 months old, hyperdiploidy correlated with long term disease free survival (>90%), whereas diploidy correlated with early treatment failure. In the group aged from 12 to 24 months hyperdiploidy correlated with 50% long term disease free survival and diploidy again correlated with early treatment failure (Look *et al* 1991). Overall, in children under 2 years old with disseminated disease, tumour cell ploidy and N-*myc* copy number can provide complementary prognostic information. Deletion of the distal portion of chromosome 1p is the most characteristic cytogenetic abnormality found in neuroblastomas, and it may be associated with the loss of a putative tumour suppressor gene (Brodeur and Fong 1989). This may involve inactivation of two alleles as in the case of retinoblastoma and Wilms' tumours (Delattre *et al* 1991). A large deletion involving the loss of appreciable material on chromosome 1p is found in about 30% of tumours and correlates strongly with N-*myc* amplification (Fong *et al* 1989). Deletions involving chromosome 1 are not restricted to neuroblastomas but are found in a variety of tumours including breast, melanoma and colon cancer (Brodeur 1990; Delattre *et al* 1991).

GENETIC PREDISPOSITION

The possibility of an inherited susceptibility to neuroblastoma is supported by the existence of familial clusters which represent a very small incidence (< 1%) of all neuroblastomas (Brodeur and Fong 1989; Delattre *et al* 1991). The tendency towards developing neuroblastoma could be inherited as in retinoblastoma and Wilms' tumour. The localisation of the gene whose constitutional mutation gives rise to germline transmission has yet to be pin-pointed. Neuroblastoma differs from retinoblastoma and Wilms' tumour in that there is no consistent constitutional chromosome abnormality or specific congenital syndrome associated with it (Brodeur and Fong 1989; Delattre *et al* 1991). A possible site for the predisposition gene may well be that of the putative tumour suppressor gene as observed in other tumours.

It has lately emerged that there are two distinct groups of neuroblastoma; type 1, consisting of those tumours which show a near-diploid karyotype with N-*myc* amplification and 1p deletion and type 2 which are hyperdiploid with no evidence of either of the above. Type 1 disease carries a poor prognosis, whereas those patients with type 2 disease have a favourable outcome (Brodeur and Fong 1989; Delattre *et al* 1991; Dominici *et al* 1989). Both types may share as a common mechanism for tumourogenesis the deletion of a tumour suppressor gene on chromosome 1, but would differ by the size of the 1p deletion involved in the second hit (Delattre *et al* 1991).

In summary, DNA content, N-*myc* amplification and 1p deletion are now regarded as strong prognostic indicators. These genetic markers could be used to predict the aggressiveness of the disease and to decide whether to use more intense therapy. Ascertaining the sequence and position of the neuroblastoma predisposition gene could assist in pre-natal diagnosis by identifying those individuals most at risk of developing neuroblastoma.

POSSIBLE ROLE OF N-*myc*

Despite the relationship between elevated N-*myc* copy number and tumour aggressiveness, it is by no means clear what role N-*myc* amplification plays in this phenomenon. Furthermore, while approximately 30% of children with neuroblastoma have N-*myc* amplification in their tumours, there are still about 50% of the remaining children with single copy who do not survive (Brodeur and Fong 1989). Evidence of other amplified oncogenes in over 100 neuroblastomas was sought but none was found (Brodeur and Fong 1989). There is no consistent evidence implicating activation of an oncogene other than N-*myc* with the poor outcome seen in a subset of patients lacking N-*myc* amplification. Clearly N-*myc* amplification alone cannot be responsible for malignant transformation in this tumour.

In order to test the ability of an oncogene to transform cells, transfection studies can be carried out on mouse or rat embryo fibroblasts. Experimental models such as these have shown the contribution of N-*myc* overexpression in tumourogenesis. Introduction of an N-*myc* expression vector immortalises primary rat embryo fibroblasts or neural precursor cells (Delattre *et al* 1991). N-*myc* can co-operate with an activated *ras* gene to induce transformation in rat embryo cells (Schwab *et al* 1985). Transgenic mice bearing the N-*myc* oncogene deregulated by juxtaposition of the immunoglobulin heavy chain enhancer may spontaneously develop B lymphoid tumours (Rosenbaum *et al* 1989). Therefore, clearly the N-*myc* oncogene can contribute to carcinogenesis.

A study of N-*myc* copy number in multiple simultaneous tumour samples and consecutive samples from individual patients showed that N-*myc* copy number did not vary between different sections of a single tumour or during treatment from the same patient over a period of time (Brodeur *et al* 1987). So far no cases of neuroblastoma with one copy at the time of diagnosis have been known to go on

to develop multiple copies (Brodeur and Fong 1989). The results suggest that tumours which develop N-*myc* amplification generally do so by the time of diagnosis and that this feature is an inherent property of a sub-set of aggressive neuroblastomas.

N-*myc* GENE PRODUCT

Nuclear proteins encoded by the *myc* family of genes appear to function in normal cells in proliferative control and in maintaining the self-renewal capacity of the cell (Westin *et al* 1982; Filmus and Buick 1985). In normal cells the expression of *myc* and other proto-oncogenes encoding nuclear proteins (e.g. *fos* and *myb*) is tightly controlled, but the expression of these in transformed cells is not so highly regulated (Hann and Eisenman 1984).

The c-*myc* gene is expressed in a variety of normal cells and encodes a nuclear protein that binds to DNA; therefore it may mediate in the pathway of mitotic signals. N-*myc* is only expressed in embryos and at a lower level in adult brain and testes (Stanton *et al* 1986). The N-*myc* and c-*myc* genes have a similar intron/exon structure and their protein products share regions of significant homology (Kohl *et al* 1986). Stanton *et al* (1986) suggested a possible function for the N-*myc* gene product. The ability of the c-*myc* product to bind to DNA may reside in the carboxy terminal of the protein, the region most conserved between c-*myc* and N-*myc* gene products. Thus it is possible that the protein encoded by N-*myc* may also bind to DNA and suggests that members of the *myc* family, whilst related, may be uniquely regulated.

N-*myc* EXPRESSION

N-*myc* is expressed at elevated levels in a limited set of neoplasms; those of neuro-ectodermal origin including neuroblastoma, retinoblastoma, small cell lung cancer and Wilms' tumour (Nisen *et al* 1988; Kohl *et al* 1984). A study by Nisen

et al (1988) on a number of neuroblastoma patients indicated that there was a rough correlation between N-*myc* RNA expression and degree of amplification of N-*myc*, but that elevated expression was not confined to tumours with amplified N-*myc*. This observation has been borne out by other investigators (Kohl *et al* 1984; Bartram and Bertold 1987). A substantial number of tumours lacking N-*myc* amplification overexpress the N-*myc* gene but this increased level of expression does not correlate strongly with a poor outcome (Seeger *et al* 1988; Nisen *et al* 1988). Increased RNA expression is a more general phenomenon found in individual specimens at all clinical stages in neuroblastoma without any obvious correlation to disease severity or outcome. It is possible that N-*myc* expression is a marker of specific stage in the neuroblastoma cell lineage at which neuroblastomas are blocked; i.e. increased N-*myc* expression correlates with poorly differentiated cells. *In situ* hybridisation studies on tumour sections show stronger signals largely in poorly differentiated tumour cells (Schwab 1984; Cohen *et al* 1988). Neuroblasts can be induced to differentiate by, for example, retinoic acid - an analogue of Vitamin A. Thiele *et al* showed a decrease in N-*myc* expression after treating neuroblastoma cell lines with the above. This decrease preceded differentiation identified by morphological changes in the form of outgrowth from neuroblasts (Thiele *et al* 1985). Southern blots of tumour cell line DNA post treatment showed no reduction in N-*myc* copy number - therefore the decreased expression was not due to this. Retinoic acid may therefore have some therapeutic value in the treatment of neuroblastoma by inducing differentiation to a benign ganglioneuroblastoma.

Christiansen *et al* (1990) found an inverse relationship between N-*myc* RNA expression and expression of the gene for Nerve Growth Factor Receptor (NGF-r). This inverse relation could help in establishing markers for prognosis. Nerve growth factors are polypeptides involved in the control of differentiation of neural cells. Christiansen *et al* found two groups of tumours. Those undifferentiated types

with elevated N-*myc* expression and missing NGF-r transcripts which correlated with early neuronal development and poor prognosis; and those more highly differentiated tumours with diminished N-*myc* expression and elevated NGF-r expression which had a better outcome (Christiansen *et al* 1990).

The restricted pattern of N-*myc* expression indicates that expression of this gene may be a property of the normal cells from which these tumours derive and may not necessarily relate to the neoplastic state (Kohl *et al* 1984). The N-*myc* gene is expressed in foetal and neonatal tissues and neuroblastoma tumours. The expression is elevated during foetal development and falls off dramatically during later development (Larcher *et al* 1991). Increased N-*myc* expression is found in human foetal brain and developing tissue. It is also observed at elevated levels in whole mouse embryos between 7.5 and 11.5 days of growth, decreasing thereafter (Mugrauer *et al* 1988). Therefore, it would appear that the N-*myc* gene plays an important role in normal neuronal development.

STRUCTURE AND SIZE OF AMPLFIED N-*myc*.

Double minute chromosomes (DM's) and homogeneously staining regions (HSR's) are cytogenetic manifestations of gene amplification. Both phenomena appear often in tumour cells, especially neuroblastoma. Double minutes were first recognised in neuroblastoma (Cox *et al* 1965) and homogeneously staining regions were described in association with both neuroblastoma and drug resistant chinese hamster cells (Biedler *et al* 1976). A small percentage of primary neuroblastoma tumours and roughly 50% of cell lines have their DNA amplified as HSR's linearly integrated into a chromomsome. It has not yet been established why HSR's are the more common form of amplified DNA in cell lines, although it is probable that DM's are lost more readily from the cell. Amplified N-*myc* was mapped to the HSR's in cell lines of neuroblastoma (Kohl *et al* 1983) and in 1984, Schwab *et al* mapped the constitutional site of the N-*myc* oncogene to the short arm of chromosome 2 (Schwab *et al* 1984). Kohl *et al* studied five HSR-containing neuroblastomas with amplified N-*myc* and observed that the site of the HSR, in each case varied but was never on chromosome 2.

Brodeur and Seeger (1986) suggest that from current evidence it would appear that a large region of chromosome 2, which includes the N-*myc* locus, becomes amplified initially as extrachromosomal DM's. This is supported by the fact that one rarely sees the HSR at the site of the single gene copy. Shiloh *et al* (1985) analyzed the organisation of sequences in the HSR on chromosome 1 of the IMR-32 neuroblastoma cell line. Probes obtained from the HSR mapped to at least three different sites on the short arm of chromosome 2. These were millions of base pairs apart. This suggests a complex mechanism in which sequences which are usually distant are spliced together and amplified in the cell line. Brodeur and Seeger suggest that the entire region of amplified sequences within the HSR probably exists as a linear array integrated into a chromosome. Furthermore N-*myc* is probably at the core of the amplified domain in human neuroblastomas but little else is known about the limits of the core or genes expressed within it. Further analysis may show other important expressed genes and analysis of the ends may reveal more about the mechanisms of gene amplification. Schwab (1991) states that the major proportion of amplified DNA in neuroblastoma is arranged as head to tail tandem repeats.

Various attempts have been made to estimate the size of the HSR containing the amplified N-*myc* oncogene and the amplified units therein. Bahr *et al* (1983) estimated the size of the HSR in a neuroblastoma cell line to be about 8% of the human haploid genome which is also supported by Brodeur *et al* (1988c) who observed that an HSR may be as large as chromosome 1, so there is potential for a very large number of copies of N-*myc*. Since, for example, 30 copies of N-*myc* (which is roughly 10 kbp long) would account for only 0.1% of an HSR then 99.9% of the HSR is something else. This suggests that the presence of amplified N-*myc* may only be a marker and that other amplified genes within the HSR may be important.

CHAPTER 4

CHEMOTHERAPY OF NEUROBLASTOMA

	PAGE
Introduction	27
Cisplatin	27
Drug Resistance	27
Mechanisms of Resistance to cisplatin	29

CHAPTER 4. CHEMOTHERAPY OF NEUROBLASTOMA

INTRODUCTION

Neuroblastoma is usually chemoresponsive and, in some instances, curable. The main reason for failure of treatment is the rapid dissemination of this disease (Pizzo *et al* 1989). The most common agents used are cyclophosphamide, cisplatin, vincristine and melphalan (Pizzo *et al* 1989). These are employed in combination; for example the OPEC regime uses vincristine (oncovine), cisplatin (pt), VP16 (etoposide) and cyclophosphamide. Although chemotherapy can result in complete responses, recurrence is common in those patients over one year old. The major barrier to the success of chemotherapy in neuroblastoma is the development of drug resistance in the tumour cells. This will be described in greater detail in the section on drug resistance (page 28).

CISPLATIN

Cisplatin is one of the most effective anti-tumour agents used in the treatment of advanced neuroblastoma in children (Sue *et al* 1988). Its mechanism of action is similar to that of classical alkylating agents (e.g. cyclophosphamide and melphalan). Cisplatin forms covalent bonds with DNA, thus inhibiting its replication and/or transcription. Cisplatin may have increased activity for some cells in G1 phase, with little or no specificity for others. Most drugs have a greater toxicity toward rapidly proliferating cells, but there are some exceptions including cisplatin. Studies of the kinetics of the formation of cross-links between cisplatin and DNA indicates that these DNA-protein cross-links remain for several hours after removal of the drug and then repair occurs (Calman *et al* 1980; Tannock 1988).

DRUG RESISTANCE

Chemotherapy can fail for a number of reasons including the proliferative state of the target cells, vascular access and poor penetration of drug into the tumour. However, one of the main barriers to successful treatment is the emergence of a drug-resistant sub-population among the tumour cells.

Gene amplification in tumour cells is often found associated with drug resistance. One of the best characterised examples of amplification is that of the gene encoding dihydrofolate reductase (DHFR), the target enzyme of methotrexate (Schimke 1982). Those tumour cells containing amplified DHFR genes express the chemo-resistant phenotype. Another example of gene amplification and drug resistance occurs in the multidrug-resistance phenomenon. It has been observed that tumour cells selected for resistance to one of a group of drugs may exhibit cross resistance to other members of that group. Examples of drugs which participate in multidrug-resistance are vincristine, adriamycin, etoposide and daunomycin (cisplatin does not participate). When membrane proteins from multidrug-resistant cells are analysed they are found to contain a glycoprotein termed P-glycoprotein. Multidrug-resistant cells have decreased uptake and increased efflux of drugs to which they are resistant and this alteration in membrane transport has recently been correlated with the expression of P-glycoprotein. The amplification of the mdr-1 gene causes over-production of the above, which seems to act as an "efflux pump" (Tannock 1988). One study of 35 neuroblastoma samples showed an inverse correlation between expression of the mdr-1 and N-*myc* genes (Nakagawara *et al* 1990). An investigation by Bourhis *et al* (1989) indicated a correlation between mdr-1 expression levels and previous chemotherapy in 41 neuroblastoma tumour samples. Although cisplatin does not participate in multidrug-resistance its effectiveness as a cytotoxic agent is often reduced by the development of drug-resistant tumour cells. Resistance may arise *in vivo* by repeated exposure to cisplatin. Studies of the mechanism of cisplatin resistance have been conducted in a variety of human and rat tumour cell lines (Sekiya *et al* 1989; Andrews *et al* 1989). Exposure of cells to cisplatin does not lead to cross resistance to other agents (apart from cisplatin analogues). This is in contrast to the multidrug-resistance phenomenon (Gibson 1989). Therefore uptake of cisplatin into cells and development of resistance may involve different mechanisms from those found in drugs such as adriamycin and vincristine which participate in the multidrug-resistance phenomenon. The concentration of cisplatin

in resistant cells is decreased by diminished uptake rather than by increased efflux as observed in the case of drugs involved in the multidrug phenomenon (Tannock 1988). A conflict exists regarding the exact mechanism of cisplatin membrane transport. The uptake of cisplatin may involve more than passive diffusion and it has been suggested that membrane transport mechanisms may be involved and that one target for cisplatin toxicity may be the plasma membrane (Andrews *et al* 1989).

MECHANISMS OF RESISTANCE TO CISPLATIN

The biochemical changes responsible for the acquisition of resistance to cisplatin are not fully understood. A variety of studies have shown a correlation between resistance and increased intra-cellular levels of glutathione (Canon *et al* 1990). Moreover, an increase in DNA repair has been reported for some resistant lines coupled with a decrease in uptake of cisplatin (Sekiya *et al* 1989). Andrews *et al* (1989) observed, from their studies on a resistant subline of human colon carcinoma, that mechanisms of resistance are complex, multi-factorial responses. Treatment with aphidicolin, an inhibitor of DNA polymerase α leads to a decrease in DNA repair capacity and partially restores the sensitivity of cisplatin resistant lines. However, its potential clinical use is limited by the fact that it is unlikely to be tumour specific (Canon *et al* 1990). Overall, the data suggest that an increase in DNA repair capacity contributes to the cisplatin resistant phenotype. Cisplatin analogues to which tumour cells are not cross-resistant may be an important step in overcoming the problems of treating neuroblastoma tumours that develop resistance to this class of drugs.

The observation that malignant cells often become resistant to drug therapy, while normal cells rarely do, suggests that certain molecular processes involved in tumourogenesis (e.g. oncogene activation) might also play a role in drug resistance. A study into the possible role of oncogenes in the development of resistance to chemotherapy was carried out on mouse NIH 3T3 cells transformed with missense

mutation activated *ras* genes (Sklar 1988a). A substantial increase in resistance to cisplatin was demonstrated. If these findings extend to human tumours, it may help to explain the role of activated oncogenes (e.g amplified N-*myc* in neuroblastoma) in the development of drug resistance (Sklar 1988a). In another study Sklar found a correlation between cisplatin resistance and levels of c-*myc* expression in murine erythrolukaemia cells (Sklar 1991). He postulated that c-*myc* levels may influence treatment success in some tumours by perhaps enhancing repair of DNA damage caused by cross linking agents such as the platinum analogues. These studies into the possible role of activated oncogenes in the development of drug resistance are given further support with an investigation by Kerr *et al.* This study showed that a mink lung epithelial cell line became significantly more resistant to doxorubicin and vincristine than the parent line following transfection with activated H-*ras*-1 (Kerr *et al* 1991).

