[^{131}I]-META-IODOBENZYLGUANIDINE TREATMENT OF NEUROBLASTOMA: EXPERIMENTAL EVALUATION OF STRATEGIES TO IMPROVE CLINICAL RESULTS

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A thesis submitted for the degree of Doctor of Philosophy
to the Faculty of Medicine, University of Glasgow

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June 1997

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Acknowledgements

There are numerous people whose advice and assistance I have called upon in the course of this research and to whom I am grateful. First and foremost to my supervisors Dr R J Mairs and Dr T E Wheldon for their advice and support throughout my research. In addition thanks to all the members of Radiation Oncology, past and present, Dr Jonathan Owens and Keith McKellar at the Radionuclide Dispensary for help with radiolabelling and RP-HPLC, and Stephen Bell and Tom Hamilton for their technical expertise.

Thanks also to the people who supported me through the 'dark winter' of writing up: Calum, Lorna and Alex all require a mention. Finally heartfelt thanks to the people who have helped in the short 'light spring' of submitting: to Anne and Marie for their patient correction of the final drafts and to Al for his cutting, pasting, collating and comma placement skills.

CONTENTS

List of figures

List of Tables

Abbreviations

A	adenosine
α	alpha
At	astatine
ATP	adenosine triphosphate
β	beta
Bi	bismuth
bp	base pairs
Br	bromine
[^{76}Br]MBBG	[^{76}Br]-meta-bromobenzylguanidine
Bq	bequerel
C	carbon or cytosine
^{o}C	degrees centigrade
cDNA	complementary deoxyribonucleic acid
Cl	chlorine
cm	centimetre
CNS	cental nervous system
CO_2	carbon dioxide
CP	cisplatin
cpm	counts per minute
CRC	Cancer Research Campaign
CT	computed tomography
Cu	copper
DDX1	DEAD box protein gene 1
DNA	deoxyribonucleic acid
dNTP	2 deoxynucleotide 5 tri-phosphate
et al	et alia
ev	electron volt
[^{131}I]FIBG	4-fluro-3-[^{131}I]iodobenzylguanidine
G	guanodine
g	gram
γ	gamma
GABA	gamma amino butyric acid
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GBq	gigabequerel
Gy	gray
h	hour
HAMA	human anti-mouse antibodies
H_2O_2	hydrogen peroxide
HPLC	high performance liqiud chromatography
H_3PO_4	phosphoric acid
HVA	homovanillic acid
I	iodine
ID	injected dose
IL-2	interleukin-2
ISI	intrasplenic imjection
IU	international units
IUDR	iododeoxyuridine

i.v.	intra-venous
kb	kilo-base
kBq	kilo-bequerel
KCl	pottassium chloride
kDa	kilo-dalton
keV	kilo electron volt
kg	kilogram
LAK	lymphokine-activated killer cells
λ	decay constant
LET	linear energy transfer
LOH	loss of heterozygosity
MABG	meta-astatobenzylguanidine
Mb	mega-base
MBq	megabequerel
MDR1	multidrug resistance gene
mg	milligram
$MgCl_2$	magnesium chloride
MIBG	meta-iodobenzylguanidine
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mRNA	messenger RNA
MRI	magnetic resonance imaging
MRP	multi-drug resistence protein
Na	sodium
n.c.a	no-carrier-added
NaI	sodium iodide
NK	natural killer
NET	noradrenaline transporter
NGF	nerve growth factor
ng	nano-gram
nM	nanomolar
nm	nanometre
nmole	nano-mole
No.	number
P	probability
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PET	positron emission tomography
pmoles	pico-moles
Rt	retention time
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chan reaction
SCID	severe combined immune deficient
sd	standard deviation
SPECT	single photon emission computed tomography
Si	silicon
Sr	strontium
T	thymidine
Taq	thermous aquaticus
TMS-BG	trimethyl-silylbenzylguanidine
TNF-α	tumour necrosis factor-alpha

Tris	tris(hydroxymethyl) aminoethane
$t_{1/2}$	half life
µg	microgram
µl	microlitre
µm	micrometre
UV	ultra violet
v / v	volume for volume
VMA	vanillylmandelic acid
w / v	weight per volume
Y	yttrium

SUMMARY

Neuroblastoma is a paediatric tumour prone to early and widespread dissemination. At present the majority of older patients with metastatic disease are destined to die from their malignancy despite recent advances in chemotherapy and the fact that it is a radiosensitive tumour. New therapeutic approaches are therefore urgently sought. One agent which has shown promise is meta-iodobenzylguanidine (MIBG). MIBG is a noradrenaline analogue which is specifically taken up by sympathetically innervated tissues and tumours derived from these. Radiolabelled MIBG has been successfully used to image tumours of neural crest origin such as neuroblastoma. In combination with conventional therapy modalities ^{131}I labelled MIBG is now being used to treat neuroblastoma patients. The results of these studies are encouraging and suggest that [^{131}I]MIBG will become an established part of the therapeutic regimen for late stage neuroblastoma patients. It is therefore essential that it is used to its optimal effect.

The work contained in this thesis is an investigation of experimental strategies aimed at improving the use of radiolabelled MIBG. Using *in vitro* and *in vivo* models of neuroblastoma a number of factors have been investigated.

It is predicted that the use of no-carrier-added [^{131}I]MIBG, rather than conventional low specific activity preparation will result in an enhanced therapeutic ratio because of different transport processes in neuroblastoma compared with most normal tissues. No-carrier-added [^{131}I]MIBG was synthesised and its stability, uptake and biodistribution investigated. Results revealed that the preparation was stable at radioactive concentrations up to 25MBq / ml. The no-carrier-added preparation behaved identically to conventional [^{131}I]MIBG prepared by iodide exchange with respect to uptake in cultured neuroblastoma cells. The accumulation of no-carrier-added [^{131}I]MIBG was significantly greater in tumour, adrenal, heart, and skin of tumour bearing mice than that of the conventional therapy preparation of [^{131}I]MIBG. These data indicate that there may be clinical advantages in the use of no-carrier-added [^{131}I]MIBG rather than conventional [^{131}I]MIBG.

As the ability of neuroblastoma cells to actively accumulate MIBG is crucial to the success of this therapy, the effect of chemotherapeutic agents on this uptake capacity needs to be investigated. Initial experiments examined the effect of cisplatin

pretreatment on the neuroblastoma cell line SK-N-BE(2c). After treating these cells with therapeutically relevant concentrations of cisplatin (2 and 20 μM), a stimulation in uptake of [^{131}I]MIBG was observed. Reverse transcription-polymerase chain reaction analysis demonstrated that this effect was due to increased expression of the noradrenaline transporter. These results suggest that appropriate scheduling of cisplatin and [^{131}I]MIBG may lead to an increase in tumour uptake of this radiopharmaceutical with consequent increases in radiation dose to the tumour.

Currently MIBG therapy consists of administration of the beta emitting ^{131}I labelled conjugate. However, the emission properties of this radioisotope are predicted to be non-ideal for the treatment of micrometastatic disease. The potential of Auger electron emitting radioiodine conjugates of benzylguanidine to treat micrometastases was therefore investigated. Three MIBG species, labelled with ^{123}I- ^{125}I- and ^{131}I, were synthesised and their *in vitro* toxicity compared in neuroblastoma cell monolayers, and small and large spheroids. The Auger electron emitting conjugates ([^{123}I]MIBG and [^{125}I]MIBG) were highly toxic to monolayers and small spheroids whilst the beta emitting conjugate, [^{131}I]MIBG, was relatively ineffective. In contrast, the Auger electron emitters were ineffective in the treatment of larger spheroids whilst the beta emitter showed greater efficacy. These findings suggest that short range emitters would be well suited to the treatment of circulating tumour cells or small clumps whilst beta emitters would be superior in the treatment of subclinical metastases or macroscopic tumours. The data provide support for a clinical strategy of combinations ('cocktails') of radioconjugates in targeted radiotherapy.

To validate the results of laboratory studies a suitable *in vivo* model, which provides a realistic reflection of the disease under investigation, is required. Studies were undertaken to develop a murine metastatic model of neuroblastoma by inoculating nude mice with human neuroblastoma cells and then tracking the fate of these cells using a PCR based assay. Preliminary results demonstrated the feasibility of the approach and indicate that human neuroblastoma cells spread to a variety of organs.

The findings presented in this thesis suggest that a number of strategies have the potential to enhance the therapeutic efficacy of MIBG targeted radiotherapy. The future availability of a suitable murine model of metastatic neuroblastoma should allow the merits of these strategies to be tested *in vivo*.

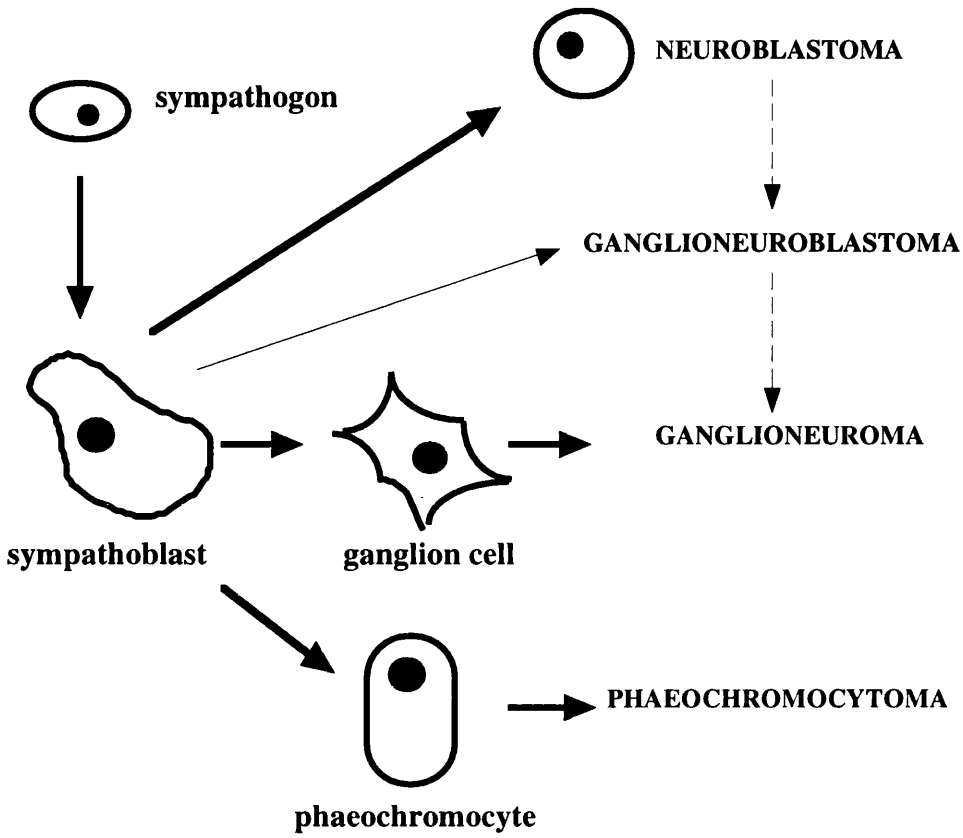
CHAPTER 1

NEUROBLASTOMA

1.1 Embryology

Neuroblastoma arises in cells of the sympathetic nervous system derived from the embryonic neural crest. In the developing embryo, these sympathagonia cells migrate from the neural crest in a ventral direction. Initially they form the sympathetic trunk in the thoracic region. They then migrate past the dorsal aorta towards the heart, lung, digestive tract and urogenital tract and form the visceral sympathetic ganglia. From these they travel to the paraganglia which are scattered in the retroperitoneal region along the aorta, and to the adrenal medulla. It is from these sympathagonia cells that tumours of the sympathetic nervous system arise (Figure 1.1). The sympathogon differentiates into sympathoblasts, the cells of origin of neuroblastoma, the more mature forms, ganglioneuroblastoma and ganglioneuroma and the chromaffin or non-chromaffin paraganglionic cells (phaeochromocytes), the progenitors of phaeochromocytoma.

Figure 1.1 Cellular origins of neuroblastoma



(Adapted from Jones and Campbell, 1976)

As figure 1.1 indicates, undifferentiated neuroblastomas have the ability to mature into fully differentiated, benign ganglioneuromas. Intermediate in their degree of differentiation are the ganglioneuroblastomas.

The embryonic nature of neuroblastoma is indicated by their occurrence early in life. In fact there is a report of the tumour's being present in a foetus *in utero* - metabolites from the tumour crossed the placenta to cause symptoms in the mother (Joute *et al.*, 1970). In such cases the tumour can be well established and even disseminated at birth. Neuroblastoma cells have also been detected at autopsy in infants dying of unrelated illnesses (Beckwith and

Perrin, 1963; Guin *et al.*, 1969). The latter observation is 14 to 40 times more common than the reported incidence of post natal neuroblastomas, which implies that many cases of these *in situ* neuroblastomas arrest and regress completely and spontaneously. Further evidence in support of these conclusions comes from mass screening programmes in infants designed to try to improve prognosis by early detection. The results of such studies indicate that the tumour is over diagnosed which implies that more cases of the disease are diagnosed than would ever manifest themselves clinically (Treuner and Schiolling, 1995).

1.2 Epidemiology

In children aged between 0 and 14 years, leukaemia, lymphoma and brain tumours are the most common forms of cancer, followed by neuroblastoma with an annual incidence of around 7 per million population in Britain. The incidence of neuroblastoma is highest during the first year of life, with a rate of around 30 per million population in Great Britain. This number decreases with increasing age: 20 at 1 year, 18 at 2 years, 13 at 3 years and 9 per million at 4 years of age. Indeed more than half of all cases are diagnosed before the third year of life. Cumulative incidence over the first 15 years of life is about 110 per million (Stiller, 1993). The male to female ratio is 1.2:1 and the white to black ratio is 3:2.

1.3 Location

1.3.1 Primary tumour

Neuroblastomas can arise anywhere in the sympathetic nervous system:- this is illustrated by comparing the anatomical location of tissues which belong to the sympathetic nervous system with the sites at which neuroblastomas have been found (Figures 1.2A + 1.2B).