CHAPTER 5

RADIOTHERAPY OF NEUROBLASTOMA

	PAGE
Introduction	32
Radiobiology	32
Genetics of Radioresistance	36
Targeted Radiotherapy	36

CHAPTER 5. RADIOTHERAPY OF NEUROBLASTOMA

INTRODUCTION

Neuroblastoma is clinically radiosensitive but is still often fatal due to its early pattern of dissemination via both lymph nodes and blood. The stage of the tumour, age at diagnosis, histology and site of tumour are all important prognostic variables (D'angio 1987). In general, the younger the patient the better the prognosis, stage for stage. The clinical response of neuroblastoma has been well documented, and a high probability of local tumour control can be attained by using doses less than 30 Gy typically given as fractions of 2 Gy on a daily basis (Dobbs *et al* 1992). The use of total body irradiation, aggressive chemotherapy and autologous bone marrow rescue is a possible treatment regime for those patients with metastatic disease.

RADIOBIOLOGY

Human tumours show a widely varying clinical response to radiation. For example, seminoma is a highly radioresponsive neoplasm, whereas melanoma is relatively refractory. The biological basis for these differences remains unexplained (Deacon *et al* 1985). Current ideas on the radiobiology of human tumours may be divided into three categories:-

(1) Cells experience different amounts of damage for the same radiation dose depending on how their DNA is packaged.

(2) Cells suffer the same damage but some are good at repair while others are less efficient.

(3) Cells suffer the same damage, are equally efficient at repairing, but differ in their tolerance to ionising radiation (Powell and McMillan 1990).

Most of the early work on radiobiology was performed on mouse tumours; however the development of *in vitro* cloning techniques has made human tumour radiobiology possible to study (Courtney and Mills 1978; Hamburger and Salmon 1977). Survival curves can now be produced for mammalian cells irradiated *in vitro*. Cells are irradiated and subsequently plated out to produce colonies. After about ten days macroscopic colonies can be seen and these are then stained and counted. Only those colonies with fifty or more cells (i.e. those cells which have undergone five or more divisions) are counted, since these cells are regarded as having the potential to undergo indefinite division. Those cells which have undergone less than five divisions are regarded as "dead" in that they have suffered radiation-induced loss of reproductive capacity. The traditional mammalian survival curve (figure 2) has a "shoulder" at low doses and only becomes exponential at higher doses.

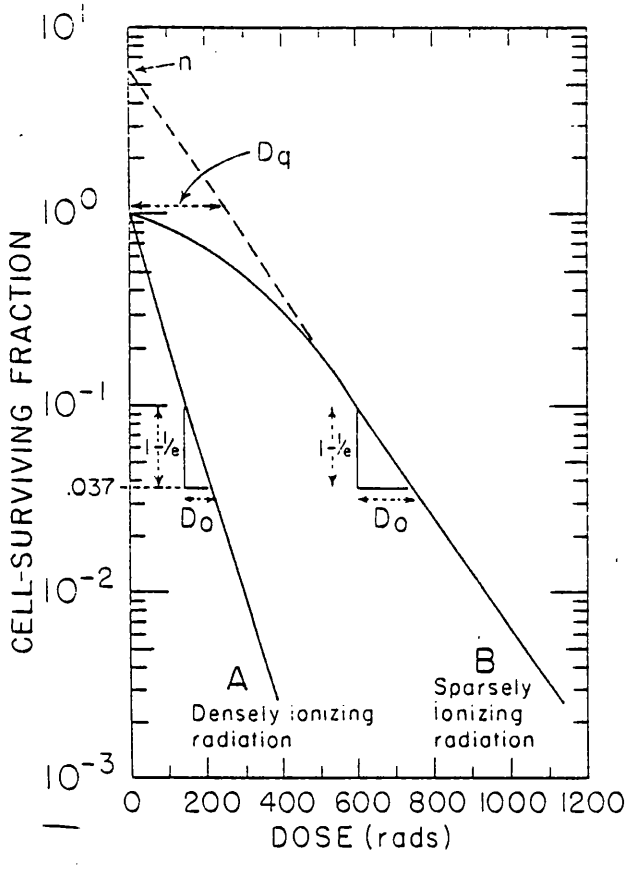

Figure 2 Typical survival curves for mammalian cells exposed to radiation. The fraction of surviving cells is plotted on a logarithmic scale against dose on a linear scale. For densely ionizing radiations, such as low-energy neutrons, the dose-response curve is a straight line and may be characterized by one parameter, the 37°C dose slope or D_o. For sparsely ionizing radiations such as x-rays, the dose-response curve has an initial shoulder followed by a portion which is straight, or almost straight. The curve is characterised by two parameters; the 37% dose slope, D_o, of the straight portion and the extrapolation number, n, which is a measure of the "width" of the initial shoulder. Alternatively, the width of the shoulder may be specified in terms of the quasithreshold dose, D_q, which is the dose at which the extrapolated straight portion of the dose-response curve cuts the dose axis.

This curve is often well fitted by an equation of the form $S = 1 - (1 - e^{-D/Do})^n$, where S= surviving fraction, D = given dose and Do, n are parameters to be estimated (Hall 1988). The extrapolation number (n), where the straight line portion of the curve crosses the Y-axis, gives an indication of the size of the shoulder and the ability to accumulate and repair sub-lethal damage. Lately, the importance of the surviving fraction at 2 Gy (SF_2) has emerged. This parameter shows the response to therapeutically relevant treatment doses. The literature on *in vitro* sensitivity of human tumours demonstrated a correlation between SF_2 and the level of clinical response of various tumour types (Fertil and Malaise 1981; Deacon *et al* 1984).

The amount of firm data on the radiobiology of childhood tumours is small and differences between cell lines are wide. Data support the view that cells of some childhood neoplasms are inherently highly radiosensitive and show a small shoulder on the survival curve (Steel and Wheldon 1991). Overall, the data on radiobiology of human neuroblastoma cells *in vitro* indicates moderate radiosensitivity with a low capacity for accumulation of sub lethal damage as indicated by the lack of an appreciable shoulder on the survival curve (Wheldon *et al* 1985; Deacon *et al* 1985; Wheldon *et al* 1986). The use of multiple small fractions of radiation is favoured in order to exploit the increased repair capacities of many normal tissues compared with neuroblastoma cells (Deacon *et al* 1985; Wheldon *et al* 1985). Since neuroblastoma grows rapidly both clinically and as a xenograft, short overall treatment times are indicated to avoid the problem of repopulation of neoplastic cells. Hence accelerated fractionation using small fraction sizes or decreased dose rate continuous irradiation would be favoured (Deacon *et al* 1985; Steel and Wheldon 1991).

THE GENETICS OF RADIORESISTANCE

The role of oncogenes in conferring radioresistance is controversial and most of the work has been done on animal cells. Russell *et al* were unable to find any significant difference between the radiosensistivity of normal mink lung epithelial cells and those transfected with either c-*myc* or H-*ras* (Russell *et al* 1992). On the other hand, a study of a radiation resistant cell line established from a patient with laryngeal cancer which progressed during a course of radiotherapy indicated that the transforming gene (in this case abnormal c-*raf*) could confer the radiation-resistant phenotype to recipient cells upon transfection. This same activated c-*raf* could induce tumours in nude mice (Kasid *et al* 1987). Investigations are underway to establish the exact nature of the genetic abnormalities within this gene and their effect. It will be important to ascertain whether this activated *raf* gene can enhance DNA repair or stimulate free-radical scavenging (Renan 1990). A recent study suggests that oncogenes may increase radioresistance by lengthening the time cells spend in G2 phase after irradiation (McKenna *et al* 1991). Other studies have implicated activated *ras* and c-*raf* oncogenes in the acquisition of tolerance to ionising radiation (Fitzgerald *et al* 1985; Sklar 1988b; Pirollo *et al* 1989). However one must view these results with some caution, since a recent study on normal rat embryo fibroblasts and a human glioblastoma line indicated that the transfection process per se is capable of inducing radioresistance (Pardo *et al* 1991).

TARGETED THERAPY

Since advanced stage neuroblastoma is refractory to conventional radiotherapy a number of studies have been carried out, both *in vitro* and in the clinic, into targeted therapy. Monoclonal antibodies bearing radioisotopes (e.g.^{125}I-UJ13A) have been utilised, directed against tumour cell surface markers (Allan *et al* 1983; Kemshead *et al* 1985). Another possible approach to targeting of neuroblastoma exploits the expression of nerve growth factor receptor which is widely found in these cells (Mairs *et al* 1991). One agent which has been particularly widely studied

is meta-iodobenzylguanidine (mIBG) in which the iodine atom has been replaced with the radioisotope ^{131}I (Beierwaltes 1987; Mairs *et al* 1991). This drug is selectively accumulated by catecholamine synthesising cells. mIBG is an analogue of the adrenoreceptor neurone blocker guanethidine and is used in many cancer centres for localisation, staging and therapy of phaeochromocytoma and neuroblastoma. Neuroblastoma cells have biochemical properties which enable preferential concentration of catecholamine precursors. Trials have been completed on mIBG therapy and one of the main centres for use is Amsterdam. Treatment with ^{131}I-mIBG has been brought to the forefront by a particular group who had some success with 50 patients who had recurrent disease (Voute *et al* 1991). Since ^{131}I is mainly a β emitter, the path length is too long to make it an ideal choice for treating small micrometastases since most of the radiation is deposited outside the cell. Various novel isotopes are being investigated at the moment with shorter ranges like ^{211}Astatine incorporated in mABG or ^{125}I. In fact some groups in the United States are investigating ^{125}I-mIBG for therapy.

One exciting, though highly speculative, possibility is that of exploiting the high copy number of N-*myc* in a sub-set of neuroblastomas by targeting the gene. This involves the use of internucleotide, linkage-protected specific, oligonucleotides conjugated to ultra short range Auger emitters such as ^{123}I or ^{125}I. Ideally, normal cells, though they accumulate the radiolabelled oligonucleotide, should suffer much less DNA damage than tumour cells with amplified N-*myc* which should (hopefully) retain greater amounts of radioactivity.

For the future, new approaches to diagnosis including cytogenetic markers like partial monosomy for chromosome 1 and N-*myc* amplification will play a role in improving treatment design. The use of targeted radiotherapy using monoclonal antibodies and catecholamine precursors are examples of novel treatments being investigated in various world centres presently.

MATERIALS AND METHODS

CELL LINES

NB1-G. This cell line was established by monolayer culture of cells released by disaggregation of a human tumour xenograft obtained from surgical excision of stage 4 abdominal neuroblastoma from a 2 year old male (Carachi *et al* 1987).

IMR-32. Cell line established from stage 4 abdominal mass in 13 month old male, diagnosed as neuroblastoma (Tumilowicz *et al* 1970).

XRNB1-G. This is a radioresistant sub-line of NB1-G produced by irradiating flasks of the parent line once per week at 2 Gy (Russell 1990).

SK-N-SH. This cell line was established from human metastatic neuroblastoma tissue (Biedler *et al* 1973).

SK-N-BE(2)C. Progressive human neuroblastoma obtained from bone marrow after therapy with adriamycin, cytoxan and radiation (Reynolds *et al* 1986).

NB2-G. Cell line from xenograft established from thoracic tumour of male patient (Stage 4 neuroblastoma) presenting to Royal Hospital for Sick Children in 1983. (Wheldon *et al* 1985).

NB100. Epidural tumour surrounding spine from a 12 year old female, diagnosed as consistent with neuroblastoma (Schlesinger *et al* 1976).

MONOLAYER CULTURE

All cell lines were maintained in 75cm^2 flasks containing 20mls of Eagle's Minimum Essential Medium with 25mM Hepes buffer, 10% foetal calf serum, 2mM glutamine, penicillin/streptomycin (100 IU/ml) and amphotericin B (2.5 μg/ml). All media and supplements were obtained from Gibco (Paisley, Scotland). Flasks were seeded with 5x10^5 cells, and kept in an atmosphere of 5% CO_2 at 37°C.

SPHEROID CULTURE

Multicellular tumour spheroids were produced by continuous stirring of approximately 2x10^6 cells in Techne (Cambridge, U.K.) stirrer vessels for 3-4 days at 37°C, 5% CO_2. Spheroids of about 300 μm diameter were assayed for response to radiotherapy or chemotherapy. Multicellular tumour spheroids are often employed as *in vitro* models for human tumour studies. These have a number of distinctive features which make them ideal for this, including heterogeneity of metabolism and cell kinetics as the spheroid size increases, and availability of a number of end points. Spheroids may be an especially good model for avascular micro-metastases *in vivo* and it may be in the context of adjuvant therapy that *in vitro* spheroid studies may be of the greatest relevance (West *et al* 1980; Mueller-Klieser 1987).

IRRADIATION PROCEDURE

Spheroids were transferred to 25 cm^2 flasks and each flask was irradiated on a ^{60}Co unit with doses from 0.5 - 5 Gy, using a 0.8 cm perspex 'build-up' to ensure maximum energy deposition. The dose rate was approximately 1 Gy per minute.

Several pilot studies were performed on each cell line to assess the plating efficiency and to ascertain the amount of radiation required to produce a reasonable (1 log) cell kill before deciding on the range of radiation to use. For example, the NB1-G line required approximately 2.5 Gy to produce 1 log cell kill. Therefore the upper limit was set at 3.5 Gy. The SK-N-BE(2)C cell line needed 4 Gy for the same effect. Therefore the upper limit was set at 5 Gy.

CHEMOTHERAPY

Spheroids were transferred to universal containers, medium was removed and the spheroids were resuspended in cisplatin (Cyanamid, U.K.) ranging in concentration from 2.5 μM - 20 μM. These were incubated at 37°C for 1 hour and the drug was removed by washing three times in Earle's balanced salt solution. Once again, several pilot experiments were done on each cell line to set a range of doses of cisplatin, as was done for radiation.

Two end points could now be pursued to estimate response to radiation and chemotherapy. These were either spheroid regrowth or colony formation following spheroid dissagregation.

SPHEROID REGROWTH

After treatment spheroids were sorted into agar coated wells of 24 well multiwell plates using one plate for each dose of radiation or chemotherapy. The plates were placed in a 5% CO_2 incubator at 37°C. The cross-sectional area of the spheroids was measured twice per week using an image analysis system (Twentyman 1982) and 0.5 mls medium was added to the wells once per week. Measurements were stopped when more than 50% of the spheroids in the plate had reached approximately 1mm in diameter. Once the spheroids reach this size their growth rate begins to plateau and the middle of the spheroid becomes necrotic. For each treatment group, plots can be made of median $\log_{10}$ volume against day of measurement to produce spheroid growth curves. Only those experimental groups where more than 50% of the spheroids regrew were used to produce growth curves. Three separate experiments were carried out for each cell line.

ESTIMATION OF CELL SURVIVAL FROM SPHEROID REGROWTH

Regrowth plots for treated spheroids initially show a decrease in the rate of growth which eventually becomes parallel to the control plot (see Fig. 3). If it is assumed that treatment has little effect on growth kinetics of the surviving cells, then by back extrapolation of the regrowth curves to zero time, an estimation can be made of median cell survival (Wheldon *et al* 1985). Using the equation S = No'/No where S = survival, No' = median $\log_{10}$ (volume) of treated spheroids on day zero and No = median $\log_{10}$ (volume) of control spheroids on day zero.

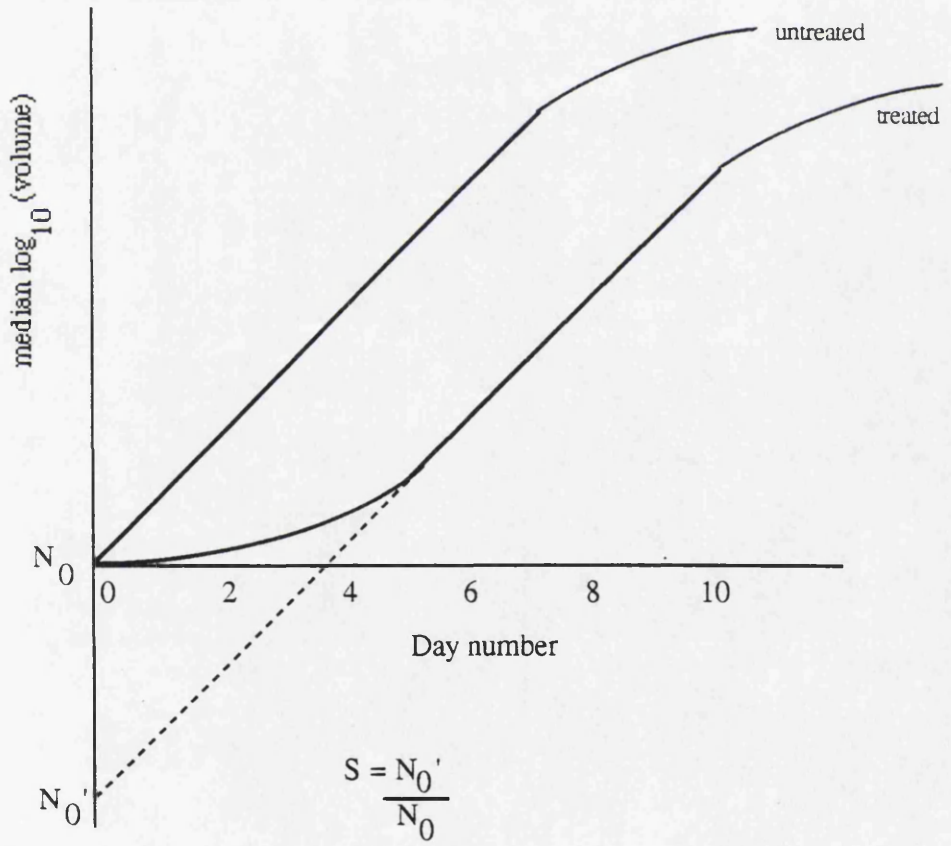

Figure 3. Estimation of cell survival from spheroid regrowth. Plot of median log volume versus time. This illustrates how the survival of treated spheroids was estimated by back-extrapolation of the treated growth curve to zero time.

ESTIMATION OF CELL SURVIVAL FROM COLONY FORMATION.

After treatment spheroids were disaggregated using a series of needles. Samples were examined microscopically to ensure a single cell suspension had been achieved. Cells were then plated out into 25cm^2 flasks at various concentrations, using three flasks per treatment group. Some cell lines (e.g. NB1-G and XRNB1-G) required feeder layers. These were produced by heavily irradiating a cell suspension at 50 Gy and placing 10^6 of these cells into each experimental flask. The flasks were then incubated at 37°C, 5% CO_2 for about 10-14 days until macroscopic colonies could be seen. These were then stained with Carbol Fuschin (10% in water) and counted. Survival curves were produced from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose. The multitarget (page 35) model was used to fit the curves.

ESTIMATION OF N-*myc* COPY NUMBER

DNA was extracted from cell lines using Proteinase K followed by phenol/chloroform extraction (Birnie and Graham 1985). DNA was digested with EcoR1, and samples were electrophoresed through 0.8% (w/v) agarose gels (using λ / Hind III fragments (Gibco, UK) as molecular weight markers) and blotted (Southern 1975) onto nylon filters (Amersham). These were simultaneously hybridised to radioactve p-Nb1 probe (1 kbp EcoR1/BamH1 insert of pBR322) (Schwab *et al* 1983; Kohl *et al* 1983) and the 5'end of the M-bcr probe (Bartram *et al* 1986). Probes were labelled with ^{32}P deoxy-cytidine triphosphate using the random primer method. The specific activity of the probes was approximately 2x10^7 counts per minute per μg of probe. The second probe was used to check the amount of DNA loaded into each well. N-*myc* copy number was assigned by densitometry of autoradiographs (Sun, U.K.). Placental DNA, which has unamplified N-*myc* was used as single copy control. Several preliminary autoradiographs were produced using serial dilutions of each cell line DNA to test

the accuracy of the densitometer and to decide how much DNA to load in each well. Then, autoradiographs containing representative signals for each cell line were scanned, and the N-*myc* copy number calculated. To set up the densitometer, a standard picture was first scanned to calibrate the instrument. Then, the autoradiograph of interest was read and an output of the optical density in each lane was produced, with any background subtracted. The copy number was then calculated for each cell line by comparison of optical density readings with single copy placental DNA.

ESTIMATION OF N-*myc* EXPRESSION

Total RNA was extracted from cell lines using the RNAzol B kit, (Biogenesis Ltd., England) and the integrity of the RNA was assessed by electrophoresing on a 0.8% (w/v) agarose gel. Hela cells were used as a negative control since they do not express N-*myc* (Nisen *et al* 1988). Total RNA was electrophoresed in a 1% agarose/formaldehyde (w/v) gel, with 10 µg of sample loaded into each lane (Davis *et al* 1986). The two major sub-units of ribosomal RNA (28S and 18S) were used as internal markers. By Northern blotting and subsequent hybridisation to radiolabelled p-Nb1 (as above), a signal was seen at 3 kb which corresponds to the size of N-*myc* messenger RNA. The level of expression in cell lines was compared by "dot-blot" analysis (Sambrook *et al* 1989). An expression value of 1 was assigned to SK-N-SH (Kohl *et al* 1984) and a value of 10 to XRNB1-G (which gave the strongest signal). All other cell lines were compared to these. Again, preliminary autoradiographs with serial dilutions of RNA were scanned before arriving at the final results.

STATISTICAL ANALYSIS

Statistical analysis was undertaken following the advice of Dr. T.E. Wheldon and Dr. J. Paul (Clinical Trials Office, Beatson Oncology Centre, Western Infirmary). Analysis was carried out to evaluate the possible correlation of N-*myc* copy number

or N-*myc* expression to SF_2 or cisplatin isodose (the dose of cisplatin required to produce 1 log cell kill), and also of N-*myc* copy number to N-*myc* expression. Data analysis was first done using the Spearman's rank correlation test to assess correlation without assuming linearity. Where a positive indication of correlation was found, least-squares analysis was undertaken to further elucidate the nature of the relationship. This analysis was carried out using the "statworks" and "cricketgraph" programs on an Applemacintosh LC computer.

N-*myc* COPY NUMBER

N-*myc* amplification as assessed by laser densitometry showed a range of copy numbers (tables 2 & 3; figs. 4 & 5). The cell lines SK-N-SH and NB100 had unamplified N-*myc*. IMR-32, NB1-G, XRNB1-G and NB2-G had copy numbers 20, 25, 25 and 30 respectively. SK-N-BE(2)C had the unusually high copy number of 800. Human placental DNA was used as single copy control.

N-*myc* EXPRESSION

Northern blot examination of RNA isolated from neuroblastoma cell lines showed that the labelled p-NB1 probe hybridised to a 3 kb mRNA (fig. 6). The level of expression, tested by "dot blot" analysis, showed cell lines SK-N-SH and NB100 to be expressing at base-line levels, with the strongest signal being shown by XRNB1-G (fig. 7). By arbitrarily assigning a value of 10 to the degree of expression in XRNB1-G, the expression levels of other cell lines were as shown in tables 2 & 3.

RADIOSENSITIVITY

The survival curves (figs. 8-14) were constructed as described previously. Radiosensitivity was assessed by the surviving fraction at 2 Gy (SF_2). This was estimated by drawing a line from 2 Gy on the x-axis onto the curve and then extending this line horizontally onto the y-axis (see figure 8). This parameter is of clinical relevance as this is a typical fractionated dose. SF_2 values of the cell lines assayed ranged from 0.13 to 0.52 (table 2).

CHEMOSENSITIVITY

Response to chemotherapy was estimated by comparison of isoeffective dose: the concentration (μM) of cisplatin required to produce one log cell kill. This was estimated by drawing a line from -1 on the y-axis onto the survival curve and extending this line vertically onto the x-axis (see figure 15). Isoeffective doses ranged from 7.5 μM for NB1-G to 13 μM for SK-N-BE(2)C (table 3). Survival curves (figs. 15-21) show that NB1-G and NB100 lack shoulders indicating that these cell lines are more sensitive to low doses of cisplatin than the others.

Cell line	N-myc copy no	N-myc expression	SF_2
SK-N-SH	1	1	0.13
NB100	1	1	0.44
IMR-32	20	3	0.25
NB1-G	25	7	0.17
XRNB1-G	25	10	0.23
NB2-G	30	3	0.24
SK-N-BE(2)C	800	2	0.52

Table 2 Comparison of N-*myc* copy number, level of N-*myc* expression and SF_2 values for the seven cell lines.

Cell Line	N-myc copy number	N-myc expression	Isoeffective dose of cisplatin(μM)
SK-N-SH	1	1	7.5
NB100	1	1	8.5
IMR-32	20	3	10
NB1-G	25	7	7.5
XRNB1-G	25	10	11
NB2-G	30	3	11
SK-N-BE(2)C	800	2	13

Table 3 Comparison of N-*myc* copy number, level of N-*myc* expression and isoeffective dose of cisplatin (μM) for the seven cell lines.

Fig. 4a

Fig. 4b

Figure 4a. Southern blot analysis of N-*myc* copy number. DNA was digested with EcoR1, electrophoresed on 0.8% (w/v) agarose gels, and analysed by Southern blot. Each well contained 3μg of DNA except SK-N-BE(2)C (50ng) and the single copy N-myc line NB100 (20μg). Placental DNA (20 μg) was used as single copy control.
After electrophoresis, the DNA was transferred to nylon membrane and hybridised simultaneously to ^{32}P-labelled p-Nb1 and 5' bcr probes. Specific activity of these was approximately $2x10^7$ c.p.m./μg. The p-Nb1 probe bound to a 2kbp fragment.

Figure 4b. The bcr probe (used to verify loading) bound to a 13kbp fragment.

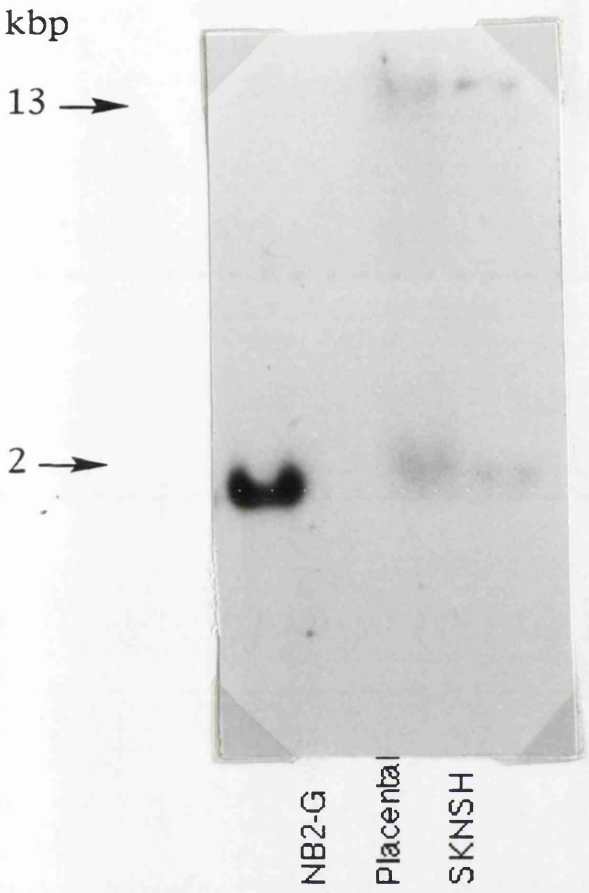

Figure 5. Southern blot analysis of N-*myc* copy number. DNA was digested with EcoR1, electrophoresed on 0.8% (w/v) agarose gels, and analysed by Southern blot. Wells contain 20μg of placental and SKNSH DNA and 3μg of NB2-G DNA. After electrophoresis, DNA was transferred to nylon membrane and hybridised simultaneously to ^{32}P-labelled p-Nb1 and 5' bcr probes. Specific activity of these was approximately $2x10^7$ c.p.m./μg. The p-Nb1 probe bound to a 2kbp fragment, and the bcr probe (used to verify loading) bound to a 13kbp fragment.

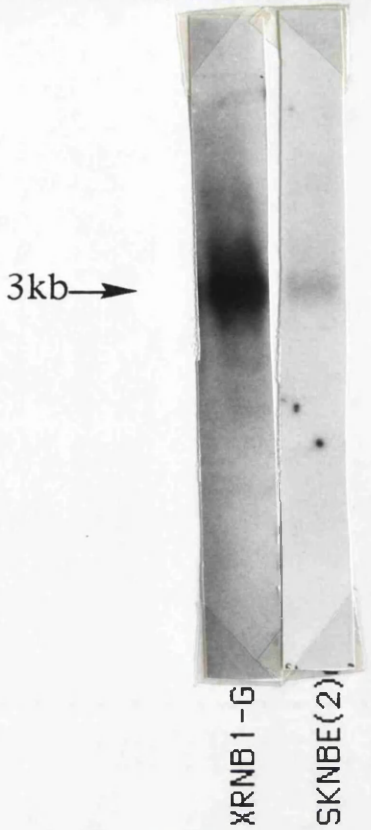

Figure 6. Northern blot analysis of N-*myc* expression. Total RNA was electrophoresed on formaldehyde/agarose gels, and analysed by Northern blot. After transferring to nylon membrane, the blot was hybridised to ^{32}P-labelled p-Nb1 probe with a specific activity of approximately 2 x 10^7 cpm per μg.
Each well contained 10μg of total RNA.

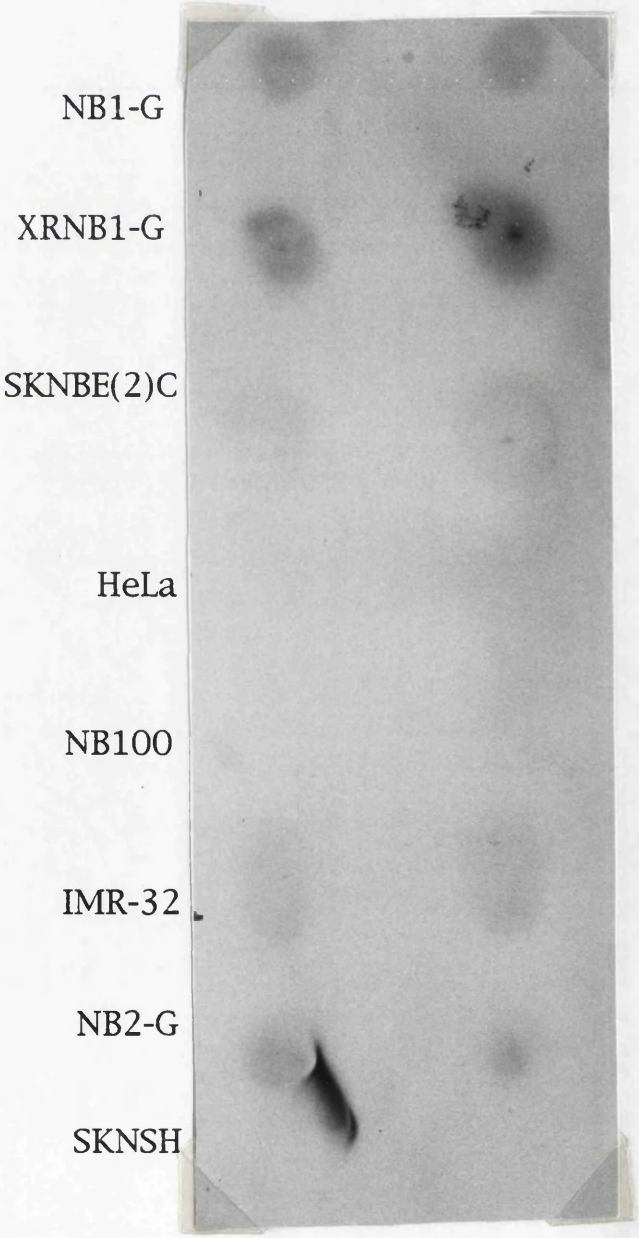

Figure 7. Dot blot analysis of N-*myc* expression. 2.5μg of total RNA was "dotted" onto nylon filter and hybridised as above. Hela cells were used as a negative control since they do not express N-myc RNA.

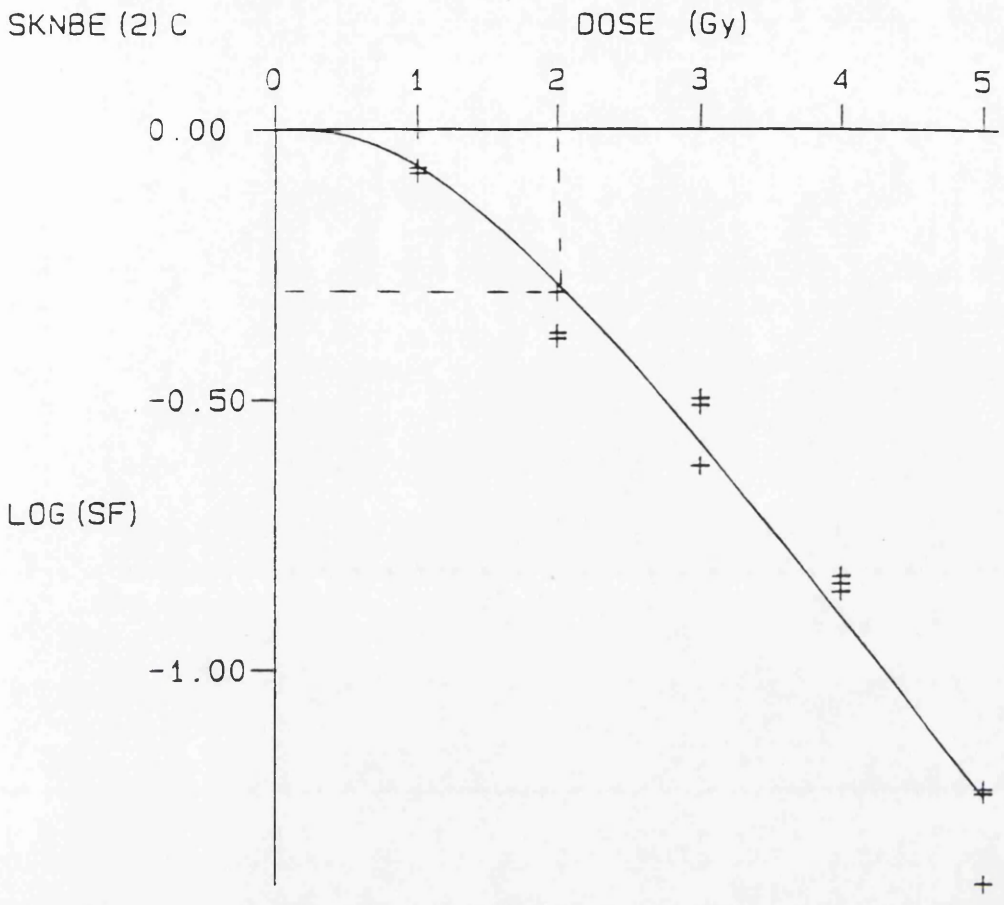

Figure 8. Survival curve for cell line SK-N-BE(2)C shows $\log_{10}$ (surviving fraction) versus dose of radiation (Gy). Spheroids were irradiated on a ^{60}Co source, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

The broken line on the plot illustrates how the surviving fraction at 2Gy (SF_2) was derived.