Figure 1.2A Anatomical location of the sympathetic nervous system

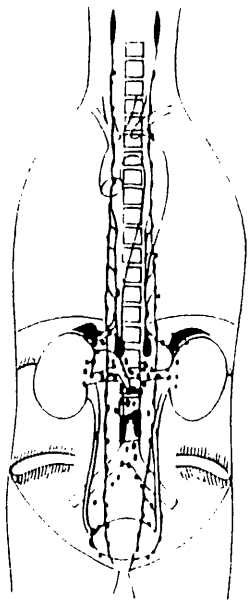
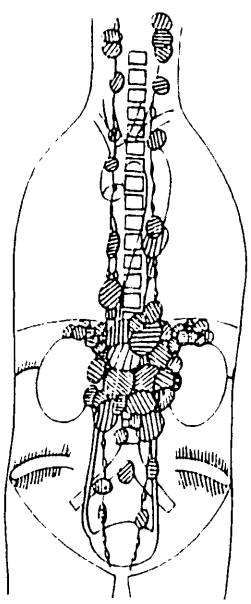


Figure 1.2B Localisation of neuroblastomas



(Adapted from Voute *et al.*, 1986)

In the majority of patients the primary tumour occurs in the retroperitoneal area (~70%) of which 35% are in the adrenal glands. Other sites include the pelvis (5%) and thorax (15%) (Voute *et al.*, 1986).

1.3.2 Metastases

Local invasion and extensive infiltration are highly characteristic of neuroblastoma. Large blood vessels often become surrounded and compressed although they are rarely invaded. In addition the tumour has a strong tendency to metastasise by lymphatic and haematologic routes. The most common blood borne sites are bone marrow, bone and liver. Spread to bone marrow occurs early relative to other childhood tumours. In bone, metastases can present as a single destructive lesion or as a diffuse infiltration of the growing ends of long bones. Liver metastases most frequently occur in infants under two years of age and can be spherical, irregular masses causing nodules on the liver or can infiltrate throughout the liver causing the organ to be massively enlarged.

1.4 Prognosis

The outlook for patients with neuroblastoma is influenced by a number of factors. The most significant is age, with younger infants having a significantly better outlook. Recent figures for children diagnosed in Britain between 1983 and 1985 quote a 77% five year survival for children under 1 year of age. This falls sharply with increasing age: 39% for those aged one, 28% for those aged two and from three to nine years less than 25% (Stiller and Bunch, 1990). Disease stage is the next most important prognostic factor. Those with low stage disease (1, 2 and 4S) usually do well. However, for those with advanced stage 3 or stage 4 disease long term survival rates are poor. A number of molecular and genetic features also correlate with prognosis (see section 1.5).

1.5 Genetic abnormalities in neuroblastoma

Neuroblastoma is a biologically and clinically complex disease. With the development of sensitive molecular techniques a variety of genetic and molecular changes have been detected many of which show a correlation with disease stage. A brief summary of the main features is given below.

1.5.1 DNA ploidy

Cytogenetic analysis of tumour cells has demonstrated that chromosomal status correlates with prognosis. In a German review study DNA aneuploidy was found in 60% of cases. In good risk groups this was a more common feature - for stage 1-3 disease 73%, and for stage 4S 60%. In contrast in patients with stage 4 disease the occurrence of aneuploidy was much lower (31%) (Christiansen *et al.*, 1995). The picture is slightly complicated by the age of the patient. In infants, tumours which are hyperdiploid or near triploid can have whole chromosome gains without any structural rearrangement in which case prognosis is more favourable. However, in older children hyperdiploid tumours often have translocations and deletions which correlate with an unfavourable outcome (Look *et al.*, 1991).

1.5.2 *N-myc* amplification

N-myc is a proto-oncogene which is expressed by neuronal cell types during embryogenesis and by a number of neoplasms of neuroectodermal origin. If deregulated it can participate in tumourogenesis. Amplification of *N-myc* is found in approximately 25 - 30% of neuroblastomas. The amplified sequence occurs either as extrachromosomal double minutes or as homogeneously staining regions on different chromosomes. *N-myc* has been mapped to the short arm of chromosome 2 (Schwab *et al.*, 1983) and it has been demonstrated that a large region from 2p24 becomes amplified. The *N-myc* gene itself is approximately 7kb in length while the amplified unit can range from 100kb to over 1Mb in length. A number of studies have investigated the

prognostic significance of amplification and have demonstrated that there is a strong correlation between amplification and poor clinical outcome (Seeger *et al.*, 1985; Brodeur and Fong, 1989; Look *et al.*, 1991). However, the situation is complicated by recent data which indicates that there are patients with amplification of *N-myc* who do respond to treatment (De Bernardi *et al.*, 1995). It has been postulated that this is because the amplicon in which *N-myc* resides contains additional genes whose over-expression may affect response to treatment. A possible candidate is the *DDX1* gene which is frequently found to be co-amplified with *N-myc* in both neuroblastoma cell lines and tumour samples (Squire *et al.*, 1995; George *et al.*, 1996).

The majority of neuorblastoma tumours showing *N-myc* amplification have high levels of N-myc expression at the RNA and protein levels (Bartram and Berthold, 1987; Slavc *et al.*, 1990). However, elevated expression can also be detected in non-amplified tumours in which case the increase is not associated with a poor prognosis (Seeger *et al.*, 1988; Nisen *et al.*, 1988). It has been suggested that expression levels have to reach a critical threshold level in order to confer an unfavourable outcome: in non-amplified tumours this threshold is not reached whereas with amplified tumours, expression levels greatly exceed the required level.

The reasons for the correlation between *N-myc* amplification and poor outcome are not clear. One explanation is that amplification confers resistance to therapy. Studies in neuroblastoma cell lines have demonstrated a correlation between *N-myc* copy number and resistance to cisplatin and etoposide (Livingstone *et al.*, 1994, 1997). Resistance to some therapeutic agents has been shown to be due to a decrease in the frequency of apoptosis (Segal-Bendirdjian *et al.*, 1995; Russell *et al.*, 1995). This is likely to be the result of mutations in one or more of the genes responsible for apoptosis and it is postulated that these in turn have an effect on the amplification of *N-myc*. An alternative theory is based on recent evidence which indicates that the N-myc protein may be a transcription factor (Wenzel and Schwab, 1995). Increased

levels of N-myc protein could result in enhanced transcription of genes which confer resistance or enhance DNA repair.

1.5.3 Deletions to the short arm of chromosome 1

Another prominent genetic alteration observed in neuroblastoma is deletion of the short arm of chromosome 1. Restriction fragment length polymorphism (RFLP) analysis has shown that the deleted region varies in size but a consensus section lies between 1p36.1 to 1p36.3. It is thought that this region harbours a tumour suppressor gene or genes, the loss of which allows the development or progression of neuroblastoma. Deletions are detected in 30-40% of neuroblastoma cases (Brodeur, 1995). The prognostic significance of this is unclear as a recent study has shown that 1p deletion alone was not associated with poor outcome (Gehring *et al.*, 1995). In combination with *N-myc* amplification however, survival chances are significantly decreased.
Another potential tumour suppresser gene is implicated by studies on chromosome 14. These have detected allelic loss of 14q in 25-50% of neuroblastomas (Suzuki *et al.*, 1989).

1.5.4 CD44 expression

The cell surface glycoprotein CD44 is believed to be involved in cell-cell interactions and cell matrix adhesion (eg Miyake *et al.*, 1990) and is expressed in a wide variety of haemopoetic and non-haemopoetic tissues. Numerous isoforms and splice variants have been characterised which show altered function: particularly higher metastatic properties. Its expression in neuroblastoma tumours and cell lines has been investigated by immunofluorescence (Gross *et al.*, 1995) which indicates that expression could be detected in most tumours in the stage 1 -3 categories and all stage 4S tumours. Expression in stage 4 tumours was however confined to a small subset. These results are supported by studies by Combaret *et al* (1995) who found CD44 expression in all the neuroblastomas with favourable prognosis (stages 1, 2 or 4S) but in only half of those with advanced stage disease

(stage 3 or 4). These results indicate that the absence of CD44 expression is a sign of tumour aggressiveness.

1.5.5 *Bcl-2* expression

Bcl-2 is an oncogene thought to contribute to malignancy by inhibiting apoptosis. It is expressed in approximately a third of neuroblastomas, and over expressed in poorly differentiated tumours (Castle *et al.*, 1993). These investigators found a significant correlation between expression and poor prognosis. Others however report its expression in a variety of tumour types, apparently independent of tumour stage (Mazzocco *et al.*, 1996).

Several investigators have speculated that its expression may contribute to clinical drug resistance by inhibiting chemotherapy-induced apoptosis. In the aforementioned study of the neuroblastomas obtained from patients after chemotherapy, more than 80% had *Bcl-2* expression (Castle *et al.*, 1993). In addition to *Bcl-2* expression the related proteins Bcl-X and BAX have also been shown to increase in tumour samples post chemotherapy (Dominici *et al.*, 1996). *In vitro* transfection experiments provide direct evidence for the role of *Bcl-2* in drug resistance (Dole *et al.*, 1994). Clones expressing high levels were resistant to cisplatin- and etoposide-induced apoptosis.

1.5.6 *TRKA* expression

The neurotrophins are a family of genes involved in the growth and differentiation of neural cells. Members of this family and their receptors have been implicated in the development of neuroblastoma (Brodeur, 1995). One of the best characterised members is nerve growth factor (NGF) whose action is mediated by the tyrosine kinase receptor TRKA. The level of expression of TRKA in neuroblastomas is significantly correlated with clinical outcome (Nakagawara *et al.*, 1993; Kogner *et al.*, 1993). Early stage tumours (1, 2 and 4S) had high levels of TRKA expression and patient survival in this group over five years was 86%. In contrast aggressive tumours had little or no

expression and cumulative 5 year survival was only 14%. It is speculated that the NGF/TRKA interaction is involved in maturation, which in patients, may be capable of stimulating neuroblastomas to regress or differentiate.

In summary, a number of genetic and molecular alterations have been detected in neuroblastoma. Many of these may have significant implications for patient prognosis. At present, *N-myc* amplification is probably the most valuable marker of tumour stage. Nevertheless, as larger patient series are investigated, the role of the other markers described will become apparent. In the future such a wide panel of markers should allow a more accurate classification of tumours, giving the clinician a better indication of the aggressiveness of the disease and therefore the ability to administer more appropriate therapeutic regimens.

1.6 Clinical Presentation

Patients with neuroblastoma can present with a variety of clinical symptoms. In mothers pregnant with tumour-bearing foetuses, symptoms are already apparent. These include hypertension, palpitations and eclampsia. These arise because of increased levels of catecholamines. In infants, the signs include distention of the abdomen, enlargment of the liver, prolongation of neonatal jaundice and subcutaneous tumours. In older children symptoms tend to depend on the primary and metastatic sites. In some, the presenting feature is bone pain which arises due to cranial or skeletal metastases. In others the presence of lymph node metastases are the first sign. If the primary tumour is in the thorax, patients can suffer coughing and dyspnoea. Primaries arising in the pelvic region cause difficulties at micturition or defecation. Other more general signs include weight loss, anorexia, diarrhoea, diabetes insipidus, hyperthyroidism and hyperhidrosis.

1.7 Diagnosis

The criteria for confirming a diagnosis of neuroblastoma have been agreed internationally (Broduer *et al.*, 1988). These are either :

1) Histological evidence from conventionally stained sections of tumour tissue accompanied by increased urine or serum catecholamines or metabolites

or

2) Presence of tumour cells in the bone marrow and increased urine and serum catecholamines and metabolites.

In common with cells from the sympathetic nervous system, these tumours synthesise and secrete a variety of catecholamines including adrenaline, noradrenaline and dopamine. Catabolism of these compounds leads to the production of several metabolites including vanillyl mandelic acid (VMA) and homovanillic acid (HVA). Measurement of the levels of VMA, HVA and dopamine present in urine and serum, confirm a histo-pathological diagnosis. Around 92 % of patients with positive biopsies have raised levels of these compounds. Supporting evidence comes from genetic analysis of tumour tissue. The presence of 1p deletions and / or *N-myc* amplification are characteristic of neuroblastoma (see sections 1.5.2 and 1.5.3).

1.7.1 Assessment of disease

The extent of disease can be assessed using magnetic resonance imaging (MRI) and computerised tomography (CT) scans which give information about the size and location of the primary tumour and large metastases. In addition MIBG scintigraphy is now recommended (see section 3.7.1). Bone marrow infiltration is detected by examining stained sections. Typical features of involvement include clumps of tumours cells, synctia, rosettes and cytoplasmic/stromal fragments (Reid, 1994). Results from these investigations give a comprehensive picture of the disease and allow an accurate

classification of stage. This is crucial in determining the appropriate therapeutic strategy.

1.7.2 Staging systems

Reliable staging systems enable optimisation of therapy and allow different treatment centres to compare clinical results and formulate more effective treatment strategies. Over the years various systems have been used by different countries. In 1988, a consensus was reached and an international system was adopted. This was updated and modified in 1993 and is described below (Brodeur *et al.*, 1993).

Stage 1	Localised tumour with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumour microscopically (nodes attached to and removed with the primary tumour may be positive).
Stage 2A	Localised tumour with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumour microscopically.
Stage 2B	Localised tumour with or without complete gross excision, with ipsilateral non adherent lymph nodes positive for tumour. Enlarged contralateral lymph nodes must be negative microscopically.
Stage 3	Unresectable unilateral tumour infiltrating across the midline, with or without regional lymph node involvement; or localised unilateral tumour with contralateral regional lymph node involvement; or midline tumour with bilateral extension by infiltration (unresectable) or by lymph node involvement.
Stage 4	Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined for stage 4s).
Stage 4S	Localised primary tumour as defined for stage 1 or 2A or 2B with dissemination limited to skin, liver, and/or bone marrow (limited to infants < 1 year of age)

Treatment of neuroblastoma depends on the severity and stage of the disease. For those presenting with localised stage 1 or 2A tumours, surgical resection is sufficient. Stage 2B and selected stage 3 patients can also be successfully treated with surgery followed, in some cases, by mild chemotherapy. More advanced disease requires a more complex multimodal approach. A brief description of the various treatments employed is given below.

1.8 Treatment

1.8.1 Chemotherapy

For aggressive stage 3 and stage 4 patients intensive chemotherapy is required. Regimens generally include a variety of agents combined to try to circumvent drug resistance. Commonly employed agents include cyclophosphamide, doxorubicin, vincristine, adriamycin, cisplatin and etoposide. Initial responses to such protocols are usually good and objective remissions are often observed (Pinkerton *et al.*, 1990; De Bernardi *et al.*, 1992; Niethammer and Handgretinger, 1995). Unfortunately these are rarely sustained and the long term results in these patients remain poor. It appears that neuroblastoma cells can become refractory to chemotherapeutic agents by induction of the multidrug resistant phenotype. Some studies on the expression of the *MDR1* gene, which encodes the P-glycoprotein multidrug efflux pump, have reported overexpression of *MDR1* transcripts in neuroblastoma tumours post chemotherapy (Bourhis *et al.*, 1989; Goldstien *et al.*, 1990). However, these results are controversial. Normal tissues such as adrenal and kidney express high levels of *MDR1* (Cordon-cardo *et al.*, 1990), and it has been suggested that the levels seen in neuroblastomas could be artificially high due to the presence of contaminating normal tissues (Favrot *et al.*, 1991). More recent investigations have examined expression of an additional resistance gene, multidrug-resistance-associated protein (MRP) (Bordow *et al.*, 1994; Norris *et al.*,, 1996). These have demonstrated a correlation between over expression and poor outcome.

1.8.2 Radiotherapy

Neuroblastoma is a radiosensitive tumour (Deacon *et al.*, 1985) and its role in the treatment of stage 3 and 4 patients is well established. Localised radiotherapy is often used after incomplete surgery to irradiate the primary site and nearby lymph nodes. It is also used in combination with chemotherapy in cases where the primary tumour is inoperable. Sufficient reductions in tumour size may allow subsequent surgery. In addition to its curative role, it can also be useful for the palliation of bone pain . In stage 4S patients, radiotherapy has been used to stimulate regression of the tumour (Gaze, 1993). Radiation can also be delivered systemically to tumour sites by targeted radiotherapy (see chapter 2). MIBG is a noradrenaline analogue which is selectively accumulated by neural crest derived tumours like neuroblastoma (Weiland *et al.*, 1980). Conjugated to ^{131}I it has shown considerable therapeutic promise (Hoefnagel, 1994). The diagnostic and therapeutic use of MIBG is discussed in detail in chapter 3. Other potential targeting agents include radiolabelled antibodies directed against cell surface markers present on neuroblastoma cells (see section 2.2.1).

1.8.3 Biological approaches

Numerous *in vitro* studies have shown that a variety of biological factors and chemical agents including retinoic acid, can induce differentiation of neuroblastoma cells (Lovat *et al.*, 1994). Clinical trials indicate that retinoic acid may be of value in some patients (Smith *et al.*, 1992; Villablanca *et al.*, 1995). Immunotherapeutic approaches using the cytokines interferon-β and interferon-γ, have also been investigated clinically. The philosophy behind these approaches is that, by administering such agents, neuroblastoma cells can be stimulated to express cell surface antigens which will be recognised and subsequently destroyed by the patient's own immune system. They may also have anti-proliferative actions *in vivo*. Clinical results with such agents have however been disappointing (Evans *et al.*, 1989). Studies with lymphokine-activated killer (LAK) cells combined with the infusion of the

lymphokine interleukin 2 (IL-2), have also been investigated (Negrier *et al.*, 1991). Unfortunately the side effects proved to be unacceptable. Several antibody-based strategies have also been evaluated using murine and humanised antibodies directed against the disialoganglioside GD2 (Cheung *et al.*, 1994; Handgretinger *et al.*, 1995), and have shown some clinical responses in patients.

1.8.4 Megatherapy

Despite the above treatment approaches the outlook for stage 4 patients over the age of 1 year remains dismal . Although it is estimated that around 60% of patients will achieve a complete remission, more than half will subsequently relapse. Long term survival rates are less than 20%. For these patients more intensive megatherapy approaches have been adopted. The principle of this strategy is to sterilise residual disease present in patients who are in apparent remission. If these tumour cells can be killed, relapse should be avoided and the patient cured. Protocols have involved the use of escalated doses of chemotherapy, in some cases accompanied by total body irradiation. More recently [^{131}I]MIBG has also been incorporated (see section 3.7.2). Bone marrow transplant may be included to circumvent the myelosuppression that such treatments induce (Pole *et al.*, 1991; Kremens *et al.*, 1994). These new regimens are considered a welcome advance on standard megatherapy protocols since they are more rationally designed and based on convincing scientific evidence (Pritchard, 1995).

In conclusion, despite the seriousness of the disease there are indications that the treatment of neuroblastoma is improving. The overall cure rate for all stages and all ages is around 50% (Novakovic, 1994). Even for those patients in the worst prognostic groups (ie stage 4, > one year at diagnosis) intensive treatment schedules offer a realistic chance of survival. The challenge lies in effectively combining the range of promising treatments in a regimen that maximises clinical effect.

CHAPTER 2

TARGETED RADIOTHERAPY

2.1 Introduction

Radiation remains one of the most effective ways of treating cancer. Sufficient doses can often be locally delivered to tumour volumes to induce regression and cure, but may be limited by normal tissue tolerance. However, disseminated disease can not be adequately treated in this way without causing lethal damage to normal tissues. A potential means of circumventing these two problems, inability to treat metastatic disease and normal tissue tolerance, is to devise a more selective means of delivering the radiation. Herein lies the concept of targeted radiotherapy: by conjugating radionuclides to appropriate carrier molecules which are selectively accumulated by tumour cells, systemically administered radiation is restricted to malignant deposits while normal, non-target tissues are spared.

2.2 Targeting vehicles

To be successful, targeted approaches need to employ suitably specific targeting vehicles. Such vehicles exploit unique properties of the tumour: metabolic, molecular or biological features which are peculiar to the malignant cells. Over the years, a range of agents have been used. Arguably the most simple and effective example of targeted radiotherapy is the administration of radioactive iodine for the treatment of thyroid carcinoma. No carrier vehicle is actually required since the thyroid gland naturally sequesters iodine. Similarly [^{89}Sr]-strontium is used for the palliation of metastatic bone pain since it is metabolised in the body like calcium, being taken up and retained in metabolically active bone (Lewington *et al.*, 1991; Lewington, 1996). However these examples are the exception to the rule. In most cases the radionuclide needs to be conjugated to an appropriate carrier molecule in order to ensure precise tumour delivery. Several carriers are currently being investigated.

2.2.1 Antibodies

The ability to produce monoclonal antibodies was the major breakthrough which revolutionised the field of radioimmunotherapy. By fusing lymphocytes, isolated from a host animal exposed to tumour cells, with myeloma cells, hybridomas are produced. Those producing the antibody of desired isotype and specificity can be selected and the cell population expanded to produce large quantities of a single antibody molecule (Kohler and Milstein, 1975). It was originally thought that unconjugated monoclonal antibodies could produce antitumour effects by stimulating the patient's own immune system. The response rates however have been low and of short duration. Results were improved by conjugating chemotherapy agents and toxins to them but these strategies encountered problems with drug resistance and non-uniform expression of the tumour antigen. Conjugation of radioisotopes circumvents these problems because, by choosing an appropriate radionuclide with a sufficient path length, adjacent tumour cells can be killed by crossfire regardless of whether or not they express the target antigen. In addition, their effectiveness is not compromised by drug resistance mechanisms. Antibodies labelled with ^{131}I or ^{90}Y have been used to treat a number of malignancies including glioma and ovarian cancer. Responses have been variable (Riva *et al.*, 1995; Stewart *et al.*, 1989).

Their most successful application is in the treatment of haematopoetic cancers (eg Applebaum *et al.*, 1992; Schwartz *et al.*, 1991). Leukaemias and lymphomas are particularly good candidates for a number of reasons: they are radiosensitive, they have well defined surface antigens against which a large number of monoclonal antibodies have been raised and the cells are likely to be more accessible to antibody than they are in solid tumours, where lack of penetration is liable to adversely effect outcome. In addition, because the majority of these patients are immunosuppressed, they are less likely to produce human anti-mouse antibodies (HAMA). The most impressive results have been obtained in B-cell lymphomas where significant response rates and

prolonged remissions have been achieved (Kaminski *et al.*, 1993; Knox, 1995; Press *et al.*, 1993, 1995).

Two monoclonal antibodies, UJ13A and 3F8, have been used to treat neuroblastoma. UJ13A is directed against the neural cell adhesion molecule whose expression is limited to tissues of neuroectodermal origin. *In vitro* studies have demonstrated that it has a high affinity for a variety of tumour cells including neuroblastoma and retinoblastoma (Allan *et al.*, 1983). Laboratory studies with UJ13A demonstrated its efficacy against neuroblastoma spheroids (Walker *et al.*, 1988) and xenografts (Jones *et al.*, 1985). However results in the clinic have been less impressive (Kemshead *et al.*, 1987). Patients developed immune reactions to the murine antibody which meant it was rapidly cleared before adequate tumour accumulation could occur. Another monoclonal antibody 3F8 has been used to treat neuroblastoma, both in its unconjugated form (see section 1.8.3) and radiolabelled with ^{131}I. Results in a group of heavily pretreated patients produced two partial responses (Cheung *et al.*, 1991).

2.2.2 Steroid hormones and growth factors

Several tumours have been shown to be hormone responsive. Breast, ovarian and endometrial cancers all express high levels of oestrogen receptors (ER). This means that they have the potential to be specifically targeted with radiolabelled oestrogens. *In vitro* studies have confirmed that ER positive cell lines are preferentially killed by ^{123}I and ^{125}I labelled oestrogens (De Sombre *et al.*, 1992; Beckmann *et al.*, 1993). Steroid hormones are good candidates for labelling with Auger electron emitting radionuclides (see section 2.4.1), since upon binding their receptors they are transported to the nucleus where they bind to hormone responsive elements of target genes. This results in the sterilisation only of receptor-positive cells because Auger electrons have an effective range of a few nanometres. Growth factors could also be useful targeting agents. Enhanced expression of the epidermal growth factor receptor

(EGFR) has been detected on several tumour types including glioblastoma (Schober *et al.*, 1995) and squamous cell carcinoma, which has led to studies with radiolabelled epidermal growth factor (EGF) (Capala and Carlsson, 1991; Sjostrom *et al.*, 1997) and transforming growth factor-alpha (TGF-α), both ligands of the EGFR (Carlsson *et al.*, 1994).

2.2.3 Thymidine analogues and oligonucleotides

Iododeoxyuridine (IUdR) and bromodeoxyuridine are analogues of the DNA precursor thymidine. They are incorporated into DNA during the S-phase of the cell cycle and consequently offer a means of selectively targeting proliferating cells. It is thought that they could be of use in the treatment of tumours such as glioma, where rapidly dividing tumour cells are surrounded by non-proliferating normal brain cells. A large number of *in vitro* studies have confirmed the toxicity of Auger electron emitter labelled IUdR (eg Makrigiorgos *et al.*, 1989). Consequently much work now centres on ways of confining its administration to the tumour site, thus avoiding distant rapidly dividing tissues, and on ways of protracting administration to achieve optimal uptake by malignant cells (Kassis *et al.*, 1990).

Oligonucleotides provide a potential means of targeting specific sequences within the DNA. A number of tumours have characteristic genetic abnormalities such as amplification of particular genes or specific chromosomal translocations (eg McDonnell *et al.*, 1993; Brodeur and Fong, 1989) which are potential targets. Oligonucleotides which form collinear triplexes with DNA can be conjugated to Auger electron emitting radionuclides to provide a potent means of inactivating genes or sterilising cells. At present, work in this field is concerned with confirming the specificity of triplex formation and ensuring effective delivery to the nuclei of target cells (O'Donoghue, 1996; Wang *et al.*, 1995).

2.2.4 Metabolic targets

Another means of targeting radiation is to exploit metabolic features of the tumour cells. Examples of this include the use of methylene blue, a phenothiazine derivative, to treat malignant melanoma. Pigmented melanoma cells contain an abundance of melanin. This can be exploited therapeutically by employing radiolabelled compounds such as thioamides and phenothiazines which have a high binding affinity for melanin. Selective localisation occurs because they are incorporated in to cells in proportion to their melanin content. Several *in vivo* studies have investigated the potential of methylene blue, . Radiolabelled with either ^{125}I or ^{211}At, inhibition of tumour growth and prevention of metastatic spread have been oberved (Link *et al.*, 1989; Link and Carpenter, 1990; Link *et al.*, 1996). Subsequent scintigraphy studies with ^{123}I and ^{131}I labelled material in patients with pigmented melanomas indicated a favourable biodistribution (Link *et al.*, 1996). Precursors of the melanin molecule could be another means of targeting malignant melanoma cells (Skellern *et al.*, 1995).

The best characterised and currently most clinically useful example of a metabolic approach, is the use of meta-iodobenzylguanidine (MIBG) to target tumours of neuroectodermal origin. This noradrenaline analogue exploits the active uptake pathway for noradrenaline present in neuroendocrine tissues. This mechanism is preserved in most neuroblastomas and phaeochromocytomas, thus allowing radiolabelled MIBG to be used for diagnostic scintigraphy and therapy of these tumours. Targeted therapy with MIBG is discussed in detail in chapter 3.

2.3 Choice of radionuclide

The second requirement for a successful targeted radiopharmaceutical is choice of the appropriate radionuclide. Some candidate radionuclides and their properties are listed in table 2.1 :

Table 2.1 Potential radionuclides for targeted radiotherapy
(adapted from Press *et al*, 1995)

Isotope	Half life	Emitted particles	Mean range of particles	Comments
^{90}Y	2.5 days	β	5.0 mm	High energy β emissions. No γ emissions.
^{131}I	8.1 days	β	0.8 mm	Well defined radiochemistry. γ emissions allow gamma camera imaging.
^{67}Cu	61.5 hours	β	0.6 mm	Emissions suitable for both imaging (γ) and therapy (β).
^{212}Bi	1 hour	α	0.05 mm	High energy, short range. Short half life limits *in vivo* use. Complex radiochemistry.
^{211}At	7.2 hours	α	0.05 mm	High energy, short range. Cyclotron generated.
^{125}I	60.1 days	Auger	1-10 nm	Extremely short range. Highly toxic when located in nucleus. Long half life.
^{123}I	13 hours	Auger	1-10 nm	Extremely short range. Highly toxic when located in nucleus. Short half life. γ emissions allow gamma camera imaging

Selection is influenced by a number of factors including chemistry of conjugation, physical half life, availability, and the range of the emitted particles. It is desirable to have straightforward chemistry which allows the targeting agent to be rapidly and stably labelled. Physical half life of the isotope is also important. Ideally it should match the biological half life of the labelled pharmaceutical to ensure maximum dose delivery. Whilst it is their particulate emissions which are of benefit therapeutically, the presence of gamma emissions can be advantageous since it allows scintigraphy to be performed (eg with ^{123}I or ^{131}I). Using tracer doses of the intended radiopharmaceutical and serial quantitative gamma camera imaging, information about the precise biodistribution of the targeting agent and estimates of tumour dose can be obtained. Particle range is particularly significant for a number of reasons which are discussed below.

2.4 Particle Range

Decay particle range is a critical feature of targeted radiotherapy. Choice is effected by 3 criteria: the subcellular fate of the targeting vehicle, the size of the targeted tumour deposit, and the heterogeneity of uptake of the targeting vehicle.