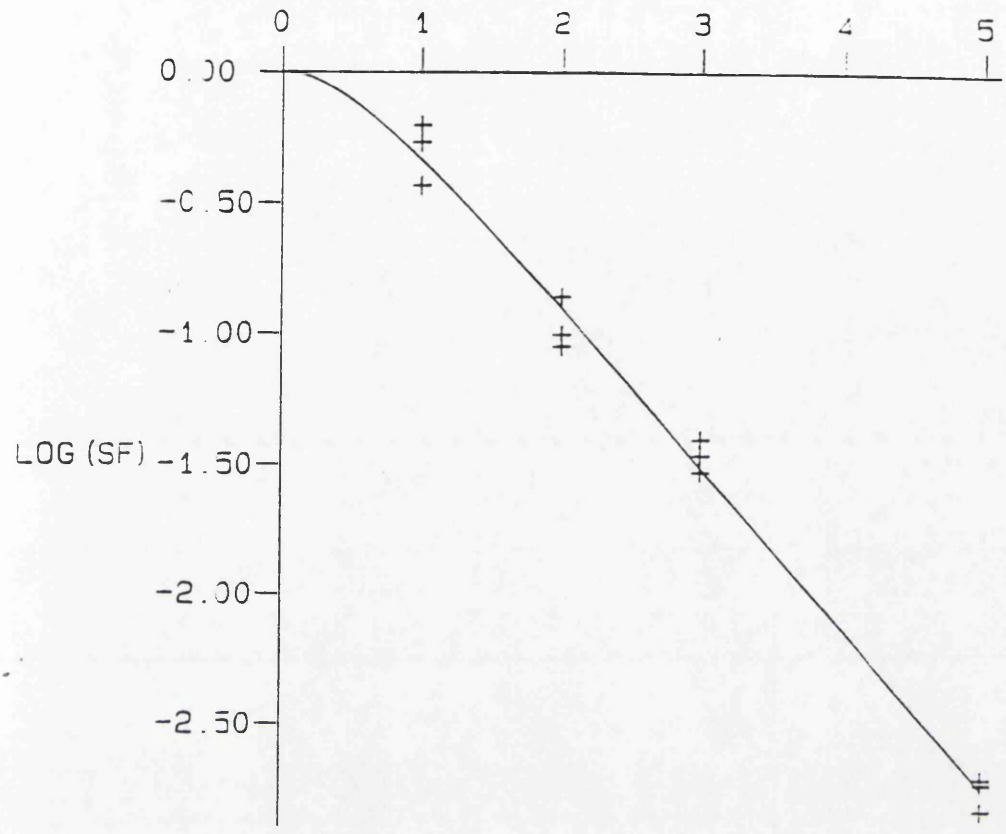

Figure 9. Survival curve for cell line SK-N-SH shows $\log_{10}$ (surviving fraction) versus dose of radiation (Gy). Spheroids were irradiated on a ^{60}Co source, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

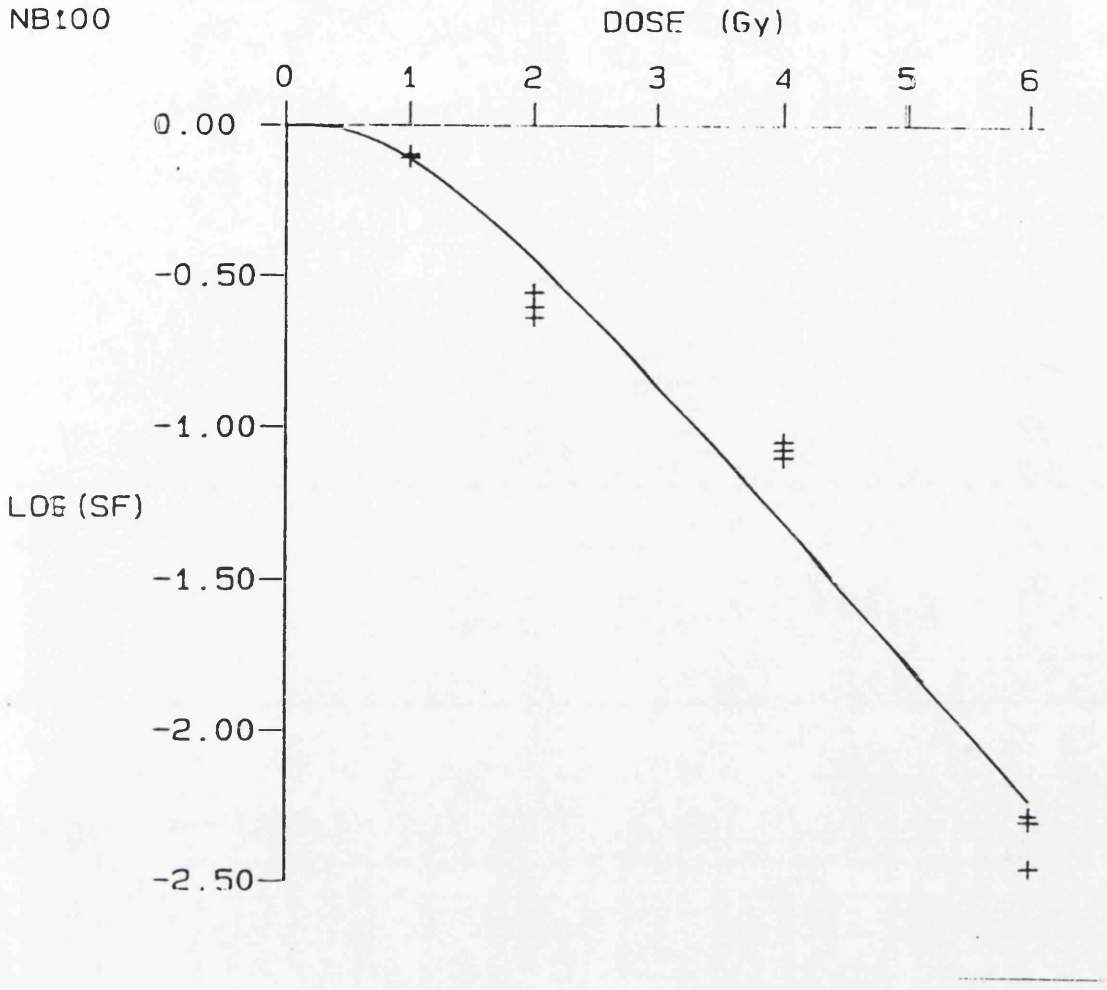

Figure 10. Survival curve for cell line NB100 shows $\log_{10}$ (surviving fraction) versus dose of radiation (Gy). Spheroids were irradiated on a ^{60}Co source, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

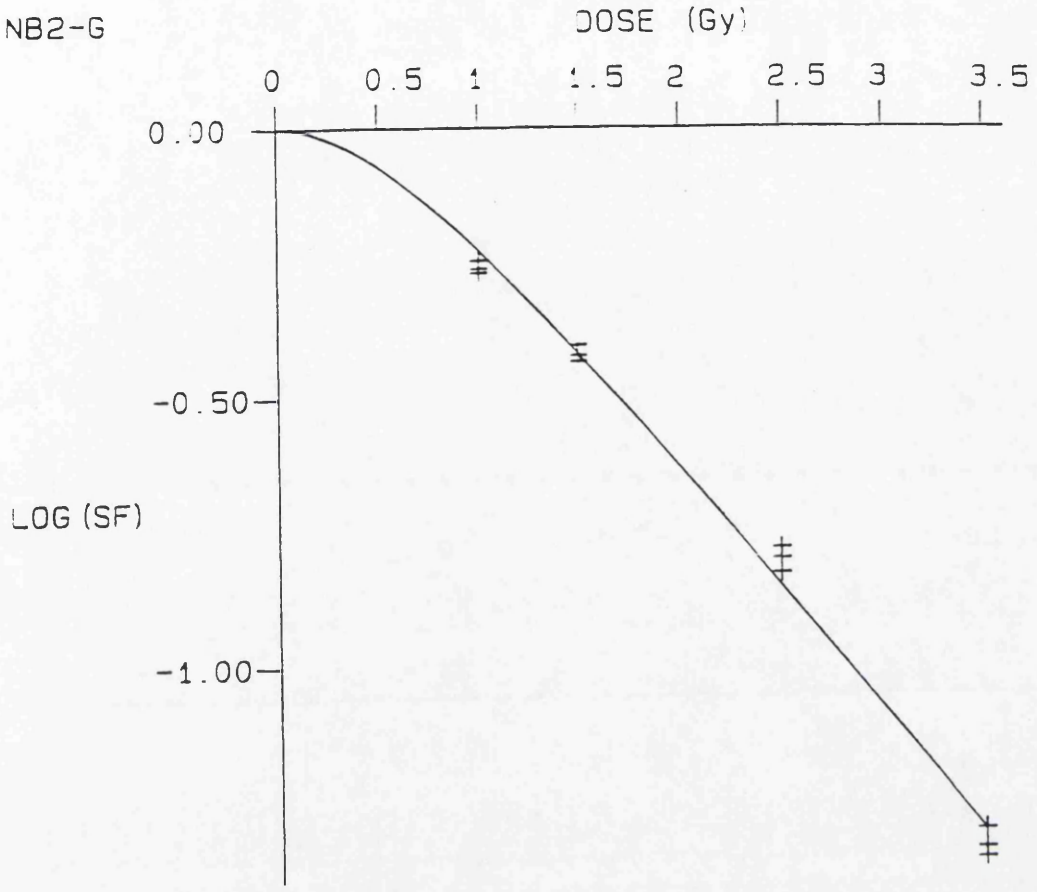

Figure 11. Survival curve for cell line NB2-G shows $\log_{10}$ (surviving fraction) versus dose of radiation (Gy). Spheroids were irradiated on a ^{60}Co source, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

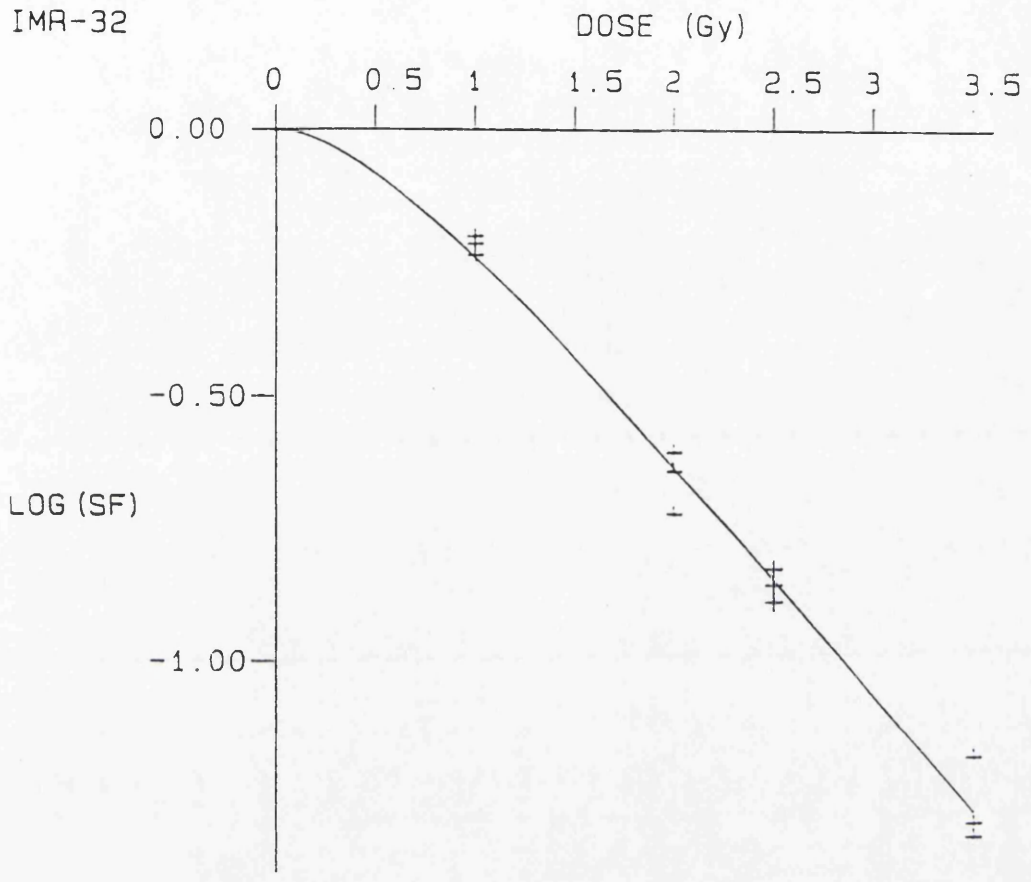

Figure 12. Survival curve for cell line IMR-32 shows $\log_{10}$ (surviving fraction) versus dose of radiation (Gy). Spheroids were irradiated on a ^{60}Co source, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

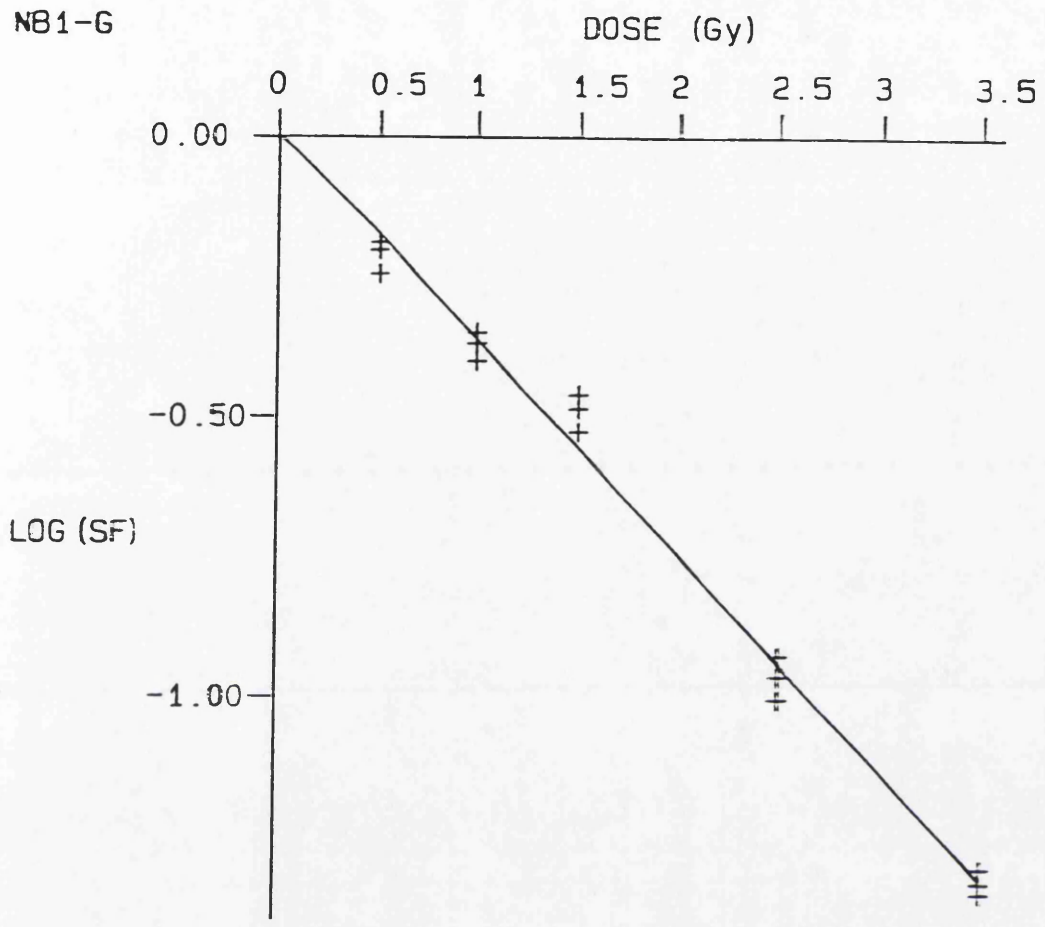

Figure 13. Survival curve for cell line NB1-G shows $\log_{10}$ (surviving fraction) versus dose of radiation (Gy). Spheroids were irradiated on a ^{60}Co source, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

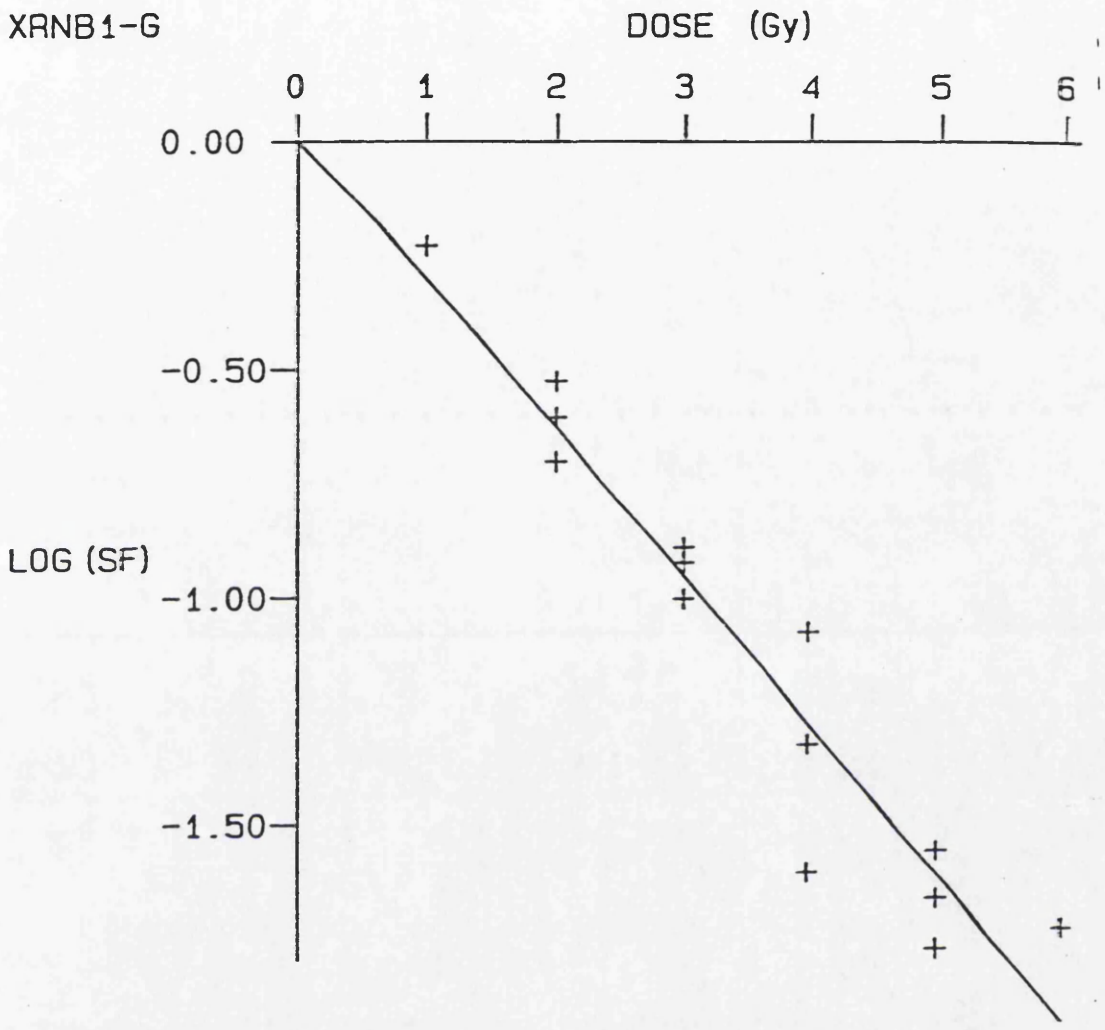

Figure 14. Survival curve for cell line XRNB1-G shows $\log_{10}$ (surviving fraction) versus dose of radiation (Gy). Spheroids were irradiated on a ^{60}Co source, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