2.4.1 Subcellular location of targeting molecule

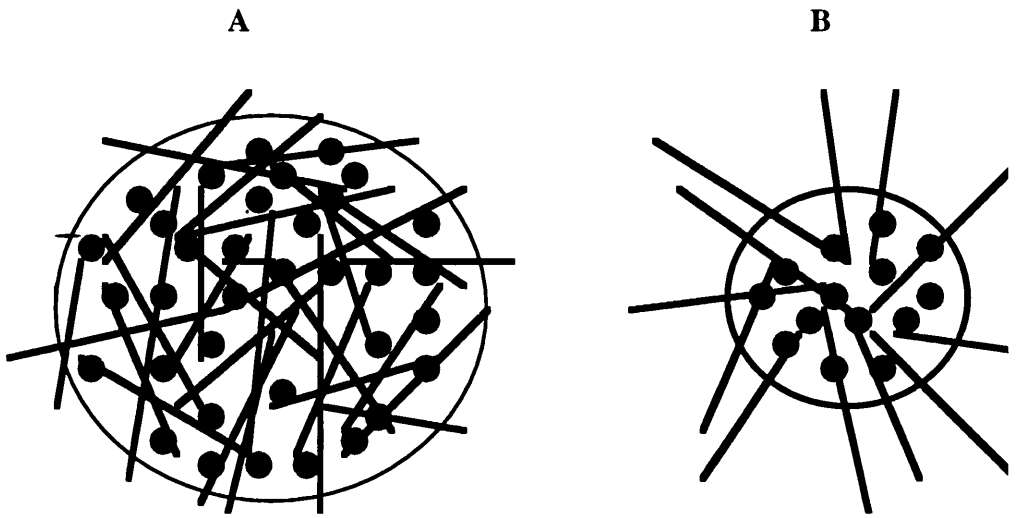
As discussed in Section 2.2, a number of potential targeting vehicles are currently under investigation. While the goal is delivery of radiation to the DNA of target cells, the optimal combination of radionuclide and delivery vehicle depends on the site of intracellular concentration. For those molecules which reach the nucleus of cells, Auger electron emitters are appropriate possibilities. These atoms decay by electron capture and internal conversion which results in the emission of extremely densely ionising radiation of very short range. As a result they are highly toxic to cells provided they are located within the nucleus. If they are located in the cytoplasm or on the cell membrane, toxicity is negligible (Kassis *et al.*, 1987). They are therefore very

effective conjugates for vehicles which are capable of reaching the nucleus and binding, or being incorporated into, DNA (sections 2.2.2 and 2.2.3). In contrast, those vehicles which remain cytoplasmic or membrane bound, should be combined with a radionuclide with longer path length decay particles. β emitting radionuclides are therefore more appropriate conjugates of antibodies which remain bound to the surface of target cells, and molecules such as MIBG which are located in the cytoplasm (Gaze *et al.*, 1991; Clerc *et al.*, 1993).

2.4.2 Microdosimetry and target size

Particle range has very important dosimetric implications for the curability of tumours. Mathematical modelling studies, which assume homogeneous radiolabelling of the tumour cell population, predict that tumour deposits which have dimensions smaller than the range of the emitted particle will be underdosed because the majority of the decay energy will be deposited outside the tumour (Humm, 1986). See figure 2.1.

Figure 2.1 Energy absorption in large and small tumours



When tumour diameter exceeds path length the majority of the energy will be absorbed within the micrometastases (A). Where the path length is greater than the diameter of the tumour a large proportion of the energy is depostited outside the micrometastases (B).

Therefore, for any given radionuclide there is an optimal tumour size for cure (Wheldon *et al.*, 1991; O'Donoghue *et al.*, 1995). In clinical terms this means that microtumours and metastases below the optimal size of the administered radionuclide will effectively be resistant to therapy and could therefore be sites of disease recurrence.

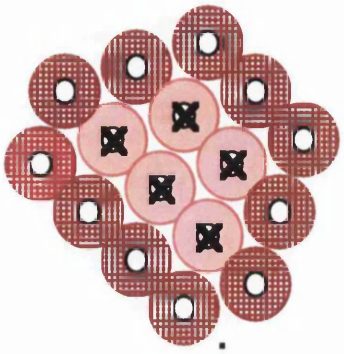
2.4.3 Heterogeneity of uptake

Targeting strategies rely on the expression by tumour cells of specific molecules. Ideally, all the malignant cells of a tumour will express sufficient levels to allow uniform distribution of the targeted radionuclide. This ideal is seldom achieved because some tumour cells may express low levels of target so that some regions of tumour will be underdosed. In addition there may be

limited penetration of the agent into solid tumours which will also contribute to the uneven distribution of energy deposition. Using long range β emitters, the problem can be overcome by crossfire: cells which themselves do not take up the drug will be irradiated by emissions from neighbouring targeted cells. However as the range of the particle emissions decreases this crossfire effect diminishes. With an alpha particle emitter like ^{211}At, which has a mean path length of 60 μm, limited crossfire can occur, killing five or six adjacent cells. With an Auger electron emitter like ^{125}I the crossfire effect is non-existent (Figure 2.2).

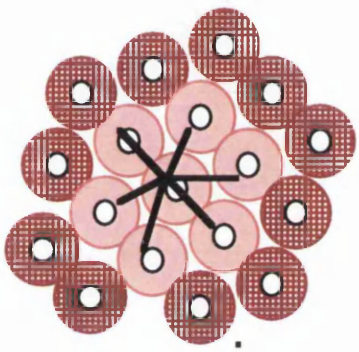
Figure 2.2 Relationship between particle range and the crossfire effect

Paler cells indicate those which have been sterilised



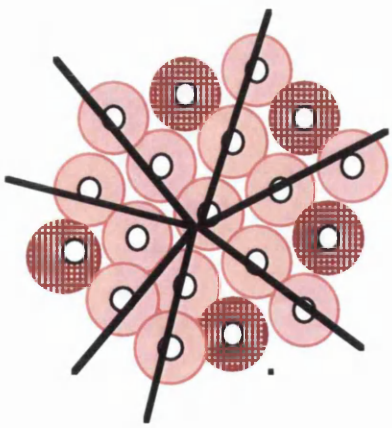
Auger electron emitter eg ^{125}I

ultra short range of < 1 μm means that isotope will be toxic only to cells which have taken it up



Alpha particle emitter eg ^{211}At

range of 60 μm means that isotope will be toxic to five or six adjacent untargeted cells



Beta particle emitter eg ^{131}I

range of 800 μm means that isotope will be toxic to several layers of untargeted cells

The limitations imposed by the size dependent aspect of targeted approaches and the problems of heterogeneous distribution of targeting agents mean that targeted radiotherapy alone is unlikely to be curative. Modelling studies predict that its optimal use will be in combined therapy approaches which incorporate agents unaffected by these problems (O'Donoghue, 1991; Amin *et al.*, 1993; Wheldon *et al.*, 1993). Such approaches are currently under investigation for the treatment of neuroblastoma where [^{131}I]MIBG is being administered in conjunction with total body irradiation and melphalan, followed by bone marrow transplantation or in conjunction with multiagent chemotherapy and stem cell rescue (see section 3.7.2).

2.5 Linear Energy Transfer

Another important feature of a given radionuclide is the quality of the radiations emitted. In contrast to diagnostic radionuclides, which are generally γ-emitters, the ideal therapeutic radionuclides are those that have higher linear transfer energy (LET). Linear energy transfer represents the average energy (measured in kiloelectron volts) locally imparted to a medium by radiation in traversing 1μm along its path or track (Hall, 1994). It is a useful quantity since it provides a means of indicating the therapeutic potential of different types of radiation. Radiations with low LET are less effective because energy exchanges with matter are widely spaced. An example is the β particles emitted by ^{131}I: each particle has only a small probability of releasing enough energy along its track to produce DNA breaks. In contrast high LET radiations are densely ionising along particle tracks which means they are much more efficient in the production of DNA breaks. For these reasons, high LET radiations are desirable for targeted radiotherapy approaches.

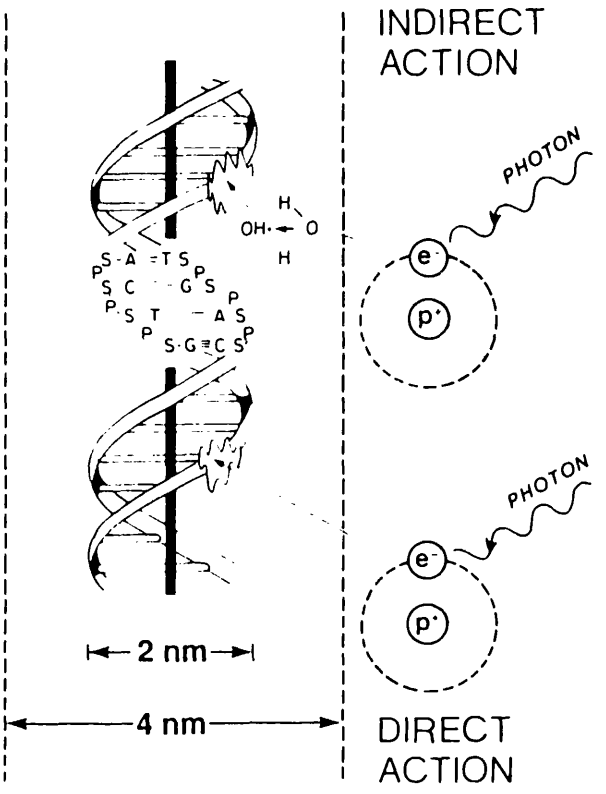
2.6 Radiobiology of targeted radiotherapy

2.6.1 Ionising Radiation

Ionising radiation is radiation which has sufficient energy to eject one or more orbital electrons from the atom or molecule in which it is absorbed. Ionising radiation results in the release of large amounts of energy within a localised area and can be classified into 2 types: electromagnetic or particulate. Examples of electromagnetic radiation include X-rays which are produced extranuclearly, and γ-rays which are produced intranuclearly when unstable isotopes decay. Such radiations are indirectly ionising: that is they themselves do not produce chemical damage but when absorbed into the material through which they pass they give up their energy to produce fast-moving charged particles which in turn may cause damage. It is particulate radiations which are relevant to targeted radiotherapy. Examples include electrons, protons, α-particles, neutrons and heavily charged ions. As long as they have sufficient kinetic energy they will directly disrupt the atomic structure of the absorber they are passing through causing chemical and biological changes.

The biological effects of radiation primarily occur as a result of damage to DNA, generally accepted to be the critical target. The actions of radiation can be considered to be direct when the target itself is ionised or excited, leading to biological damage. Alternatively they may be indirect, in which case the radiation interacts with other atoms or molecules in the cell to produce free radicals which in turn diffuse and cause damage to critical targets (Figure 2.3).

Figure 2.3 Direct and indirect actions of radiation



(Adapted from Hall, 1994)

2.6.2 DNA Damage after radiation

When cells are irradiated, breaks in the DNA backbone occur. If these are single strand breaks they pose few problems for the cell since they can be readily repaired using the opposite strand as a template. Where breaks occur in both strands, but are well separated, repair can again occur relatively easily since the cell treats them as two independent breaks. However, where the breaks in the two strands are opposite each other, or are separated by only a few base pairs, a double strand break may result. As there is no intact template for their repair, these double strand breaks may be misrepaired, disrupting the structure of the chromosome. Densely ionising radiations produce a wide variety of complex lesions, including base damage, as well as double strand breaks. This multiple damage occurs over a relatively short distance in the DNA molecule and is much more difficult for the cell to repair. It is for these reasons that alpha and Auger electron emitters are suitable isotopes for targeted radiotherapy.

2.6.3 Radiobiological features

The five R's of radiobiology which characterise conventional radiotherapy: repair, repopulation, redistribution, reoxygenation and radiosensitivity, are generally applicable to targeted strategies.

Dose rates in targeted radiotherapy vary considerably, rising for the first 24-48 hours as the tumour accumulates radiopharmaceutical and then falling off as the drug is subsequently cleared from the body and isotopic decay occurs. Since average dose rates are generally low targeted cells have the potential to repair damage and thus survive treatment. This is not so significant for high LET radiations, which cause more irreparable damage, but could dramatically affect treatment outcome for low LET radiation.

When tumour cells are treated with any cytotoxic agent (including radiation), those cells which survive treatment can be triggered to divide faster than

before: a process known as repopulation. This is particularly important in fractionation schedules, where it allows normal tissues to recover, assuming there is enough time between doses. But if the delay is too long, repopulation will also occur in the tumour. In targeted therapy the majority of the dose is delivered in the first week of treatment, therefore repopulation is not thought to be important (Wheldon, 1994). Redistribution can allow cells, initially at an insensitive phase in the cell cycle, to reassort into more susceptible parts of the cycle. It is postulated that as the dose rate falls, this would result in an inverse dose-rate effect : the dose rate is so low that cells blocked in G2 of the cell cycle are released and can proceed into sensitive phases of the cycle again (Wheldon, 1994).

Reoxygenation is not thought to be important in targeted approaches because the therapy duration is not thought to be long enough to allow reoxygenation of tumours. However some *in vivo* studies which combined hypoxic radiosensitisers with radiolabelled anitbobodies have demonstrated enhanced anit-tumour effects (Langmuir and Mendonca, 1992; Wilder *et al.*, 1994).

The effect of radiosensitivity will depend on the type of tumour being treated: within the clinic, tumours exhibit a range of sensitivities towards radiation. Melanomas and glioblastomas generally respond poorly to radiotherapy, while lymphomas and neuroblastomas are typically radioresponsive. The most common tumours, squamous cell carcinomas and adenocarcinomas, fall between these two extremes.

CHAPTER 3

META-IODOBENZYLGUANIDINE (MIBG)

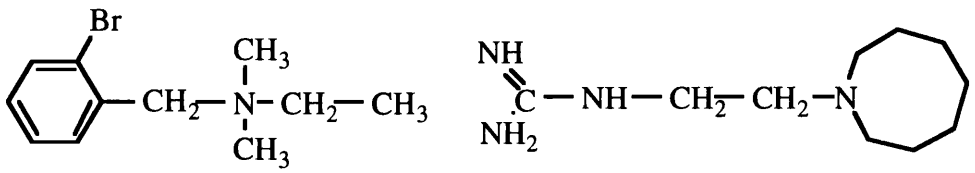
3.1 Historical Background

Meta-iodobenzylguanidine (MIBG) was the result of a long search to develop agents which could be used to image the adrenal medulla . Initial studies using carbon-14 (^{14}C) labelled catecholamines and their precursors demonstrated that such compounds could be concentrated by the adrenal medulla in animals (Morales *et al.*, 1967) and subsequently in neuroblastoma and phaeochromocytoma tumours in humans. Unfortunately these compounds could not be radio-iodinated and were therefore unsuitable for scintigraphic imaging (Ice *et al.*, 1975). Subsequent research then involved the evaluation of iodinated bretylium analogues, iododopamine and a large number of aralkylguanidines (Korn *et al.*, 1977). Of these agents, the most promising was the aralkylguanidine iodobenzylguanidine which was used to successfully image the adrenal medulla of a dog (Wieland *et al.*, 1980). The meta-iodo isomer was more stable than the ortho- and para-isomers in terms of *in vivo* deiodination and was used to positively locate human phaeochromocytomas in 1981 (Sisson *et al.*, 1981). Since then it has been used for the imaging of a number of neural crest tumours including neuroblastomas, carcinoid tumours and medullary thyroid carcinomas. It is now also employed in the therapy of neuroblastoma.

3.2 Structure

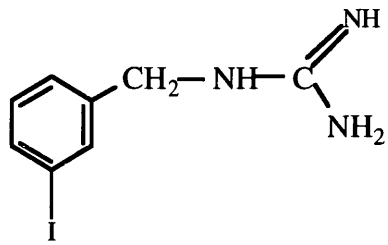
MIBG is composed of a ring structure and a guanadinium side group analogous to the adrenergic neurone blockers guanethidine and bretylium (Figure 3.1).

Figure 3.1 Chemical structure of related adrenergic neurone blockers and MIBG



Bretylium

Guanethidine



meta-iodobenzylguanidine

The compound is highly polar and does not pass through the blood brain barrier.

3.3 Synthesis and radiolabelling

Commercial production of MIBG involves the synthesis of the non-radiolabelled drug followed by radio-iodination. Unlabelled material is produced by mixing meta-iodobenzylamine hydrochloride with cyanimide and then heating at 100°C for 4 hours. Addition of potassium bicarbonate precipitates MIBG-bicarbonate which is then dissolved in hot sulphuric acid. As this cools crystals, of MIBG sulphate form (Weiland *et al.*, 1980).

Several radiolabelling procedures have been developed all of which involve exchange reactions, where stable iodine is substituted for radioiodine.

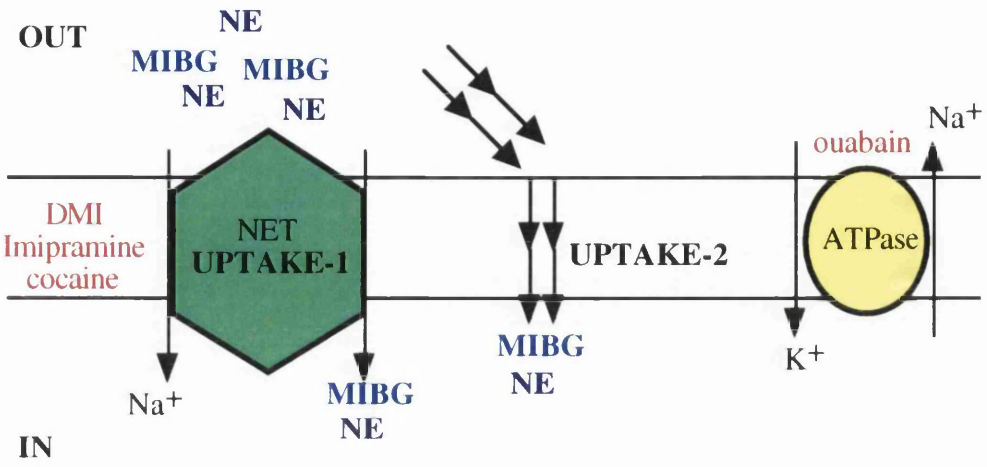
Methods vary in terms of the reagents used to facilitate exchange - ammonium sulphate and Cu^{1+} and Cu^{2+} ions have all been used (eg Eisenhut *et al.*, 1985; Doremalen *et al.*, 1985; Franceschini *et al.*, 1991). Cu^{1+}-catalysed reactions are now the method of choice because a better radiochemical yield is obtained, the material has a higher specific activity, and the reaction time is shorter.

3.4 *In vitro* Studies

3.4.1 Cellular Uptake

In cells of the sympathetic nervous system noradrenaline is synthesised and stored as a complex with ATP and protein (chromagranin) in chromaffin storage granules. In response to nervous stimulation these vesicles fuse with the plasma membrane and release noradrenaline into the synaptic cleft, where it mediates its effects through post synaptic α and β adreno-receptors. Signalling is terminated by recapturing released noradrenaline in an active process sometimes referred to as Uptake-1. This occurs via a transporter protein located in the presynaptic membrane. This specific uptake mechanism occurs only in neuronal tissues and tumours derived from these, and has a number of characteristic features: it is temperature dependent, it requires sodium and chloride ions, it is sensitive to ouabain (a Na^+ / K^+ -ATPase inhibitor), it is energy dependent (absence of glucose or the presence of metabolic inhibitors such as 2-deoxy-D-glucose or sodium azide reduces uptake) and it is sensitive to competitive inhibitors such as desmethylimipramine and cocaine (Tobes *et al.*, 1985; Gasnier *et al.*, 1986) (Figure 3.3).

Figure 3.2 Mechanisms governing MIBG uptake



NET = noradrenaline transporter, NE = noradrenaline. Agents which inhibit various aspects of the process are shown in red.

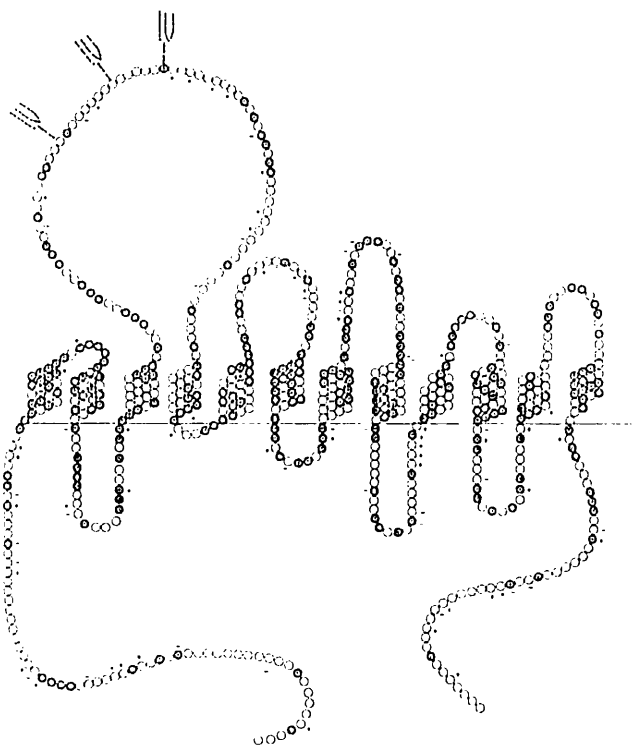
MIBG utilises this transporter to gain entry to neuroectodermal tumours. The transporter has a high affinity for MIBG and saturates at concentrations of around 1 μM (Smets *et al.*, 1989). A second non-specific mechanism exists which has a much lower affinity for noradrenaline and MIBG. Unlike transporter mediated uptake the process is energy independent, shows no ouabain sensitivity and does not saturate at MIBG concentrations up to 5 mM (Jaques *et al.*, 1984). This process occurs in normal as well as malignant tissues and is sometimes referred to as uptake-2.

3.4.2 The noradrenaline transporter

The noradrenaline transporter is responsible for the active uptake of MIBG by neuroblastoma cells. The saturability and specificity of this mechanism has been characterised by studies involving competitive and non-competitive inhibitors of MIBG accumulation. Recently the molecular aspects of this transporter have been investigated. The human transporter has been cloned and

the organisation and sequence of the coding region established (Pacholczyk *et al.*, 1991; Porzgen *et al.*, 1995). Analysis of the cDNA sequence predicts a protein of 617 amino acids with 12-13 hydrophobic regions thought to correspond to 12 membrane spanning domains. Subsequent studies with polyclonal antibodies directed against hydrophilic peptide sequences confirm the proposed topology (Bruss *et al.*, 1995) (see Figure 3.3).

Figure 3.3 Schematic representation of the noradrenaline transporter



(Adapted from Pacholczyk *et al.*, 1991)

Darker circles represent amino acid sequences homologous with the GABA transporter. Glycosilation sites and charged residues are also denoted.

The protein is 55-58kDa and is heavily glycosylated - a feature essential for its function (Melikian *et al.*, 1994). It is a member of a family of structurally related Na^+ and Cl^- dependant neurotransmitter transporters (Bruss *et al.*, 1993). Expression of the transporter has been examined in a number of neuroblastoma cell lines using RT-PCR and shown to correlate directly with the ability to accumulate MIBG (Mairs *et al.*, 1994; Lode *et al.*, 1995).

3.4.3 MIBG uptake in cell lines

Pharmacological studies of MIBG uptake have demonstrated that it can be inhibited by monoamines such as dopamine and serotonin (Lashford *et al.*, 1991). This has led to the suggestion that MIBG may be promiscuous with respect to receptor binding (Lode *et al.*, 1995). Studies on MIBG uptake in platelets support this idea since fluvoxamine, a specific inhibitor of serotonin transport, was capable of inhibiting MIBG accumulation (Rutgers *et al.*, 1993). However studies of noradrenaline transporter expression levels in neuroblastoma cell lines and lines transfected with the cDNA encoding the various transporters contradict this. In an elegant study of the mechanism of MIBG uptake, DNA sequences encoding the human noradrenaline transporter (hNET), the bovine dopamine transporter (bDAT) and the rat serotonin transporter (r5HTT) were expressed in HeLa cell lines ordinarily negative for these transporters. Only transfectants expressing the hNET were capable of actively accumulating MIBG. This uptake was blocked by noradrenaline and by the uptake-1 inhibitors desmethylimipramine and paroxetine. No significant uptake was detected in lines expressing bDAT or r5HTT (Glowniak *et al.*, 1993). In addition, expression of the dopamine and serotonin transporters could not be detected in neuroblastoma cell lines (Lode *et al.*, 1995). The above studies support the idea that MIBG active uptake in neuroblastoma cells occurs solely via the noradrenaline transporter, and is completely independent of the dopamine and serotonin transporters. Dopamine and serotonin are merely capable of competitive inhibition of the noradrenaline transporter by virtue of their structural similarities to noradrenaline.

3.4.4 Storage

In normal adrenal medulla cells, incorporated MIBG, like noradrenaline, is transported into storage vesicles via an energy dependent mono-amine transporter in the vesicular membrane. Reserpine, an inhibitor of this transport mechanism, causes a rapid depletion of MIBG from phaeochromocytoma cells indicating that storage in these cells is granular. In contrast neuroblastoma cells are insensitive to the effects of reserpine demonstrating that storage in these cells and tumours is predominantly extragranular (Smets *et al.*, 1989). Histological examinations of storage granules present in neuroblastoma SK-N-SH cells and phaeochromocytoma PC12 cells confirm that low numbers are present in neuroblastoma cells (Smets *et al.*, 1990). Supporting evidence for these storage differences comes from the effects of a variety of pharmacological agents (Table.3.1)

Table 3.1 Effect of pharmacological agents on MIBG retention
(Adapted from Wafelman, 1994)

	% of stored MIBG depleted (compared to controls)	
Agent	PC-12	SK-N-SH
Reserpine	77%	15%
Imipramine	22%	82%
Acetylcholine (ACh)	12%	3%
Potassium (K^+)	23%	4%
ACh or (K^+) + nifedipine	0%	0%

Abscence of vesicular accumulation has also been observed in other neuroblastoma cell lines (Mairs *et al.*, 1991; Lashford *et al.*, 1991). Acetylcholine and potassium, which induce exocytosis from vesicles, cause

depletion of MIBG from phaeochromocytoma cells but not from neuroblastoma cells. This process requires calcium ions, which explains why calcium channel blockers such as nifedipine inhibit depletion from phaeochromocytoma cells. Such drugs have been shown to prolong MIBG retention in phaeochromocytoma tumours and it was suggested that they may do likewise in neuroblastoma (Blake *et al.*, 1988). However *in vitro* studies demonstrated that nifedipine had no effect and verapamil only a mild effect on MIBG retention at concentrations which would be cardiotoxic *in vivo* (Mairs *et al.*, 1991). In conclusion these studies confirm an extragranular fate for MIBG in neuroblastoma cells. Rather than vesicular storage of MIBG, neuroblastoma cells maintain intracellular levels by rapid reuptake of diffusing drug, since inhibitors of the transport process (eg imipramine) cause a dramatic depletion of cellular levels.

3.5 Cytotoxicity

3.5.1 Unlabelled MIBG

The objective of the administration of MIBG is to deliver radiation selectively to neuroectodermal tumour sites. Toxic effects on tumour cells are the result of the decay of radioactive conjugates. Some investigators have suggested that unlabelled MIBG itself could also be toxic to cells. At drug concentrations of 7-70 μM growth inhibition has been reported in a variety of cell lines both neural and non-neuronal. Antitumour effects have also been observed in tumour bearing animals (Smets *et al.*, 1988). This toxicity is believed to be caused by inhibition of mitochondrial respiration at complex I (Loesberg *et al.*, 1991) although additional cellular processes may also be involved (Cornelissen *et al.*, 1995). However it is unlikely that these effects make an appreciable contribution to the anti-tumour effect of MIBG *in vivo*. Peak plasma concentrations in patients undergoing [^{131}I]MIBG are around 0.1μM (Ehinger *et al.*, 1987) and even allowing for locally elevated concentrations in tumours it is unlikely that they will reach the high molar levels required to achieve anti-proliferative effects.

3.5.2 Radiolabelled MIBG

The toxicity of radio-iodinated MIBG has been demonstrated in a number of *in vitro* systems. Bruchelt *et al* (1988) showed that uptake-competent SK-N-SH cells were killed in a dose dependent manner by [^{131}I]MIBG , while in SK-N-LO cells which lack the noradrenaline transporter, no appreciable toxicity was apparent. Similar results were obtained using [^{125}I] labelled material. A similar study by Guerreau *et al* (1990) demonstrated that [^{125}I]MIBG treatment reduced colony formation to less than 60% of controls in SK-N-SH cells at concentrations greater than 150 kBq / ml. Investigations using multicellular spheroids have demonstrated that treatment with [^{131}I]MIBG inhibits regrowth of neuroblastoma spheroids (Gaze *et al.*, 1992; Weber *et al.*, 1992)

3.6 *In vivo* studies

3.6.1 Animal models of neuroblastoma

In vivo studies with [^{131}I]MIBG require reproducible models of neuroblastoma. Several such models of disease have been reported using a variety of neuroblastoma cell lines: Senekowitsch *et al* (1989) obtained solid tumours by simple subcutaneous injection of SK-N-SH cells into nude mice. Rutgers *et al* (1991) inoculated mice intrasplenically with SK-N-SH to induce tumour growth on the liver and spleen. Tumour fragments from these sites were then implanted subcutaneously and could subsequently be serially passaged. This technique has been employed to establish xenografts from SK-N-BE(2c) cells (Gaze *et al.*, 1994). One non-murine neuroblastoma xenograft model has been reported (Nilsson *et al.*, 1993). Subcutaneous injection of SH-SY5Y cells into athymic rats produced rapidly growing tumours.