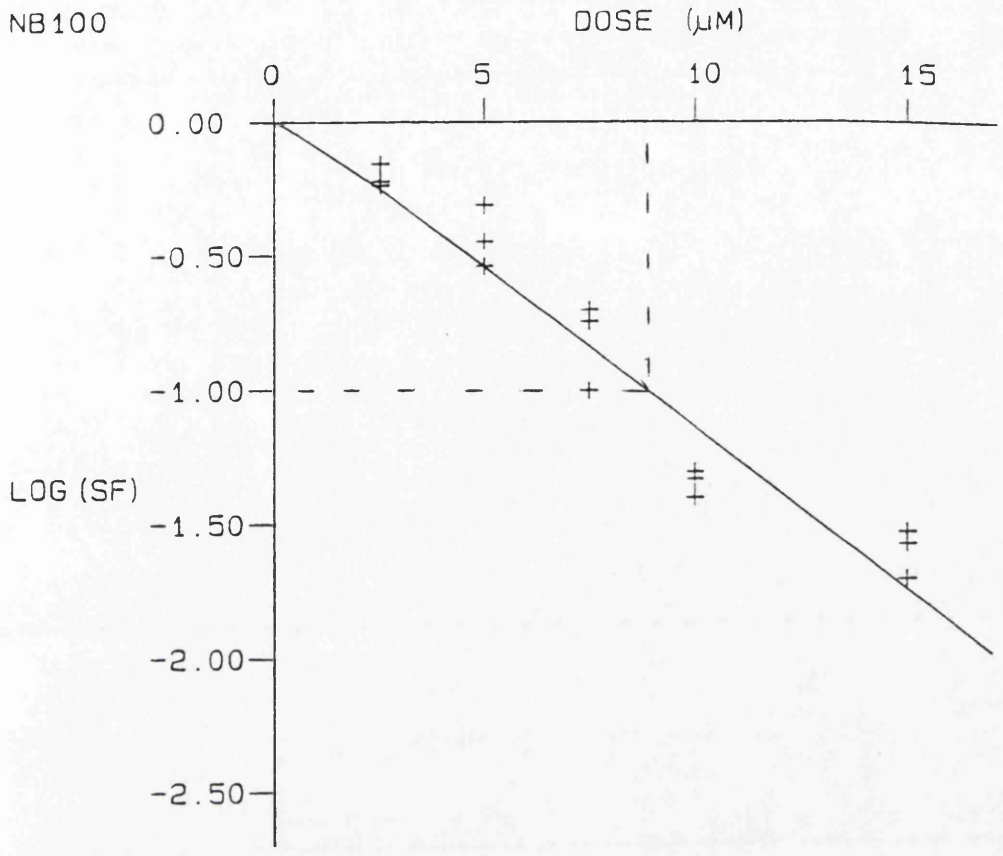

Figure 15. Survival curve for cell line NB100 shows $\log_{10}$ (surviving fraction) versus dose of cisplatin (μM). Spheroids were incubated with cisplatin, washed three times in Earles balanced salt solution, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

The broken line on the plot illustrates how the isoeffective dose of cisplatin was derived, i.e. the dose required to produce 1 log cell kill.

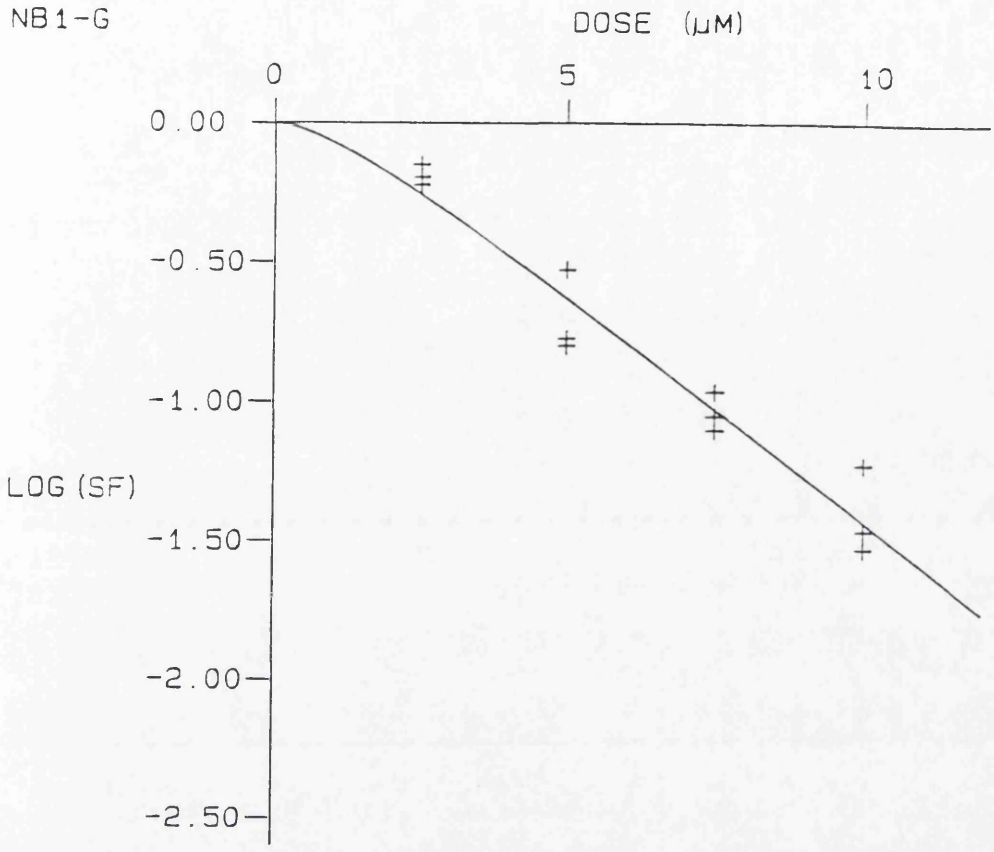

Figure 16. Survival curve for cell line NB1-G shows $\log_{10}$ (surviving fraction) versus dose of cisplatin (μM). Spheroids were incubated with cisplatin, washed three times in Earles balanced salt solution, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

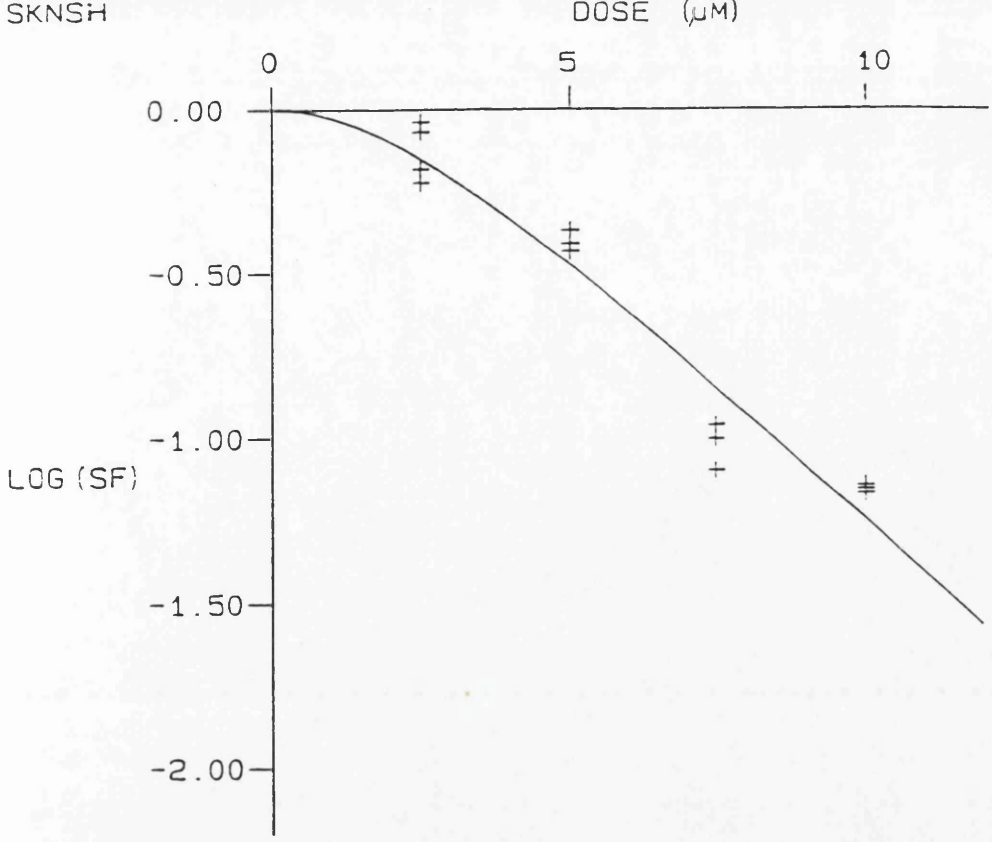

Figure 17. Survival curve for cell line SK-N-SH shows $\log_{10}$ (surviving fraction) versus dose of cisplatin (μM). Spheroids were incubated with cisplatin, washed three times in Earles balanced salt solution, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

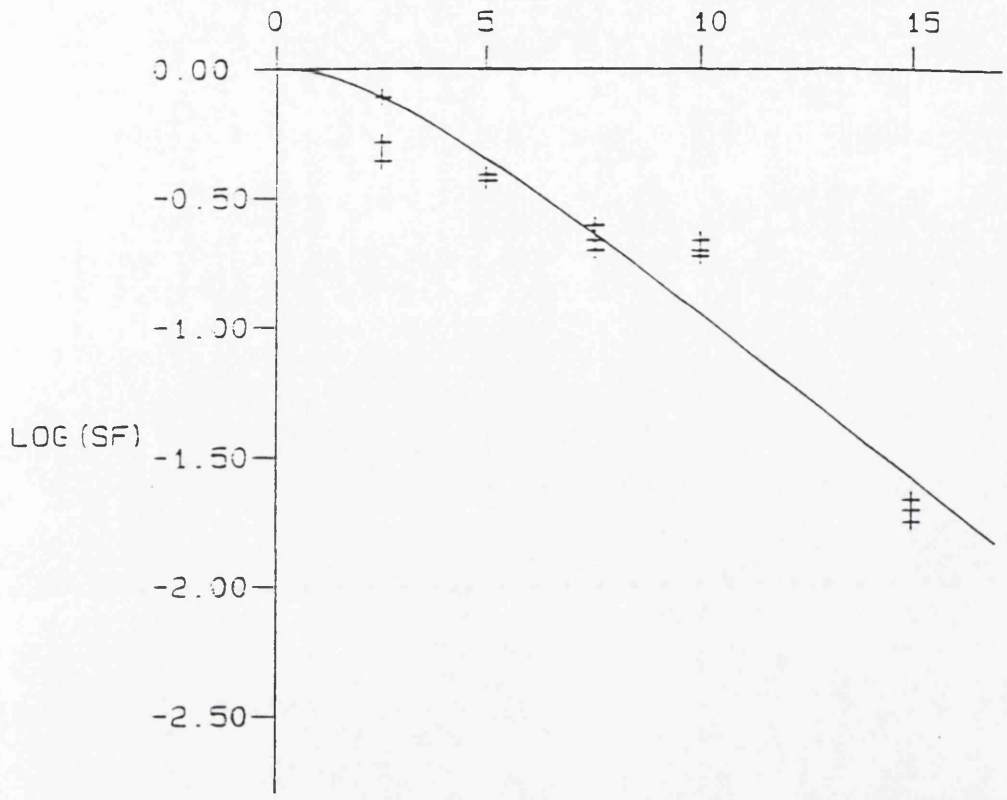

Figure 18. Survival curve for cell line IMR-32 shows $\log_{10}$ (surviving fraction) versus dose of cisplatin (μM). Spheroids were incubated with cisplatin, washed three times in Earles balanced salt solution, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

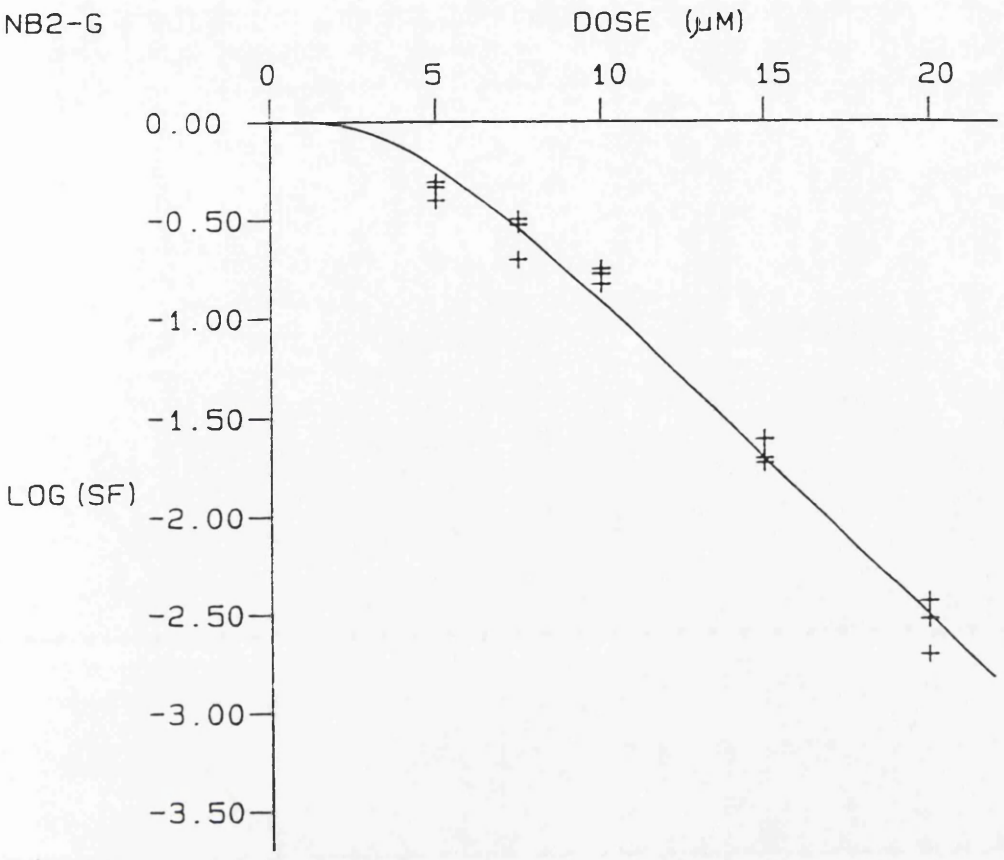

Figure 19. Survival curve for cell line NB2-G shows $\log_{10}$ (surviving fraction) versus dose of cisplatin (μM). Spheroids were incubated with cisplatin, washed three times in Earles balanced salt solution, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

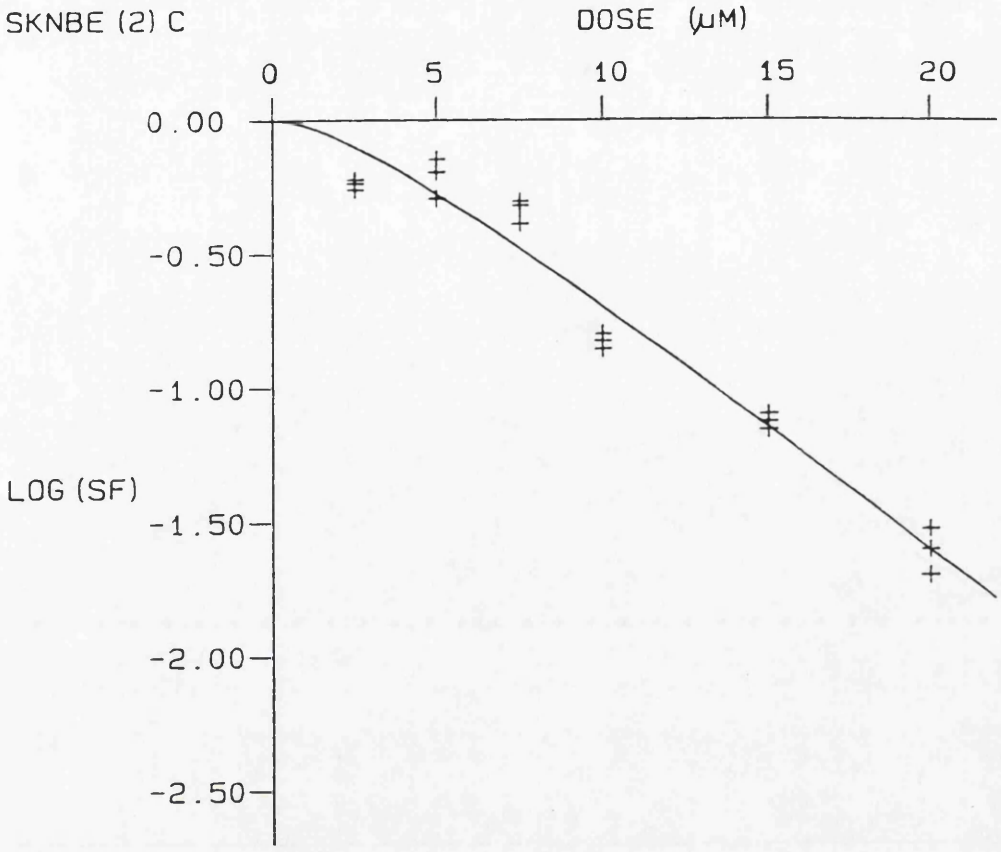

Figure 20. Survival curve for cell line SK-N-BE(2)C shows $\log_{10}$ (surviving fraction) versus dose of cisplatin (μM). Spheroids were incubated with cisplatin, washed three times in Earles balanced salt solution, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