3.6.2 Biodistribution and treatment

It is encouraging that these various models all give similar results in terms of MIBG distribution.

Senekowitsch *et al* (1989) found that uptake capacity by subcutaneous xenografts was maximal at 6 hours post injection. Other sympathetically innervated sites such as heart also showed high levels of uptake. Using activities of 185 MBq per mouse tumour cure was achieved.

Rutgers *et al* found maximal uptake occurring in tumours and in the adrenal glands - the natural targets for MIBG. Therapy experiments using 30 - 50 MBq of [^{131}I]MIBG demonstrated large variations in tumour uptake and retention between animals, however a positive correlation was established between estimated radiation absorbed dose and tumour response. SK-N-BE(2c) xenografts were found to behave similarly in terms of uptake and retention (Gaze *et al.*, 1994). Comprehensive biodistribution data indicated that maximal accumulation of [^{131}I]MIBG in adrenal glands and in tumours occurred at 24 hours. In normal tissues there was a biphasic clearance. As in the other studies attempts to conduct therapy experiments with this xenograft model were hampered by the huge variation in uptake between animals, consequently no real correlation between administered dose and tumour response was observed. However growth delay could be detected at high activities (105 MBq per mouse). Biodistribution studies with [^{123}I]MIBG in xenografts in athymic rats revealed a similar pattern to that observed in murine models (Nilsson *et al.*, 1993).

3.7 Clinical use of MIBG

3.7.1 Diagnostic Scintigraphy

Radiolabelled MIBG has been used for diagnostic imaging since 1980 and now has a firmly established role in the clinical investigation of a variety of

neuroendocrine tumours. Imaging can be carried out using either [^{131}I] or [^{123}I] labelled MIBG: [^{131}I]-labelled material protocols typically involve the administration of 18.5-37 MBq, with imaging at 24, 48 and 72 hours post injection. Where the label is [^{123}I] much larger activities can be administered since the absorbed radiation dose per MBq is much lower than for [^{131}I]. Generally 185-370 MBq can be injected and images obtained at 24 and 48 hours. There has been debate about which isotope is superior. [^{123}I] has a higher photon flux and a 159keV photon, features which enhance the quality of images and allow SPECT studies to be performed. However tumour detection rates appear to be similar for both (Lynn *et al.*, 1985; Sinon *et al.*, 1992; Gelfand *et al.*, 1994).

MIBG imaging for neuroblastoma is highly sensitive and specific. A multicentre review of more than 700 scans quotes an overall sensitivity of 92% and a specificity of nearly 100% (Hoefnagel, 1994). It has proved particularly effective in detecting tumour deposits in bone and bone marrow where infiltration can be diffuse and difficult to confirm histologically (Schulkin *et al.*, 1992; Osmanagaoglue *et al.*, 1993).

In addition to confirming diagnosis, the scan also provides information about MIBG clearance and dosimetry which can be used to calculate the therapeutic dose appropriate to individual patients. There have been some discrepancies however between diagnostic predictions of MIBG accumulation and actual tumour uptake as measured by counting of radioactivity in tumours surgically excised following radiolabelled MIBG administration (Moyes *et al.*, 1989). Alternative methods of estimating uptake have therefore been investigated. RT-PCR analysis of noradrenaline transporter expression could be a viable alternative. By determining levels of expression in tumour biopsy samples it may be possible to obtain accurate estimations of uptake (Mairs *et al.*, 1994).
Other neuroendocrine tumours can be imaged with MIBG. Phaeochromocytomas arise in chromaffin cells and tend to affect adults rather than children. Invariably the tumour is located on the adrenal medulla and the patient presents with clinical symptoms which arise because of the excessive

catecholamines which the tumours secrete. While most tumours are benign, on rare occasions they can be malignant. MIBG scintigraphy has been shown to accurately locate the majority of phaeochromocytomas with an overall sensitivity of 86% and specificity of 97% (Campeau *et al.*, 1991). Other tumours which can be imaged with MIBG include carcinoids, medullary thyroid carcinomas, ganglioneuromas and paraganglioma.

MIBG can also be used for cardiac imaging. Unlike noradrenaline, MIBG is not metabolised by monoamine oxidase or catechol-o-methyltransferase. In addition, MIBG release from non-neuronal stores is rapid. Therefore the localised distribution of adrenergic nerve elements can be visualised. The stability of MIBG and its rapid loss from non-target sites allows its use in the assessment of adrenergic neurone function in the heart. Lower cardiac uptake is observed in conditions where noradrenaline content and/or uptake is reduced, such as myocardial infarction and congestive heart failure (eg Glowniak *et al.*, 1989; Merlet *et al.*, 1992; Shakespeare *et al.*, 1993). Several studies have also used MIBG to monitor the effects of the cytotoxic antibiotics on heart function. The anthracyclines are an important group of chemotherapy agents which have a wide spectrum of anti-tumour activity. However an unfortunate side effect of their repeated administration is significant cardiotoxicity. The degree of injury shows wide variation between individuals which makes appropriate dose scheduling difficult. MIBG scintigraphy provides a sensitive means of monitoring cardiac damage in patients during and after anthracycline therapy (Wakasugi *et al.*, 1993; Valdes Olmos *et al.*, 1995).

3.7.2 MIBG Therapy

Following the demonstration of its use for the specific and sensitive detection of tumours the potential of MIBG to deliver therapeutic doses of radiation was evaluated.

Initially therapeutic administration was limited to neuroblastoma patients with progressive, chemotherapy-resistant disease. Encouraging results were obtained with an overall response rate of 35% (Hoefnagel *et al.*, 1994). [^{131}I]MIBG has subsequently been employed in many centres at an earlier stage in therapy either as first line treatment or in combination with other treatment modalities. Treatment generally involves the administration of 3 to 11 GBq of [^{131}I]MIBG (specific activity ~1.1GBq / mg) which is injected intravenously over 1 to 4 hours. Prior to administration a full drug history is taken from the patient since a number of commonly prescribed agents and over-the-counter medicines can interfere with MIBG uptake (Solanki *et al.*, 1992). To protect the thyroid gland patients are given oral potassium iodide to competitively block uptake of free [^{131}I].

Treatment is usually well tolerated although some patients experience mild nausea and vomiting (Sisson *et al.*, 1988). This may be as a result of the binding of MIBG to noradrenaline receptors in the emesis-controlling region of the brain since noradrenaline levels have been implicated in the nausea and vomiting experienced by patients undergoing chemotherapy (Fredrikson *et al.*, 1994). The most significant side effect is thrombocytopenia which can be severe and long lasting. There is debate about how this condition arises. It is possible that it is caused by crossfire irradiation of stem cells or megakaryocytes from adjacent tumour sites within the bone marrow which have actively accumulated [^{131}I]MIBG (Gelfand, 1993). If this is the case then a correlation between bone marrow involvement and the degree of thrombocytopenia would be expected. However such a relationship has not been clearly identified (Hoefnagel and Lewington, 1994) and indeed marrow suppression has been reported in patients who showed no bone marrow involvement (Garaventa *et al.*, 1991). An alternative explanation is that toxicity arises directly in platelets themselves, or more likely their nucleated precursors the megakaryocytes. *In vitro* studies have shown that human platelets are capable of actively accumulating MIBG via the serotonin transporter (Rutgers *et al.*, 1993) although this finding is controversial since transfection studies involving the rat serotonin transporter demonstrated no

MIBG accumulation (Glowniak *et al.*, 1993) (see section 3.4.3). A third more plausible explanation is that the acute myelosuppression is a manifestation of previous treatments since it is often most severe in intensively pretreated patients (Sisson *et al*, 1988; Mastrangelo *et al.*, 1995).

"De novo" administration of [^{131}I]MIBG to patients prior to surgery and chemotherapy has been pioneered in Amsterdam with an impressive response rate of 69% (De Kraker *et al.*, 1995). This approach was adopted because previous experience had demonstrated that [^{131}I]MIBG had been most effective in patients with a large tumour burden at the time of treatment. In addition to its antitumour effects MIBG treatment was also capable of significantly reducing pain. Another novel approach which has been evaluated by the Amsterdam group is the combination of [^{131}I]MIBG with hyperbaric oxygen treatment. This approach is based on the well known observation that the availability of molecular oxygen can enhance the effects of radiation (Gray *et al.*, 1953). Treatments which are capable of increasing tumour oxygenation are therefore predicted to enhance radiosensitivity. Clinical approaches to decrease tumour hypoxia have included the use of hypoxic cell sensitizers and hyperbaric oxygen. In this study patients were given therapeutic doses of [^{131}I]MIBG, and were then placed in a hyperbaric chamber for up to 5 days. Preliminary results with stage 4 patients indicate moderately higher survival rates compared to [^{131}I]MIBG treatment alone (Voute *et al.*, 1995).

Other studies have investigated the integration of [^{131}I]MIBG into combined therapy approaches. There are theoretical grounds for combining MIBG with additional therapies which are unaffected by the same size constraints as [^{131}I]MIBG (section 2.4.3). A pilot study in Glasgow combined [^{131}I]MIBG with total body irradiation and high dose mephalan to treat small micrometastases (predicted to be underdosed by [^{131}I] labelled MIBG alone) and localised external beam radiotherapy for larger measurable deposits. The clinical experience demonstrated the feasibility of such an approach, although further assessment is needed to confirm its efficacy (Gaze *et al.*, 1995)

Mastrangelo and colleagues have described the use of cisplatin and [^{131}I]MIBG in stage IV patients (Mastrangelo *et al.*, 1995). Cisplatin was chosen because of its low haematological toxicity and its purported synergy with radiation (Douple *et al.*, 1985; Dewit, 1987). Encouraging results were obtained in a group of heavily pretreated, relapsed patients and ongoing work is now investigating the combination in earlier stages of disease.

Administration of therapeutic doses of [^{131}I]MIBG have also proved efficacious in the treatment of phaeochromocytomas, carcinoids and medullary thyroid carcinomas with objective responses of 56%, 16% and 32% respectively (Hoefnagel, 1994). As with neuroblastoma patients [^{131}I]MIBG is also very effective in relieving bone pain caused by skeletal metastases.

CHAPTER 4

AIMS OF THIS STUDY

Clinical experience with [^{131}I]MIBG has demonstrated its therapeutic effectiveness and it is now confirmed as a valuable addition to the armoury of agents employed to treat neuroblastoma. Nonetheless it has become clear that [^{131}I]MIBG therapy alone is unlikely to be curative. The aims of this study were therefore to identify ways in which its use could be enhanced. To this end, research has focused on four lines of investigation:

4.1 No-carrier-added MIBG

A potential means of improving results with [^{131}I]MIBG is to investigate ways of enhancing tumour uptake of the drug. This problem can be addressed chemically, by improving the synthesis and radiolabelling procedures used to produce MIBG. As indicated in section 3.3, currently available material is produced by iodide exchange, an inefficient process which results in a product unavoidably contaminated with cold carrier molecules. As these will compete with radiolabelled MIBG for tumour sites of active uptake, high molar amounts of drug are required to deliver a therapy dose. This situation is non-ideal since it reduces the therapeutic ratio and increases radiation damage to non-target tissues. Alternative radiolabelling procedures have now been developed which result in production of carrier free [^{131}I]MIBG. In chapter 5 the synthesis and evaluation of this preparation in *in vitro* and *in vivo* models of neuroblastoma is described.

4.2 Modulation of MIBG uptake

As described in section 3.7.2, several centres have adopted the use of MIBG targeted radiotherapy in combination with more firmly established treatment modalities. However experimental demonstration of beneficial combinations is lacking and the effect upon MIBG uptake of the earlier delivery of chemotherapeutic agents has not been established. Initial studies to address this issue are described in chapter 6 where the effect of cisplatin pretreatment on MIBG uptake capacity of neuroblastoma cells *in vitro* is investigated.

4.3 Alternative radiohaloconjugates of MIBG

Due to the dissipation of substantial amounts of long range β-decay energy outwith the targeted tumour, MIBG labelled with ^{131}I is expected to be less effective for the treatment of neuroblastoma deposits of submillimetre dimensions than of larger tumours (section 2.4). The use of alternative radiolabels which emit particles with a shorter range, may be more appropriate for targeting neuroblastoma micrometastases. In chapter 7, the toxic potential of two Auger electron emitting conjugates of MIBG, [^{123}I]MIBG and [^{125}I]MIBG, are compared with [^{131}I]MIBG in neuroblastoma cell monolayers and spheroids which are used as an *in vitro* model of micrometastases.

4.4 Development of an *in vivo* model of neuroblastoma

To adequately investigate the effectiveness of individual therapies, and to develop ways in which these treatments can be optimally combined, a realistic *in vivo* model of metastatic neuroblastoma is required. To establish such a model, groups of nude mice have been inoculated with neuroblastoma cells and monitored for the appearance of disease using RT-PCR based methodology. The results of this approach are reported in chapter 8.

CHAPTER 5

NO-CARRIER-ADDED MIBG

5.1 Introduction

5.1.1 The requirement for no-carrier-added MIBG

The principles governing the development of no-carrier-added (n.c.a) MIBG are related to the kinetics of its uptake and the manner in which currently available MIBG is synthesised.

As described in section 3.4.1, uptake of MIBG occurs via two mechanisms: a specific, high affinity, saturable mechanism mediated via the noradrenaline transporter and responsible for active MIBG accumulation by neuroendocrine tumours and a second, non-specific mechanism, passive diffusion. This accounts for uptake in most normal non-target tissues, with the exception of sympathetically innervated organs such as the heart and the adrenal glands. Because of these mechanisms the molar concentration of MIBG critically affects its distribution. At low MIBG concentrations the specific mechanism predominates (Mairs *et al.*, 1991). Drug accumulation is therefore limited to those sites expressing the noradrenaline transporter. As the molar concentration increases however, this mechanism saturates and an increasing proportion of the drug is accumulated by passive diffusion - so that normal, non-target sites take up more radiolabelled drug. Therefore it is postulated that one way of confining radiolabelled MIBG to tumour tissues is to administer it at the lowest possible chemical concentration.