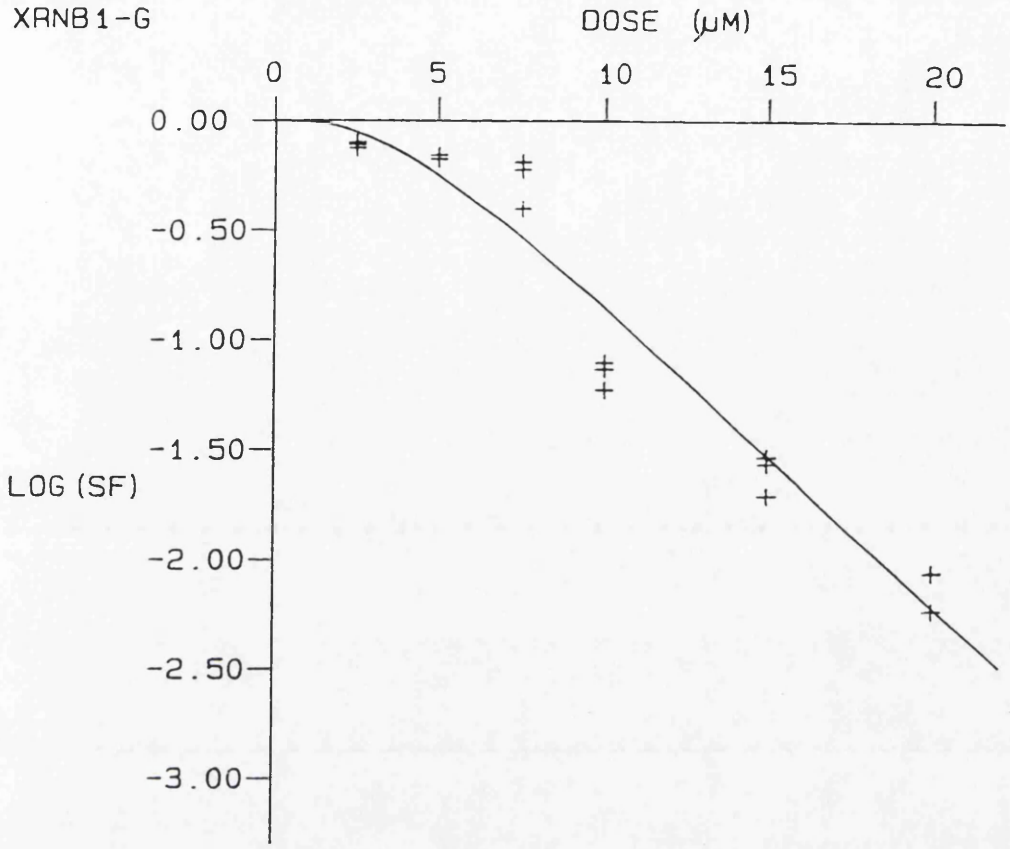

Figure 21. Survival curve for cell line XRNB1-G shows $\log_{10}$ (surviving fraction) versus dose of cisplatin (μM). Spheroids were incubated with cisplatin, washed three times in Earles balanced salt solution, disaggregated to form a single cell suspension, and plated to form colonies. Survival curves were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose.

RELATIONSHIP BETWEEN N-*myc* COPY NUMBER AND SF_2

The relationship between SF_2 and the $\log_{10}$ (N-*myc* copy number) is shown in figure 22. A logarithmic scale was used in order to accomodate the high copy number of 800. Spearman's rank correlation test (see materials and methods) was applied in order to evaluate the significance of association. Spearman's statistic = 0.327; p = 0.474. This shows no significant relation between the two, therefore no further statistical analysis was done.

RELATIONSHIP BETWEEN N-*myc* EXPRESSION AND SF_2

Figure 23 shows the relationship between SF_2 and N-*myc* expression for the cell lines. Spearman's rank correlation test gave the following results: Spearman's statistic = -0.255; p = 0.582. This shows no significant relationship between the two and, therefore, no further statistical analysis was done.

RELATIONSHIP BETWEEN N-*myc* COPY NUMBER AND ISOEFFECTIVE DOSE OF CISPLATIN

The relationship between $\log_{10}$ (N-*myc* copy number) and isoeffective dose of cisplatin indicated the following: Spearman's statistic = 0.750; p = 0.052. This indicates borderline significance, therefore a least squares fit analysis was done to investigate the relationship between N-*myc* copy number and isoeffective dose. This showed R = 0.708; p = 0.05. The relationship between isoeffective dose and the log of N-*myc* copy number (fig. 24) gave an R value (least squares analysis) = 0.806; $0.01 < p < 0.05$. These results indicate a possible relation between isoeffective dose and N-*myc* copy number. Therefore, the relationship could be of approximate linearity between isoeffective dose and log N-*myc* copy number.

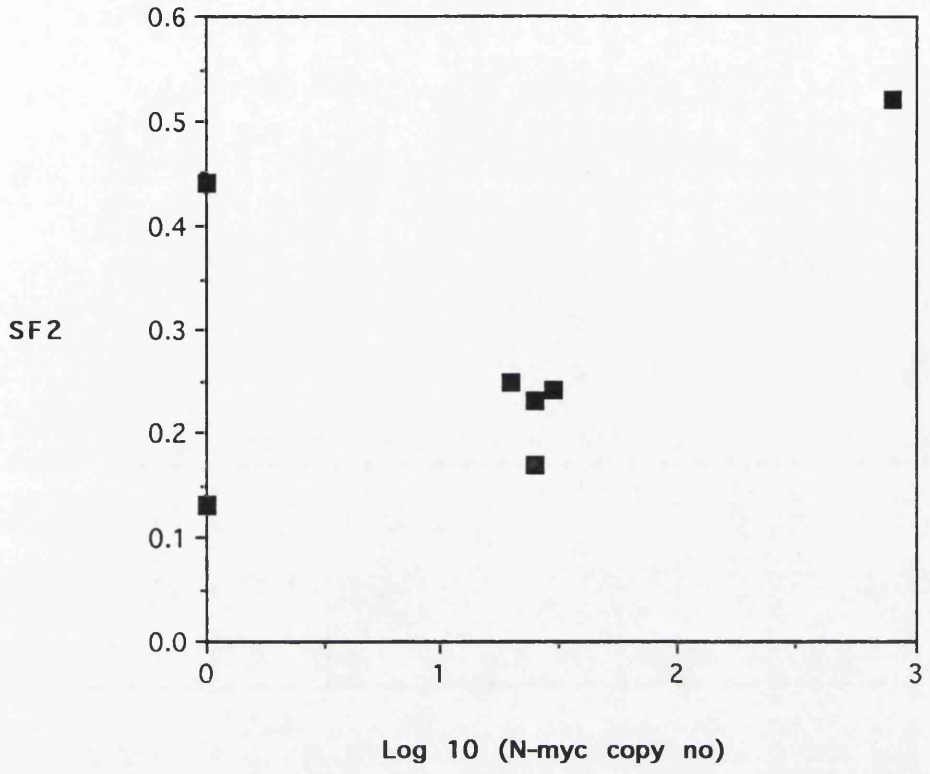

Figure 22. Relationship between N-*myc* copy number and SF_2. Plot of surviving fraction at 2Gy (SF_2) versus $\log_{10}$ (N-*myc* copy number) for seven cell lines. Each point represents the result of at least three separate experiments for each cell line. SF_2 values were derived from radiation survival curves.

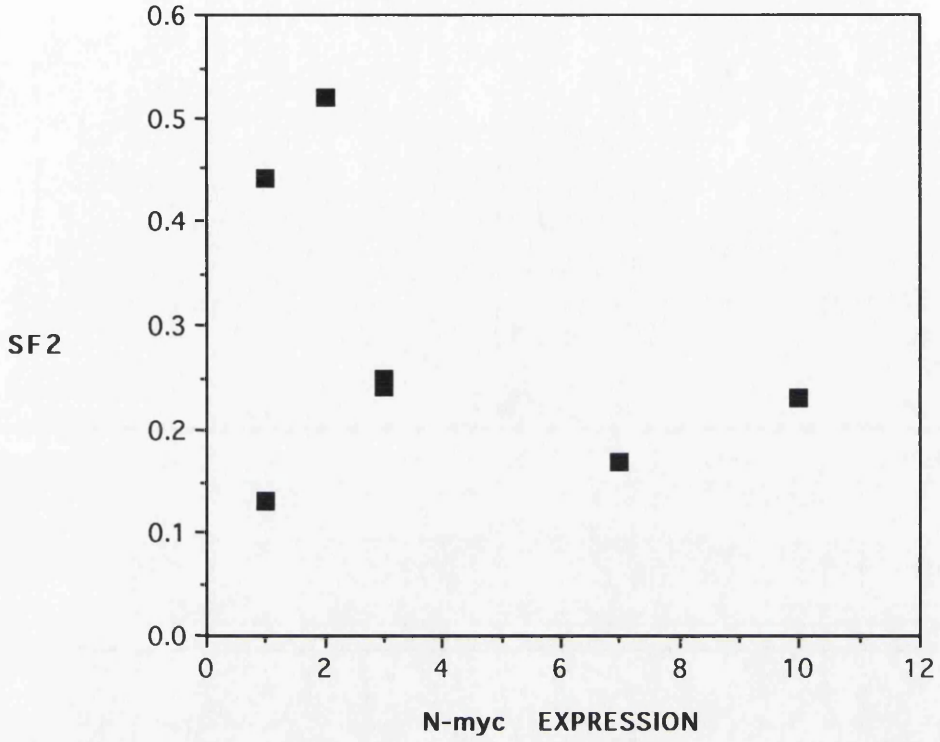

Figure 23. Relationship between N-*myc* expression and SF_2. Plot of surviving fraction at 2Gy (SF_2) versus level of N-*myc* expression for seven cell lines. Each point represents the result of at least three separate experiments for each cell line. SF_2 values were derived from radiation survival curves.

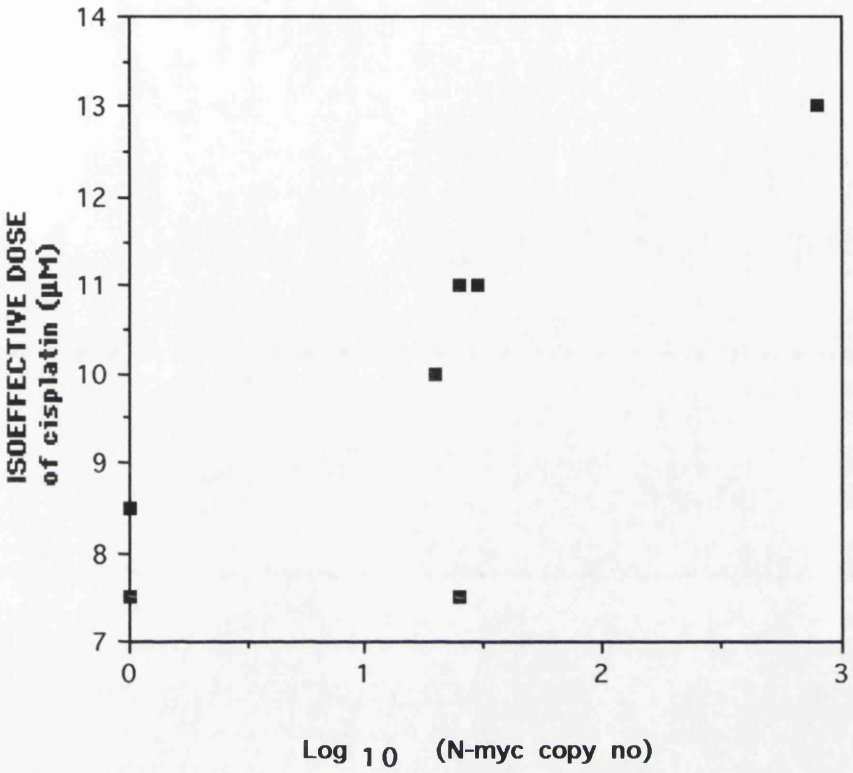

Figure 24. Relationship between N-*myc* copy number and isoeffective dose of cisplatin. Plot of isoeffective dose (μM dose required to produce 1 log cell kill) of cisplatin versus $\log_{10}$ (N-*myc* copy number) for seven cell lines. Each point represents the result of at least three separate experiments for each cell line. Isoeffective dose was derived from survival curves.

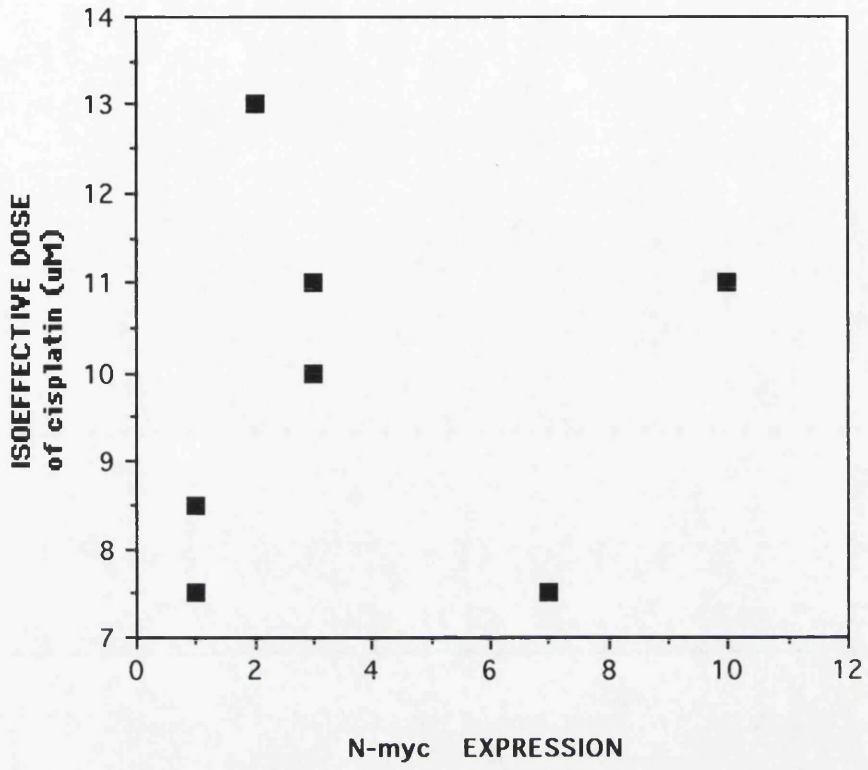

Figure 25. Relationship between N-*myc* expression and isoeffective dose of cisplatin. Plot of isoeffective dose (μM dose required to produce 1 log cell kill) of cisplatin versus level of N-*myc* expression in seven cell lines. Each point represents the result of at least three separate experiments for each cell line. Isoeffective dose was derived from survival curves.

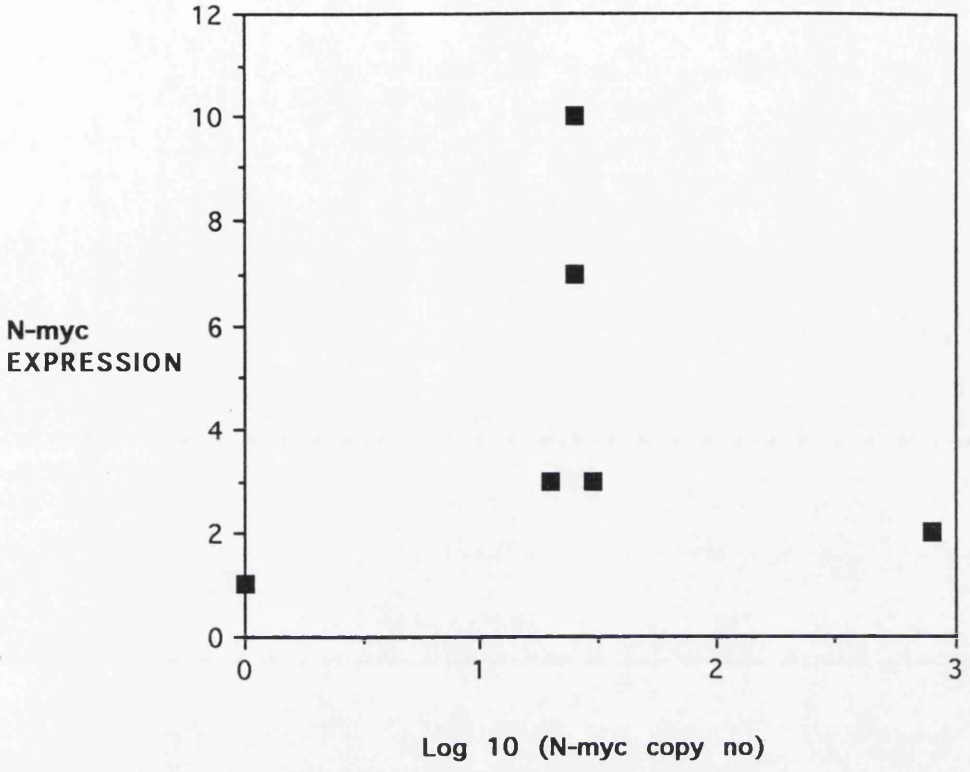

Figure 26. Relationship between N-*myc* expression and N-*myc* copy number. Plot of the level of N-*myc* expression versus $\log_{10}$ (N-*myc* copy number) for seven cell lines. The point on the Y axis represents two coincident points.

RELATIONSHIP BETWEEN N-*myc* EXPRESSION AND ISOEFFECTIVE DOSE OF CISPLATIN

Figure 25 shows the relationship between isoeffective dose of cisplatin and N-*myc* expression. Spearman's rank correlation gave Spearman's statistic = 0.222; p = 0.632. This shows no significant correlation between the two therefore, no further statistical analysis was done.