Unfortunately currently available [^{131}I]MIBG preparations are non-ideal. Because the radiolabelling process involves the exchange of stable iodine for radioiodine (section 3.3) the final product is inevitably contaminated with an excess of unlabelled carrier MIBG molecules. This inefficient synthesis results in an estimated ratio of radiolabelled to non-radiolabelled MIBG molecules of only 1 in 2000. Therefore unnecessarily high molar concentrations of the drug are administered. Unlabelled MIBG molecules compete with radiolabelled ones for uptake sites, lowering the delivery of radioactivity to the tumour and increasing the radiation dose to non-target organs. This hypothesis is supported

by the observations of Mock and Tuli (1988), who found that at high loading doses the absolute cardiac uptake of [^{123}I]MIBG in rats declined as specific activity decreased, and the results of Bruchelt *et al* (1988), who showed that greater toxicity to SK-N-SH neuroblastoma cells was obtained with high specific activity [^{131}I]MIBG (0.74 - 1.1 GBq / mg) than with low specific activity [^{131}I]MIBG (7.4 - 11.1 MBq / mg). Therefore a synthetic route other than iodide exchange is required which will generate a carrier-free product. This should enhance the differential between target and non-target uptake, increasing tumour dose while sparing normal tissues.

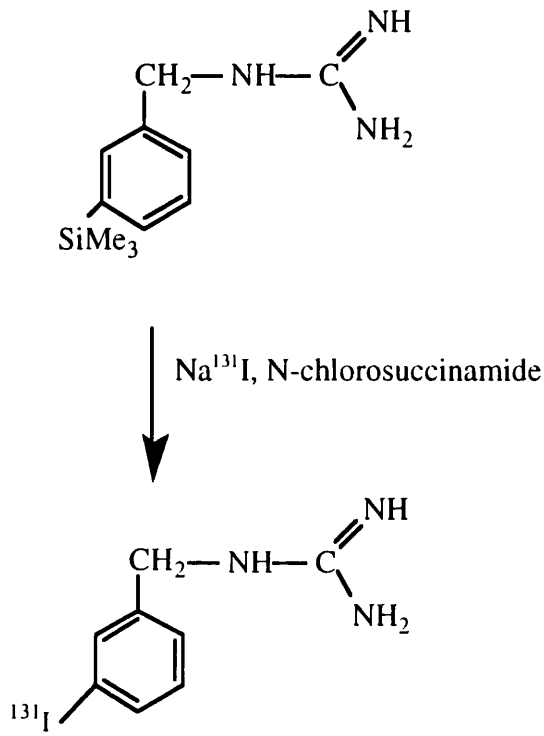
5.1.2 No-carrier-added synthesis of MIBG

Several chemical methods have been described for the production of n.c.a MIBG. Although they differ mechanistically they have in common the production of a reactive precursor of the MIBG molecule which can be quickly and efficiently radiolabelled in a single step.

Mairs *et al* (1994) employed a novel synthetic route to produce MIBG via the meta-diazo derivative of benzylguanidine. Meta-aminobenzylguanidine was synthesised by refluxing meta-nitrobenzylamine in the presence of cyanamide. This was then reacted with sodium nitrite to form a highly reactive meta-diazo derivative. When iodide ions were added to this precursor, site specific iodination occurred to form MIBG. Although successful in producing carrier free product, the efficiency of the reaction was poor and the radiochemical yield was only 13.4%.

Vaidyanathan and Zalutsky (1993) employed silicon chemistry which has been utilised for the radiohalogenation of a variety of compounds (eg Wilbur *et al.*, 1982). Using 3-bromotoluene as a starting compound they produced 3-trimethylsilylbenzylguanidine (TMS-BG). The C-Si bond is susceptible to electrophilic cleavage which allows iododesilylation to occur in the presence of an appropriate oxidant, such as N-chlorosuccinimide (Figure 5.1).

Figure 5.1 Synthesis of [^{131}I]MIBG by the iododesilylation of 3-methylsilybenzylguanidine



By performing the reaction in trifluroacetic acid , at room temperature, yields of 85-90% were obtained. Since the iododesylation process gives greater radiochemical yield it is the favoured route for producing n.c.a MIBG.

5.1.3 Laboratory studies with n.c.a MIBG

Having synthesised n.c.a [^{131}I]MIBG, Vaidyanathan and Zalutsky (1993) carried out preliminary studies to determine uptake and biodistribution of the novel preparation. *In vitro* binding studies in SK-N-SH neuroblastoma cells demonstrated that uptake of n.c.a drug remained constant over a 2-log activity range, while that of conventional [^{131}I]MIBG prepared by exchange methods decreased by a factor of seven. *In vivo* biodistribution studies in mice allowed an assessment of n.c.a uptake in normal tissues. Sympathetically innervated

tissues showed significantly higher uptake of n.c.a material. An indication of the potential therapeutic benefit of n.c.a [^{131}I]MIBG was obtained by calculating the target to non-target ratio of drug accumulation. Using the adrenals as a target tissue and the liver as an example of a non-target tissue, the ratio of uptake 24 hours after injection was 7.54 for the exchange preparation and 28 for n.c.a [^{131}I]MIBG.

5.1.4 Aims of this study

The results of preliminary investigations of n.c.a [^{131}I]MIBG are encouraging and lend support to the hypothesis that the molar mass of drug present does effect the uptake and biodistribution of MIBG. The aims of this study were to carry out a more detailed evaluation of the n.c.a preparation to determine its therapeutic potential. Experiments were undertaken to examine its radiochemical stability, uptake, and biodistribution in mice bearing neuroblastoma xenografts.

5.2 Materials and Methods

5.2.1 Synthesis of n.c.a MIBG

Chemicals were purchased from Aldrich Chemical Company (Dorset, UK). HPLC grade solvents were obtained from Rathburn Chemicals (Peebleshire,UK). Carrier free ^{131}I-NaI was purchased from Amersham International (Buckinghamshire,UK). The precursor, TMS-BG, was kindly provided by Dr Vaidyananthan (Department of Radiology, Duke University). n.c.a MIBG was synthesised by iododesilyation of TMS-BG according to the previously published method (Vaidyanathan *et al.*, 1993; Mairs *et al.*, 1994). Peracetic acid, the oxidising agent, was prepared by mixing 130 µl of 30% (v/v) H_2O_2 with 50 µl of glacial acetic acid. This was kept at room temperature for 2 hours prior to use. 0.1 mg of TMS-BG was dissolved in 40 µl of trifluroacetic acid to give a final concentration of 10 nmol / µl. This was added to a Wheaton Reactivial (Pierce, Cambridge) containing the desired activity of [^{131}I]-NaI, followed by 20 µl of peracetic acid. The vial was capped, the contents mixed gently and then left at room temperature for 5 minutes. The MIBG product was then purified by Reverse Phase HPLC using a Waters Bondapak C18 column (10 µm, 3.9 mm x 100 mm) with H_2O: tetrahydrofuran: triethylamine: H_3PO_4 (96.5:2.0:1.0:0.5 v/v/v/v) solvent system at a flow rate of 3ml / minute using a Waters 600E pump, with Waters 490 UV detection and a sodium iodide radiodetector. Tetrahydrofuran was removed from the HPLC fraction containing the [^{131}I]MIBG with a stream of nitrogen for 15 minutes. The remaining solution was desalted using a C18 Sep-Pak (Waters, Millipore). After loading the sample, the Sep-Pak was washed with 2 x 5 ml water and 2 x 500 µl of 5 mM sodium acetate (pH 4.5). The [^{131}I]MIBG was then eluted with 250 µl aliquots of methanol. The methanol was removed with a stream of nitrogen and the activity reconstituted in PBS. HPLC was used to estimate the specific activity of the n.c.a preparation. Using MIBG standards the limit of detection corresponded to a specific activity of approximately 3 x 10^{16} Bq / mol. As a cold MIBG trace could not be detected during synthesis, the specific activity of n.c.a [^{131}I]MIBG was estimated to be at least 3 x 10^{16} Bq / mol. This is at least

100 times higher than that of conventional exchange prepared [^{131}I]MIBG (specific activity 3 x 10^{14} Bq / mol).

5.2.2 Stability of n.c.a [^{131}I]MIBG

N.c.a MIBG was synthesised as detailed above. To determine the free iodine content, samples were analysed by reverse phase HPLC, using the solvent system described above but with a Waters analytical C-18 Nova-pak column (3.9 x 150 mm) at a flow rate of 1ml / min. Under these conditions Rf values for free [^{131}I] and [^{131}I]MIBG were 1.5 minutes and 5.5 minutes respectively. Data were collected and analysed using Waters baseline software. To determine the effect of storage temperature, samples were stored at room temperature and frozen at -20^{0}C. To investigate the effect of radioactive concentration, the level of free iodine in 25 MBq / ml and 100 MBq / ml samples were compared.

5.2.3 Cell Culture

The human neuroblastoma cell line SK-N-BE(2c) was used for these studies. This established cell line was originally derived from the bone marrow of a patient with progressive neuroblastoma following treatment with radiotherapy and chemotherapy (Beidler *et al.*, 1978) and has a high capacity for uptake of MIBG (Mairs *et al.*, 1994).

Cells were grown in a 5% CO_2 atmosphere at 37^{O}C in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin / streptomycin (100 IU / ml), amphotericin B (2 μg / ml) and glutamine (200 mM). All media and supplements were obtained from Gibco (Paisley, UK). Flasks were subcultured every 5-7 days when the monolayers became confluent.

5.2.4 Effect of inhibitors on n.c.a [^{131}I]MIBG uptake

SK-N-BE(2c) cells were seeded in 6-well plates at an initial density of 2.5 x 10^{5} cells per well and incubated for 48 hours. RPMI Medium was then removed,

replaced by 2.5 ml of fresh medium containing the desired inhibitor: desmethylimipramine, imipramine, amitriptyline, dopamine, serotonin or noradrenaline (Sigma, Poole, Dorset) at the appropriate concentration, and the cells incubated for 30 minutes. Medium was then removed, replaced by 2.5 mls fresh medium containing inhibitor plus n.c.a [^{131}I]MIBG and uptake measured over 2 hours. Medium was then removed, the cells washed with PBS, and radioactivity extracted by 2 aliquots of 10% (w/v) trichloroacetic acid (Aldrich, UK). Activities of extracts were then counted in an automated gamma well counter (Canberra Packard, Berkshire, UK). The level of uptake was expressed as a percentage of control cells incubated in the absence of inhibitor.

5.2.5 Biodistribution of [^{131}I]MIBG in mice bearing neuroblastoma xenografts

All animal work was carried out in accordance with the UK Coordinating Committee for Cancer Research guidelines on experimental neoplasia in animals under the authority of a project licence granted by the Home Office under the Animals (Scientific Procedures) Act, 1986.

5.2.5.1 Establishment of neuroblastoma xenografts

Six week old female athymic nude mice of strain MF1 nu/nu (Harlan Olac, Bicester, UK) were used for these studies. Xenografts were established according to the technique described by Rutgers *et al* (1991). SK-N-BE (2c) cells were cultured as described in section 5.2.3. Prior to intrasplenic injection (ISI) cells were trypsinised, counted and resuspended in sterile PBS and stored at 4°C until use. Under sterile conditions mice were anaesthetised using hypnorm / hynovel (Janssen Animal Health, Kent). An incision was made through the skin and peritoneum and the spleen partly exposed. Approximately 3 x 10^6 SK-N-BE (2c) cells in 200ul PBS were injected slowly into the upper pole of the spleen. Peritoneum and skin were then sealed with histoacryl tissue adhesive (Davis and Geck, Hampshire) and the wound sealed with 9 mm autoclip wound clips. Following a latent period of 3-12 weeks, hepatic and splenic tumours developed at which point mice were sacrificed and the tumours

removed. Tumour fragments of 2-3mm in diameter were then implanted subcutaneously in the subcostal flank of 6 to 8 week old mice. Subcutaneous xenografts developed in 2-3 weeks and could be serially passaged into fresh animals. Mice were used for pharmacokinetic and biodistribution experiments 2-3 weeks after implantation when subcutaneous tumours had grown to 5 - 10 mm in diameter.