RELATIONSHIP BETWEEN N-*myc* EXPRESSION AND N-*myc* COPY NUMBER

Figure 26 shows the relationship between N-*myc* expression and $\log_{10}$ (N-*myc* copy number). Spearman's rank correlation test showed the following: Spearman's statistic = 0.463; p = 0.295. This indicated no significant relation between the two and, therefore, no further statistical analysis was carried out.

DISCUSSION

Data for radiation survival curves show a range of SF_2 values from 0.13 to 0.52 (table 2). From published data, an average value for the SF_2 of neuroblastoma cell lines is approximately 0.2, whereas the relatively radioresistant melanomas, osteosarcomas and glioblastomas average 0.5 (Deacon et al 1984; Steel and Wheldon 1990). Therefore, NB100 and SK-N-BE(2)C, which have SF_2 values of 0.44 and 0.52 respectively (table 2) indicate unusually high radioresistance for neuroblastomas. These two cell lines have n values of 5 and 3 respectively, indicating a shoulder on the initial portion of the survival curve and some ability to repair sub-lethal damage. This contrasts with NB1-G which has an n value of 1.

Table 4 indicates the N-*myc* copy number found for cell lines in this study, compared to the available information from other studies: -

Cell Line	This Study	Others
IMR-32	20	15-20 Schwab et al 1983 25 Kohl et al 1983
SKNSH	1	1 Kohl et al 1983 1 Schwab et al 1983
NB100	1	1 Kohl et al 1983
NB1-G	25	20-30 Carachi et al 1987
SK-N-BE(2)C	800	-
SK-N-BE(2)	-	170 Rosen et al 1986

Table 4 Comparison of N-*myc* copy numbers found in this study with the available published data.

Tables 2 and 3 show that the level of expression is not necessarily reflected in the degree of amplification of N-*myc*. This has been shown by others as mentioned previously on page 23. It would appear that N-myc expression is not a good prognostic indicator.

These studies have demonstrated that neither N-*myc* amplification nor its expression bears a strong relationship to the survival of cultured human neuroblastoma cells following their treatment with ionising radiation. Cisplatin studies showed no relation between N-*myc* expression and response to treatment. However, there may be a correlation between N-myc copy number and cisplatin resistance. This represents the first demonstration of a possible explanation for treatment failure in neuroblastoma patients with amplified N-*myc*. Whether this is due to N-*myc* enhanced DNA repair following cisplatin-induced cross-link damage or due to decreased cisplatin uptake remains to be investigated. In particular, the relationship between isoeffective dose of cisplatin and the logarithm of N-*myc* copy number showed a significant correlation. This would suggest that beyond a certain threshold value for N-*myc* copy number, prognosis is not reflected by degree of amplification. For example, a cell line with 800 copies of N-*myc* would not be ten times more chemoresistant than one with 80 copies.

There have been very few investigations into N-*myc* copy number and expression before and after chemotherapy in neuroblastoma patients. Those studies which have been carried out indicate an increase in N-*myc* expression after treatment, but no consistency in alteration of N-*myc* copy number (Rosen *et al* 1986; Tonini *et al* 1987; Kuroda *et al* 1991). These were not extensive studies and only involved a few patient samples. Until larger studies of this type has been carried out, the relationship between N-*myc* gene and drug resistance will remain unclear.

Investigations into the N-*myc* protein have shown it to be 62-64 kd in size and located in the nuclear fraction of human neuroblastoma cells (Igekaki *et al* 1986) Immunohistochemical techniques can be used to stain for the protein, and a

correlation has been found between positive staining and a poor prognosis (Hashimoto *et al* 1989). As with the c-*myc* protein, the N-*myc* gene product appears to bind to DNA and, along with other members of the *myc* family, appears to function in the control of cellular proliferation. It is possible that the N-*myc* protein interferes with the interaction between cisplatin and DNA, thereby reducing its cytotoxicity. Alternatively N-*myc* protein may enhance DNA repair following cisplatin-induced cross-link damage. Although the function of the N-*myc* protein is unknown in either neoplastic or normal tissues, it has been suggested that it may regulate gene expression during embryonic development since *myc* proteins have been observed to contain two structural domains previously seen in transcription factors (Collum and Alt 1990; Nakajiama *et al* 1989). Rosolen *et al* (1990) used synthetic anti-sense oligonucleotides to specifically inhibit N-*myc* m-RNA without affecting c-*myc* and showed a resulting decrease in growth in the CHP100 cell line (neuroepithelioma: single copy N-*myc*).

As discussed earlier (page 25) amplified N-*myc* may constitute as little as 0.1% of an HSR; therefore there may be other important genes residing here. It is possible that cisplatin resistance could be the result of expression of unknown genes which are co-amplified with N-*myc*. Since N-*myc* over-expression per se in single copy tumours does not seem to be associated with a poor outcome the question has been raised as to whether or not N-*myc* is the critical gene expressed from the amplified domain in tumours with N-*myc* amplification (Brodeur 1990). Indeed, it is possible that N-*myc* is a marker for another gene or genes which are responsible for poor prognosis (Brodeur 1990).

CONCLUSION AND IDEAS FOR THE FUTURE

The purpose of this study was to investigate the relationship between N-*myc* amplification/expression and resistance to radiotherapy and cisplatin in neuroblastoma cell lines. The results suggest that there may be a relationship between N-*myc* copy number and resistance to cisplatin. This could have important clinical relevance since cisplatin, in combination with other drugs, is one of the main treatments used to cure patients with neuroblastoma. Despite the fact that no correlation was found between copy number and resistance to radiotherapy, there is no doubt that N-*myc* copy number is a good prognostic indicator. For the future, there may be a number of issues worth investigating. For example, this study only looked into cisplatin sensitivity whereas a number of drugs could be used, e.g. vincristine or cyclophosphamide. It would be worthwhile exploring whether N-*myc* amplification confers resistance to these drugs. It would also be of interest to study a cell line with a hundred or so copies of N-*myc*. Lately, the importance of chromosome 1p deletion has emerged and this may be a candidate for a tumour suppressor gene (see page 19) or the site of a possible predisposition gene. In those patients with N-*myc* amplification, perhaps extra copies of N-*myc* are somehow increasing the turnover rate of the tumour cells. The antibody Ki67, which binds to an epitope uniquely expressed in dividing cells, may be a useful tool, for *in vivo* studies of the growth of patients' tumours. N-*myc* amplification may be enhancing metastatic spread, and it may be worthwhile carrying out an investigation to test this hypothesis.

Allan, P.M., J.A. Garson, E.I. Harper, and others. "Biological characterisation and clinical applications of a monoclonal antibody recognising an antigen restricted to neuroectodermal tissues." Int. J. Cancer 31 (1983): 591.

Andrews, P.A., M.P. Murphy, and S.B. Howell. " Characterisation of Cisplatin- resistant COLO 316 human ovarian cancer cells." Eur J Can Clin Oncol 25(4) (April 1989): 619-625.

Bahr, G., F. Gilbert, G. Balaban, and W. Engler. "Homogeneously staining regions and double minutes in a human cell line:Chromatin organisation and DNA content." J. Natl. Cancer Inst. 71 (1983): 657-661.

Bartram, C.R., A. De Klein, A. Hagemeijer, F. Carbonell, E. Kleihauer, and G. Grosveld. "Additional c-abl/bcr rearrangements in a cml patient exhibiting two Ph1 chromosomes during blast crisis." Leukemia Research 10 (2 1986): 221-225.

Bartram, C. and F. Berthold. "Amplification and Expression of the N-myc gene in Neuroblastoma." Eur. J. Paediatr. 146 (1987): 162-165.

Beierwaltes, W.H. "Update on basic research and clinical experience with metaiodobenzyl guanidine." Med. Paediatr. Oncol. 15 (1987): 163.

Benard, J., J. Bourhis, and G. Riou. "Clinical significance of MDR in human cancers." Anti Can Res. 10 (1990): 1297-1302.

Biedler, J.L., L. Helson, and B.A. Spengler. "Morphology and growth,tumourogenicity and cytogenetics of human neuroblastoma cells on continuous culture." Can. Res. 33 (1973): 2643-2652.

Biedler, J.L. and B.A. Spengler. "Metaphase chromosome anomaly:Association with drug resistance and cell-specific products." Science. 191 (1976): 185-187.

Biedler, J.L. and B.A. Spengler. "A novel chromosome abnormality in human neuroblastoma and antifolate-resistant Chinese hamster cells in culture." J. Natl. Cancer. Inst. 57 (1976): 683-695.

Birnie, GD. and S.V. Graham. Nuclear Structures: Isolation and characterisation. Chapter 11. ed. A.D. Mac Gillivray and G.D. Birnie. London.: Butterworths., 1985.

Bishop, J.M. "Cellular oncogenes and retroviruses." Ann. Rev. Biochem. 52 (1983): 301-354.

Bourhis, J., J. Benard, O. Hartmann, L. Boccon-Gibod, J. Lemerle, and G. Riou. "Correlation of mdr-1 gene expression with chemotherapy in neuroblastoma." J. Natl. Can. Inst. 81 (18 1989): 1401-1405.

Brodeur, G.M., R.C. Seeger, M. Schwab, H.E. Varmus, and J.M. Bishop. "Amplification of N-myc in untreated human neuroblastomas correlates with advanced stage disease." Science 224 (1984): 1121-1124.

Brodeur, G.M., R.C. Seeger, H. Sather, A. Dalton, S.E. Siegel, K.Y. Wong, and D. Hammond. "Clinical Implications of Oncogene Activation in Human Neuroblastomas." Cancer 58 (1986): 541-545.

Brodeur, G.M. and R.C. Seeger. "Gene amplification in human neuroblastomas:Basic mechanisms and clinical implications." Cancer Genet. Cytogenet. 19 (1986): 101-111.

Brodeur, G.M., A. Hayes, A.A. Green, J.T. Casper, J. Wasson, S. Wallach, and R.C. Seeger. "Consistent N-myc copy number in simultaneous or consecutive neuroblastoma samples from 60 individual patients." Can. Res. 47 (Aug 15 1987): 4248-4253.

Brodeur, G.M., R.C. Seeger, A. Barrett, F. Berthold, R.P. Castleberry, P. D'Angio, and 20 others. "International criteria for diagnosis, staging and response to treatment in patients with neuroblastoma." J. Clin. Oncol 6 (1988a): 1874-1881.

Brodeur, G.M., C.T. Fong, M.G. Morita, F.A. Hayes, and R.C. Seeger. "Molecular analysis and clinical significance of N-myc amplification and chromosome 1 abnormalities in human neuroblastomas." Progr. Clin. Biol. Res. 271 (1988b): 3-15.

Brodeur, G.M., F. Gilbert, A. Nakagawara, and R.C. Seeger. "Discussion." In Advances in neuroblastoma research 2., ed. A.E. Evans, G.J. D'Angio, and A.G. Knudson. 51-56. New York: Wiley-Liss, 1988c.

Brodeur, G.M. and C-t. Fong. "Molecular biology and genetics of human neuroblastoma." Cancer Genet. Cytogenet. 41 (1989): 153-174.

Brodeur, G.M. "Neuroblastoma:clinical significance of genetic abnormalities." Cancer Surveys 9 (4 1990):

Burck, K.B., E.T. Liu, and J.W. Larrick. "Chapters 5,6 & 10." In Oncogenes:an introduction to the concept of cancer genes., ed. K.B. Burck, E.T. Liu, and J.W. Larrick. New York.: Springer-Verlag., 1988.

Calman, K.C., J.F. Smyth, and M.H.N. Tattershall. "chapter 5." In Basic principles of cancer chemotherapy., ed. K.C. Calman, J.F. Smyth, and M.H.N. Tattershall. 49-78. London & Basingstoke.: MacMillan press, 1980.

Canon, J-L., Y. Humblet, and M. Symann. "Resistance to cis-platin:how to deal with the problem?" Eur. J. Can. 26 (1) (Jan 1990): 1-3.

Carachi, R., T. Roza, D. Robertson, T.E. Wheldon, L. Wilson, A. Livingstone, V. Van Heyningen, G. Spowart, P. Middleton, J.R. Gosden, J.T. Kemshead, and J.P. Clayton. "Biological properties of a tumour cell line (NB1-G) derived from human neuroblastoma." Br. J. Cancer. 55 (1987): 407-411.

Christiansen, H., N.M. Christiansen, F. Wagner, M. Altmannsberger, and F. Lampert. "Neuroblastoma: Inverse relationship between expression of N-myc and NGF-r ." Oncogene 5 (1990): 437-440.

Cohen, P.S., R.C. Seeger, T.J. Triche, and M.A. Israel. "Detection of N-myc gene expression in neuroblastoma tumours by in situ hybridisation." Am. Jnl. Pathology. 131 (3 1988): 391-397.

Collum, R. and F.W. Alt. "Are myc proteins transcription factors?" Cancer Cells 2 (1990): 69-75.

Combaret, V., Q. Wang, M.C. Favrot, P. Thiesse, I. Philip, and E. Bouffet. "Clinical Value of N-myc oncogene amplification in 52 patients with Neuroblastoma included in recent therapeutic protocols." Eur. J Cancer Clin Oncol 25 (11 1989): 1607-1612.

Courtenay, V.D. and J. Mills. "An in vitro colony assay for human tumours grown in immune-suppressed mice and treated in vivo with cytotoxic agents." Br. J. Cancer 37 (1978): 261-268.

Cox, D., C. Yuncken, and A. Sproggs. "Minute chromatin bodies in malignant tumours of childhood." Lancet 2 (1965): 55-58.

D'Angio, G.J. "Paediatric Tumours." In Principles and practice of radiation oncology., ed. Perez and Brady. 1199. J.B. Lippincott, 1987.

Davis, L.G., M.D. Dibner, and J.F. Battey. Basic Methods in Molecular Biology. New York, Amsterdam,London.: Elsevier., 1986.

Deacon, J., M.J. Peckham, and G.G. Steel. "The radioresponsiveness of human tumours and the initial slope of the cell survival curve." Radiotherapy and Oncology 2 (1984): 317-323.

Deacon, J, M.,, P.A. Wilson. and M.J. Peckham. "The radiobiology of human neuroblastoma." Radiotherapy and Oncology 3 (1985): 201-209.

Delattre, O., J. Michon, J.M. Zucker, and G. Thomas. "Acquired genetic alterations in neuroblastoma: Clinical implications." Cancer Journal. 4 (4). (July- August. 1991): 238-243.

Der, C.J., T.G. Krontiris, and G.M. Cooper. "Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses." Proc. Natl.Acad. Sci. USA 79 (1982): 3637-40.

Dias, P., P. Kumar, H.B. Marsden, H.R. Gottamaneni, J. Heighway, and S. Kumar. "N- myc gene is amplified in alveolar rhabdomyosarcomas (RMS) but not in embryonal RMS." Int. J. Cancer. 45 (1990): 593-596.

Dobbs, J., A. Barrett, and D. Ash. Practical Radiotherapy Planning. 2nd ed., London: Edward Arnold, 1992.

Dominici, C., A. Negroni, A. Romeo, M.A. Castello, A. Clerico, M. Scopinaro, F. Mauro, and G. Raschella. "Association of near diploid DNA content and N-myc amplification in neuroblastomas." Clin. Exp. mets. 7 (2 1989): 201-211.

Fertil, B. and E.P. Malaise. "Inherent radiosensitivity as a basic concept for human tumour radiotherapy." Int. J. Radiat. Oncol. Biol. Phys. 7 (1981): 621-629.

Filmus, J. and R.N. Buick. "Relationship of c-myc expression to differentiation and proliferation of HL-60 cells." Cancer. Res. 45 (1985): 822-825.

Fitzgerald, T.J., L.A. Rothstein, B.S. Daugherty, M. Mc Kenna, K. Kase, and J.S. Greenburger. "Activated N-ras oncogene enhances irradiation repair of mammalian cells in vitro less effectively at a low dose rate." Am J

Clin Oncol 8 (1985): 517-522.

Fong, C.T., N.C. Dracopoli, P.S. White, P.T. Merrill, R.C. Griffith, D.E. Housman, and G.M. Brodeur. "Loss of heterozygosity for chromosome 1p in human neuroblastomas:correlation with N-myc amplification." Proc. Natl. Acad. Sci. USA 86 (1989): 3753-3757.

Gerlach, J., N. Kartner, D. Bell, and V. Ling. "Multidrug resistance." Cancer Surveys 5 (1986): 25-46.

Gibson, N.W. "Alkylating agents:mechanisms and modulation. chapter 1." In Cancer and chemotherapy:concepts,clinical investigations and therapeutic advances., ed. F.M. Muggia. Kluwer academic., 1989.

Goldstein, L.J., A.T. Fojo, K. Ueda, W. Crist, A. Green, G.M. Brodeur, I. Pastan, and M.M. Gottesman. "Expression of the multidrug resistance,MDR1, gene in neuroblastomas." J. Clin. Oncol. 8 (1990): 128-136.

Hall, E.J. Radiobiology for the Radiobiologist. ed. E.J. Hall. Maryland: Harper and Row, 1988.

Hamburger, A.W. and S.E. Salmon. "Primary bioassay of human tumour stem cells." Science 197 (1977): 461-463.

Hann, S.R. and R.N. Eisenman. "Proteins encoded by the human c-myc oncogene: differential expression in neoplastic cells." Mol. Cell. Biol. 14 (1984): 2486-2497.

Hashimoto, H., Y. Daimaru, M. Enjoji, and A. Nakagawara. "N-myc gene product expression in neuroblastoma." J. Clin. Pathol 42 (1989): 52-55.

Hayes, F.A. and E.I. Smith. "Neuroblastoma." In Principles and practice of paediatric oncology:chapter 28, ed. P.A. Pizzo and D.G. Poplack. 607-622. Lippincott, J.B., 1989.

Hiyama, E., K. Hiyama, T. Yokoyama, and T. Ishii. "Immunohistochemical analysis of N-myc protein expression in neuroblastoma: correlation with prognosis of patients." J. Paediatr. Surg. 26 (7). (July. 1991): 838-843.

Hollstein, M., G. Sidronsky, B. Vogelstein, and C.C. Harris. "p53 mutations in human cancers." Science. 253 (5 July 1991): 49-53.

Igekaki, N., J. Bukovsky, and R.H. Kennett. "Identification and characterization of the N-myc gene product in human neroblastoma cells by monoclonal antibodies with defined specificities." Proc. Natl. Acad. Sci. USA 83 (1986): 5929-5933.

Kasid, U., A. Pfeifer, R.R. Wiechselbaum, A. Dritschilo, and G.E. Mark. "The raf oncogene is associated with a radiation-resistant human laryngeal cancer." Science 237 (1987): 1039-1043.

Kemshead, J.T. "Monoclonal antibodies - their use in the diagnosis and therapy of paediatric and adult tumours derived from the neuroectoderm." In Monoclonal antibodies for cancer detection and therapy., ed. R.W. Baldwin and V.S. Byers. 281. London: Academic press., 1985.

Kerr, D.I., J.A. Plumb, R.I. Freshney, M.Z. Khan, and D.A. Spandidios. "The Effect of H-ras Oncogene Transfection on Response of Mink Lung Epithelial Cells to Growth Factors and Cytotoxic Drugs." Anticancer Res. 11 (1991): 1349-1352.

Klein, G. "The role of gene dosage and genetic transpositions in carcinogenesis." Nature 294 (1981): 313-318.

Kohl, N., N. Kanda, R.R. Schreck, G. Bruns, S.A. Latt, F. Gilbert, and F.W. Alt. "Transposition and Amplification of Oncogene-Related Sequences in Human Neuroblastomas." Cell 35 (December (Part 1) 1983): 359-367.

Kohl, N.E., C.E. Gee, and F.W. Alt. "Activated expression of the N-myc gene in human neuroblastomas and related tumours." Science 226 (14 December 1984): 1335-1337.

Kohl, N.E., E. Legouy, R.A. DePinho, P.D. Nisen, R.K. Smith, C.E. Gee, and F.W. Alt. "Human N-myc is closely related in organisation and nucleotide sequence to c-myc." Nature 319 (Jan 1986): 73-77.

Kretschmar, C.S. "Neuroblastoma." In Comprehensive Textbook of Oncology, ed. A.R. Moossa, S.C. Schimpff, and M.C. Robson. 1499-1513. 2. Baltimore: Williams & Wilkins, 1991.

Krontiris, T.G. and G.M. Cooper. "Transforming activity of human tumour DNAs." Proc. Natl. Acad. Sci. USA 78 (1981): 1181-84.

Kuroda, H., T. Sugimoto, K. Veda, S. Tsuchida, Y. Horii, and J. Inazowa. "Different drug sensitivity in two different cell lines established from the same patient before and after chemotherapy." Int. J. Cancer. 47 (1991): 732-737.

Larcher, J.C., J.L. Vayssiere, L. Lossouarn, F. Gros, and B. Croizet. "Regulation of c and N-myc expression during induced differentiation of murine neuroblastoma cells." Oncogene 6 (4 1991): 633-638.

Lee, W.H., A.L. Murphree, and W.F. Benedict. "Expression and amplification of the N-myc gene in primary Retinoblastoma." Nature. 309 (1984): 450-460.

Look, A.T., F.A. Hayes, J.J. Shuster, E.C. Douglass, R.P. Castleberry, L.C. Bowman, E.I. Smith, and G.M. Brodeur. "Clinical relevance of tumour cell ploidy and N-myc gene amplification in childhood neuroblastoma: a Paediatric oncology group study." J. Clin.Oncol. 9 (4). (1991): 581-591.

Mairs, R.J., W. Angerson, M.N. Gaze, T. Murray, J.W. Babich, R. Reid, and C McSharry. "The distribution of alternative agents for targeted radiotherapy within human neuroblastoma spheroids." Br. J. cancer 63 (1991): 404-409.

Mc Kenna, W.G., G. Iliakis, M.C. Weiss, E.J. Bernhard, and R.J. Muschel. "Increased G2 delay in radiation-resistant cells obtained by transformation of primary rat embryo cells with the oncogenes H-ras and v-myc." Radiation Res. 125(3) (1991): 283-7.

McQuaid, S. and A. O-Meara. "N-myc oncogene amplification in paediatric tumours." Ir. J. Med. Sci. 159 (6). (1990):

Minden, M.D. "Oncogenes,chapter 5." In Basic Science of Oncology., ed. I.F. Tannock and R.P. Hill. 72-78. Pergamon Press., 1987.

Mueller-Klieser, W. "Multicellular Spheroids:A review on cellular aggregates in cancer research." Jnl. Can Res. and Clin. Oncol. 113 (1987): 101-122.

Mugrauer, G., F.W. Alt, and P. Ekblom. "N-myc expression during organogenesis in the developing mouse as revealed by in situ hybridisation." J. Cell. Biol. 107 (4 1988): 1325-1336.

Nakagawara, A., K. Ikeda, T. Tsuda, K. Higashi, and T. Okabe.

"Amplification of N-myc oncogene in stage 2 and 4-S neuroblastomas may be a prognostic indicator." Journal of paediatric surgery. 22 (5 1987): 415-418.

Nakagawara, A., K. Kadomatsu, S-I. Sato, K. Kohno, H. Takano, K. Akazawa, Y. Nose, and M. Kuwano. "Inverse correlation between expression of multidrug resistance gene and N-myc oncogene in Human Neuroblastomas." Can Res. 50 (may 15 1990): 3043-3047.

Nakajiama, H., M. Ikeda, N. Tsuchida , S. Nishimura, and Y. Taya. "Inactivation of the N-myc gene product by single amino acid substitution of leucine residues located in the leucine-zipper region." Oncogene 4 (1989): 999-1002.

Nisen, P.D., P.G. Waber, M.A. Rich, S. Pierce, J.R. Garvin, F. Gilbert, and P Lanzkowsky. "N-myc oncogene RNA expression in Neuroblastoma." J Natl Cancer Inst 80 (1988): 1633-1637.

O'Brien, K.P., R.A. Weisgrau, C.T. Black, and J. Halpern. "Neuroblastoma:chapter 3." In Handbook of paediatric oncology., ed. R.A. Gottlieb and D. Pinkel. 15-19. Little, Brown & co, 1989.

Parada, L.F., C.J. Tabin, C. Shih, and R.A. Weinberg. "Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene." Nature 297 (1982): 474-78.

Pardo, F.S., R.G. Bristow, A. Taghian, A. Ong, and C. Borek. "Role of transfection and clonal selection in mediating radioresistance." Proc. Natl. Acad. Sci. USA. 88 (December 1991): 10652-10656.

Pirollo, K.F., R. Garner, S.Y. Yuan, L. Li, W.A. Blattner, and E.H. Chang. "Raf invovement ih the simultaneous genetic transfer of the radioresistant and transforming phenotypes." Int. J. Radiat. Biol 55 (1989): 783-796.

Pizzo, P.A., M.E. Horowitz, D.G. Poplack, D.M. Hays, and L.E. Kun. "Solid tumours of childhood : chapter 47." In Cancer: principles and practice of oncology., ed. V.T. De Vitta, S. Hellman, and S.A. Rosenberg. 1624-1631. London: Liipincot, J.B., 1989.

Powell, S. and T.J. McMillan. "DNA damage and repair following treatment with ionising radiation." Radiother. & Oncol. 19 (2) (1990): 95-108.

Reddy, E.P., R.K. Reynolds, E. Santos, and M. Barbacid. "A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene." Nature 300 (1982): 149-152.

Renan, M.J. "Cancer genes: current status,future prospects,and applications in radiotherapy/oncology." Radiother. and Oncol. 19 (1990): 197-218.

Reynolds, C.P., J.L. Biedler, B.A. Spengler, D.A. Reynolds, R.A. Ross, E.P. Frenkel, and R.C. Smith. "Characterization of human neuroblastoma cell lines established before and after therapy." J.Natl. Cancer Inst. 76 (1986): 375-387.

Rosen, N., C.P. Reynolds, C.J. Thiele, J.L. Biedler, and M.A. Israel. "Increased N-myc expression following progressive growth of human neuroblastoma." Cancer Res. 46 (1986): 4139-4142.

Rosenbaum, H., E. Webb, M. Adams, S. Cory, and A.W. Harris. "N-myc transgene promotes B lymphoid proliferation, elicits lymphomas and reveals cross regulation with c-myc." EMBO 8 (1989): 749-755.

Rosolen, A., L. Whitesell, N. Ikegaki, R.H. Kennett, and L.M. Neckers. "Antisense inhibition of single copy N-myc Expression results in decreased cell growth without reduction of c-myc protein in a neuroepithelioma cell line." (1990): 6316-6322.

Rowley, J.D. "Biological implications of consistent chromosome rearrangements in leukaemia and lymphomas." Cancer Res. 44 (1984): 3159-3168.

Russell, J. "Isolation of a radioresistant mutant, obtained from a radiosensitive human neuroblastoma cell line." Br. J. Radiol. 63 (1990):

Russell, J., M.Z. Khan, D.J. Kerr, and D.A. Spandidos. "The effect of transfection with the oncogenes H-ras and c-myc on the radiosensitivity of a mink epithelial cell line." Radiation Research 130 (1992): 113-116.

Sambrook, J., E.F. Fritsch, and T. Maniatis. In Molecular Cloning - A Lab Manual, ed. J. Sambrook, E.F. Fritsch, and T. Maniatis. 7.53. 1. Cold Spring Harbour, 1989.

Schimke, R.T. "Gene Amplification: Summary." In Gene Amplification, ed. R.T. Schimke. 317-333. Cold Spring Harbour, N.Y.: Cold Spring Harbour Laboratory Press, 1982.

Schlesinger, H.R., J.M. Gerson, P.S. Moorhead, H. Maguire, and K. Hummeler. "Establishment and characterisation of human cell lines." Can. Res. 36 (September. 1976): 3094-3100.

Schwab, M., K. Alitilo, K.-H. Klempnauer, H.E. Varmus, J.M. Bishop, F. Gilbert, G. Brodeur, M. Goldstein, and J. Trent. "Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour." Nature 305 (1983): 245-248.

Schwab, M., H.E. Varmus, J.M. Bishop, K.H. Grzeschik, S.L. Naylor, A.Y. Sakaguchi, G. Brodeur, and J. Trent. "Chromosome localisation in normal human cells and neuroblastomas of a gene related to c-myc." Nature 308 (1984): 288-291.

Schwab, M. "Enhanced expression of N-myc consequent to amplification of DNA may contribute to malignant progression of neuroblastoma." Proc. Natl. Acad. Sci. USA. 81 (1984): 4940-4944.

Schwab, M., H.E. Varmus, and J.M. Bishop. "Human N-myc gene contributes to neoplastic transformation of mammalian cells in culture." Nature 316 (11 July 1985): 160-162.

Schwab, M. "Is there a neuroblastoma oncogene?" In Advances in neuroblastoma research 3, ed. A.E. Evans, G.J. D'Angio, A.G. Knudson, and R.C. Seeger. 1-9. New York: Wiley-Liss, 1991.

Seeger, R.C., G.M. Brodeur, H. Sather, A. Dalton, S.E. Siegel, K.Y. Wong, and D. Hammond. "Association of multiple copies of N-myc oncogene with rapid progression of neuroblastomas." N Eng J Med 313 (1985): 1111-1116.

Seeger, R.C., R. Wada, G.M. Brodeur, T.J. Moss, R.L. Bjork, L. Sousa, and D.J. Slamon. "Expression of N-myc by neuroblastomas with one or multiple copies of the oncogene." Progr. Clin. Biol. Res. 271 (1988): 41-49.

Sekiya, S., T. Oosaki, S. Andoh, N. Suzuki, M. Akaboshi, and H. Takamizawa. "Mechanisms of resistance to

cis-diamminedichloroplatinum (II) in a rat ovarian carcinoma line." Eur. J. Can. Clin. Oncol. 25(3) (March 1989): 429-437.

Shih, C., L.C. Padhy, M. Murray, and R.A. Weinberg. "Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts." Nature 290 (1981): 261-64.

Shiloh, Y., J. Shipley, G.M. Brodeur, G. Bruns, B. Korf, T. Donlon, R.R. Schreck, R. Seeger, K. Sakai, and S.A. Latt. "Differential amplification,assembly and relocation of multiple DNA sequences in human neuroblastomas and neuroblastoma cell lines." Proc. Natl. Acad. Sci. USA. 82 (1985): 3761-3765.

Sklar, M.D. "Increased resistance to cisplatin in NIH-3T3 cells transformed by ras oncogenes." Can Res. 48 (1988a): 793-797.

Sklar, M.D. "The ras oncogene increases the intrinsic resistance of NIH 3T3 cells to ionising radiation." Science 239 (1988b): 645-647.

Sklar, M. "Modulation of cis-platinum resistance in Friend erythroleukaemia cells by c-myc." Cancer. Res. 51 (8) (1991): 2118-23.

Southern, E.M. "Detection of specific sequences among DNA fragments seperated by gel electrophoresis." J. Mol. Biol. 98 (1975): 503-527.

Stanbridge, E.J. "A case for human tumour suppressor genes." Bioessays 3 (1985): 252-55.

Stanton, L.W., M. Schwab, and J.M. Bishop. "Nucleotide sequence of human N-myc gene." Proc. Natl. Acad. Sci 83 (1986): 1772-1776.

Steel, G.G. and T.E. Wheldon. "Radiobiology of paediatric tumours." In Paediatric Oncology: clinical practice and controversies., ed. P.N. Plowman and C.R. Pinkerton. 73-86. London: Chapman and Hall, 1991.

Stehelin, D., H.E. Varmus, J.M. Bishop, and P.K. Vogt. "DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA." Nature 260 (1976): 170-173.

Sue, K., A. Nakagawara, S-I. Okuzono, T. Fukushige, and K. Ikeda. "Combined effects of vitamin E (alpha-tocopherol) and cis-platin on the growth of murine neuroblastoma in vivo." Eur. J. Can. Clin. Oncol. 24 (11) (Nov 1988): 1751-1758.

Tabin, C.J., S.M. Bradley, C.I. Bargmann, and et al. "Mechanism of activation of a human oncogene." Nature 300 (1982): 143-149.

Tannock, I.F. "Biological properties of anti-cancer drugs : chapter 17." In Basic science of oncology., ed. I.F. Tannock and R.P. Hill. 278-291. Pergamon press, 1988.

Tannock, I.F. "Experimental chemotherapy:chapter 19." In Basic science of oncology, ed. I.F. Tannock and R.P. Hill. 308-325. Pergamon press, 1988.

Thiele, C.J., C.P. Reynolds, and M.A. Israel. "Decreased expression of N-myc precedes retinoic acid induced morphological differentiation of human neuroblastomas." Nature 313 (31st Jan. 1985): 404-406.

Thomas, H. and J. Waxman. "Oncogenes and cancer:chapter 1." In The molecular biology of cancer., ed. J. Waxman and K. Sikora. 1-20. London: Blackwell scientific., 1989.

Tonini, G.P., G. Verdona, A. Garaventa, and P. Cornaglia-Ferraris. "Antiblastic treatment does not affect N-myc gene amplification in neuroblastoma." Antican Res. 7 (1987): 729-732.

Tsuda, H., Y. Shimosato, M.P. Upton, J. Yokota, M. Terada, M. Ohira, T. Sugimara, and S. Hirohashi. "Retrospective Study on Amplification of N-myc and c-myc genes in paediatric solid tumours and its association with prognosis and tumour differentiation." Laboratroy Investigation 59 (3 1988): 321-327.

Tumilowicz, J.J., W.W. Nichols, J.J. Cholon, and A.E. Greene. "Definition of a continuous human cell line derived from neuroblastoma." Can. Res. 30 (1970): 2110-2118.

Twentyman, P.R. "Growth delay in small EMT6 spheroids induced by cytotoxic drugs and its modification by Misonidozole pre-treatment under hypoxic conditions." Brit. J .Can 45 (1982): 565.

Varmus, H.E. "The molecular genetics of cellular oncogenes." Ann Rev. Genet. 18 (1984): 553-612.

Voute, P.A., J. de Kraker, and J.M.V. Burgers. "Tumours of the sympathetic nervous system: chapter 22." In Cancer in children

(U.I.C.C.), 2nd edition., ed. P.A. Voute, A. Barrett, H.J.G. Bloom, J. Lemerle, and M.K. Neidhart. 238-251. 2nd. ed., Vol. Springer- Verlag., 1986.

Voute, P.A. "General introduction: chapter 1." In Molecular biology and genetics of childhood cancers : approaches to neuroblastoma., ed. M. Sluyser and P.A. Voute. 7-12. W. Sussex: Ellis Horwood, 1988.

Voute, P.A., C.A. Hoefnagel, J. de Kraker, R.V. Olmos, D.J. Bakker, and A.J. van de Kleij. "Results of treatment with 131 I-Metaiodobenzylguanidine in patients with neuroblastoma.Future prospects of zetotherapy." In Advances in neuroblastoma research 3., ed. A.E. Evans, G.J. D'Angio, A.G. Knudson, and R.C. Seeger. 439-455. New York: Wiley-Liss, 1991.

Watson, J.V. and K. Sikora. "Oncoproteins and their function in the transformed cell:chapter2." In The molecular biology of cancer., ed. J. Waxman and K. Sikora. 21-37. London: Blackwell Scientific., 1989.

Weinberg, R.A. "The action of oncogenes in the cytoplasm and nucleus." Science 230 (1985): 770-776.

West, G.W., R. Weichselbaum, and J.B. Little. "Limited penetration of methotrexate into human osteosarcoma spheroids as a proposed model for solid tumour resistance in adjuvant chemotherapy." Can.Res. 40 (1980): 3665-3668.

Westin, E.H., F. Wong-Staal, E.P. Gelmann, and et al. "Expression of cellular homologues of retroviral onc genes in human haematopoietic cells." Proc. Natl. Acad. Sci. USA. 73 (1982): 2490-2494.

Wheldon, T.E., A. Livingstone, L. Wilson, J. O'Donoghue, and A. Gregor. "The radiosensitivity of human neuroblastoma cells estimated from regrowth curves of multicellular tumour spheroids." Brit. J. Radiol. 58 (July 1985): 661-664.

Wheldon, T.E., J. O'Donoghue, A. Gregor, A. Livingstone, and L. Wilson. "Radiobiological considerations in treatment of neuroblastoma by total body irradiation." Radiotherapy and Oncology 6 (1986): 317-326.