5.2.5.2 Biodistribution studies

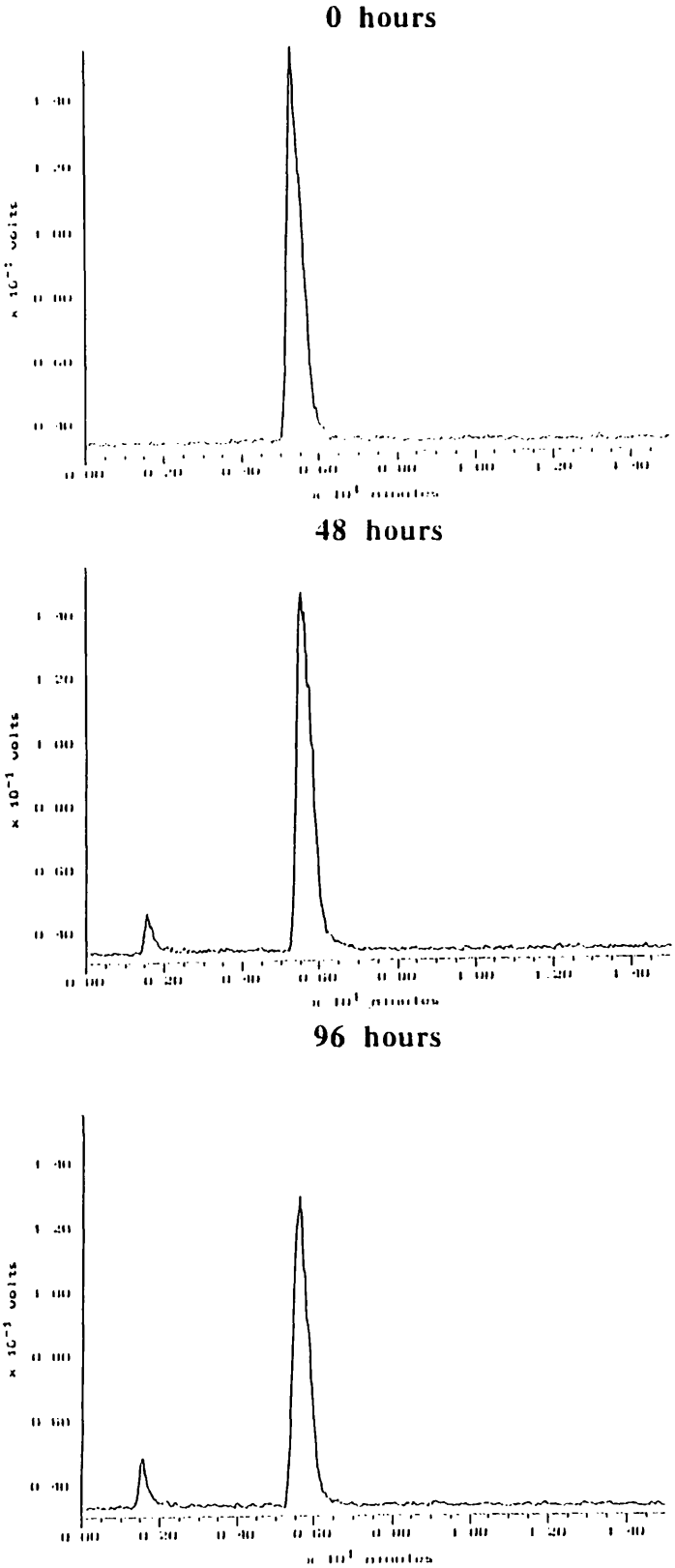
Groups of 7 tumour bearing mice were used for each timepoint. The distribution of n.c.a [^{131}I]MIBG (specific activity > 0.11 TBq / mg) was compared with that of commercial therapy [^{131}I]MIBG (ex-[^{131}I]MIBG) prepared by conventional exchange methods (specific activity > 1.1 GBq / mg). Mice were injected intraperitoneally with 5 MBq of the appropriate preparation. The precise activity administered to each mouse was measured using a Curiementor-2 ionisation chamber radionuclide meter (Radiation Components, Bracknell, UK). At the appropriate time after [^{131}I]MIBG administration (1, 16, 24, 48 or 72 hours), mice were sacrificed. Samples of blood, tumour, heart, lung, adrenal glands, kidney, spleen, skin, thyroid gland and skeletal muscle were excised and carefully dissected from fat or connective tissue. These were then placed in screw-capped Eppendorf tubes and weighed. The radioactivity present in the tissue samples was then determined by counting in an automated gamma well counter. The counts from each sample (in cpms) were converted to absolute activities (MBq) by comparison with the measurements obtained from a series of standards of known activity. The concentration of radiopharmaceutical in each organ and the tumour was expressed as the percent of the injected dose per gram of tissue. Correction was made for the radioactive decay which had occurred since the time of injection.

5.3 Results

5.3.1 Stability of n.c.a [^{131}I]MIBG

Sample HPLC elution profiles of n.c.a [^{131}I]MIBG dissolved in PBS at a concentration of 20.7 MBq / ml and stored at -20^{o}C are shown in Figure 5.2.

Figure 5.2 HPLC elution profiles of no-carrier-added [^{131}I]MIBG at 0, 48 and 96 hours after synthesis

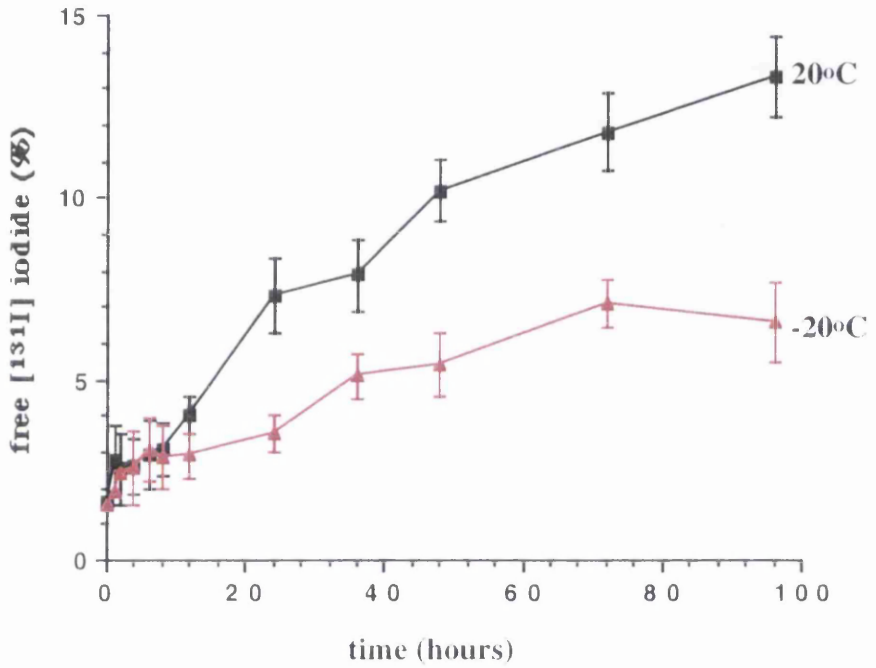


The major radioactive peak eluted with a retention time identical to that of an authentic cold MIBG sample. The minor radioactive peak, which was apparent after 48 hours, eluted close to the void volume of the column. This is assumed to be free ^{131}I, since the retention time matched that of [^{131}I]-NaI. These results indicate that the major radiochemical impurity is free ^{131}I. No other radiolabelled breakdown product was detected.

5.3.1.1 The effect of storage temperature

A comparison of the rates of deiodination of [^{131}I]MIBG stored at -20°C and 20°C is shown in Figure 5.3.

Figure 5.3 Effect of storage temperature on the stability of n.c.a [^{131}I]MIBG



n.c.a [^{131}I]MIBG was reconstitued in PBS to a radioactive concentration of 20.7 MBq / ml before storage at -20°C and 20°C. Data presented are the means and standard deviations of three determinations.

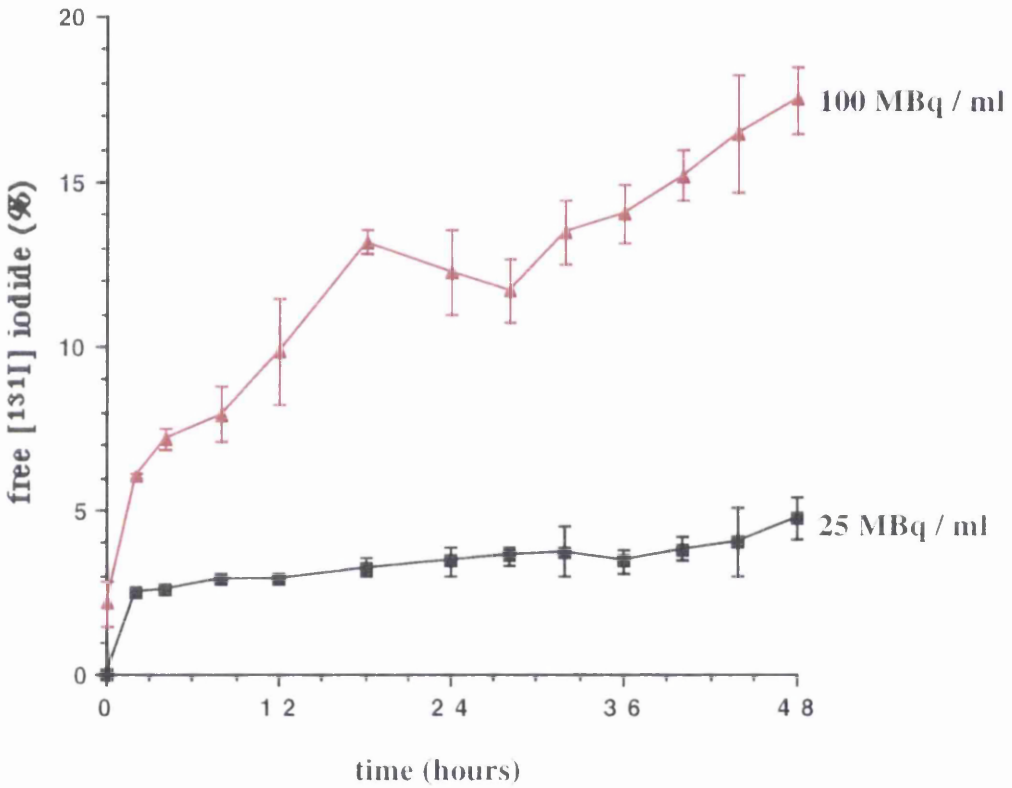
Stability was enhanced by maintaining the preparation at -20°C. After 12 hours there was a statistically significant ($P < 0.05$) difference in the percentage of free ^{131}I present in the samples stored at 20°C compared with frozen material. By

thirty six hours after synthesis, the percentage of free iodide was no greater than 5% in frozen material while levels in excess of 8% were detected in material stored at 20°C.

5.3.1.2 The effect of radioactivity concentration

To determine the effect of radioactivity concentration on deiodination, the percentage of free ^{131}I was monitored in two solutions of n.c.a [^{131}I]MIBG, 25 MBq / ml and 100MBq / ml, over 48 hours. The results are shown in Figure 5.4.

Figure 5.4 Effect of radioactive concentration on the stability of n.c.a [^{131}I]MIBG



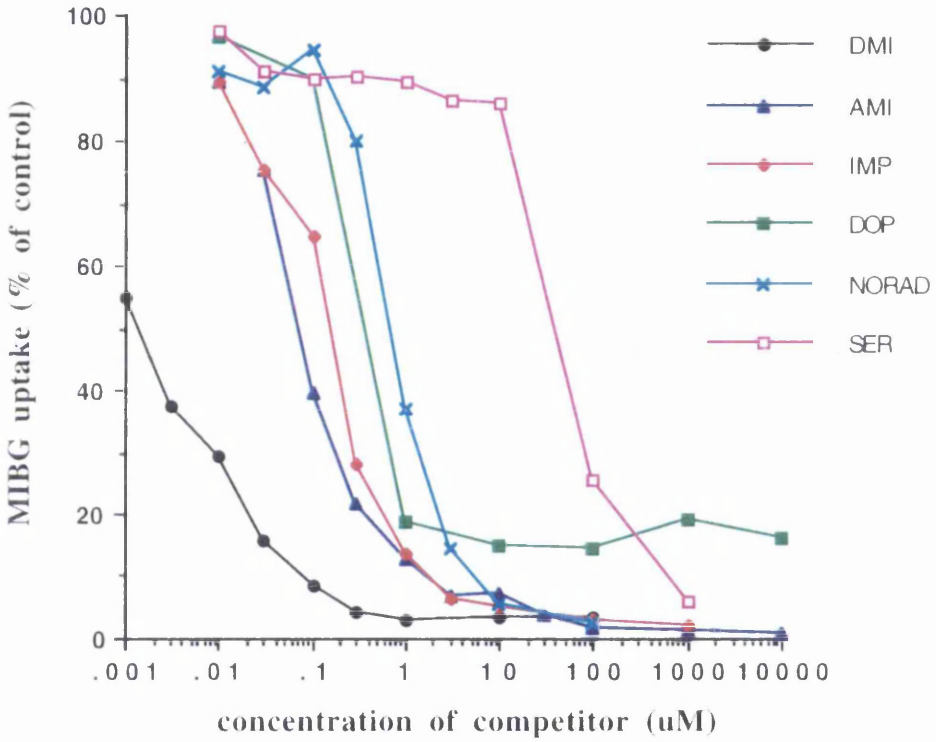
n.c.a [^{131}I]MIBG was reconstituted in PBS to the desired radioactive concentration and stored at -20°C. Data presented are the means and standard deviations of three determinations.

Activity concentration had a marked effect on the stability of the n.c.a material. At 4 hours after synthesis the percentage free ^{131}I in the 25 MBq / ml solution was 2% compared with 7% for the 100 MBq / ml solution. At all storage times after 4 hours, the level of free iodide in the 100 MBq / ml samples was significantly higher ($P < 0.01$) than that present in the lower concentration material.

5.3.2 Effect of uptake-1 inhibitors on the accumulation of n.c.a [^{131}I]MIBG by SK-N-BE(2c) cells

The effect of inhibitors on active MIBG uptake are shown in Figure 5.5.

Figure 5.5 Inhibition of uptake of n.c.a [^{131}I]MIBG by monoamine inhibitors



The concentrations required to reduce MIBG uptake by 50% were 1.5 nM for desmethylimipramine (DMI), 80 nM for amitriptyline (AMI), 170 nM for imipramine (IMP), 500 nM for dopamine (DOP) , 800 nM for noradrenaline (NORAD) and 60 µM for serotonin (SER).

5.3.3 Biodistribution of n.c.a [^{131}I]MIBG

The *in vivo* biodistribution of n.c.a [^{131}I]MIBG was compared with commercial therapy [^{131}I]MIBG (ex-[^{131}I]MIBG) in nude mice bearing SK-N-BE(2c) xenografts. Mice were sacrificed at various time points after intraperitoneal injection, and the radioactivity measured in tissues of interest. The results are shown in Tables 5.1 and 5.2, and Figures 5.6 to 5.10.

Table 5.1 Biodistribution of commercial therapy [^{131}I]MIBG (ex-[^{1311}I]MIBG) in tumour bearing mice expressed as % injected dose per gram of tissue

Organ	1hour	16hours	24hour	48hours	72hours
tumour	0.74 (0.49)	0.80 (0.49)	1.08 (0.41)	0.39 (0.26)	0.25 (0.08)
muscle	0.71 (0.15)	0.16 (0.09)	0.08 (0.02)	0.07 (0.03)	0.03 (0.02)
liver	2.91 (1.09)	0.76 (0.31)	0.45 (0.22)	0.22 (0.08)	0.12 (0.07)
spleen	2.26 (0.78)	0.59 (0.27)	0.68 (0.18)	0.29 (0.18)	0.09 (0.03)
skin	1.78 (0.71)	0.60 (0.32)	0.43 (0.20)	0.22 (0.09)	0.08 (0.05)
lung	1.63 (0.96)	0.77 (0.29)	0.30 (0.18)	0.11 (0.03)	0.06 (0.02)
heart	3.08 (0.81)	0.35 (0.07)	0.39 (0.21)	0.19 (0.06)	0.14 (0.07)
kidney	1.78 (0.61)	0.51 (0.29)	0.32 (0.13)	0.14 (0.05)	0.09 (0.04)
thyroid	1.43 (0.52)	1.00 (0.32)	0.99 (0.36)	0.42 (0.29)	0.15 (0.07)
blood	1.15 (0.38)	0.10 (0.05)	0.08 (0.05)	0.04 (0.02)	0.02 (0.02)
adrenals	4.20 (1.09)	3.01 (0.74)	2.62 (0.70)	1.33 (0.56)	0.88 (0.32)

+ $P < 0.05$ ++ $P < 0.01$ +++ $P < 0.001$

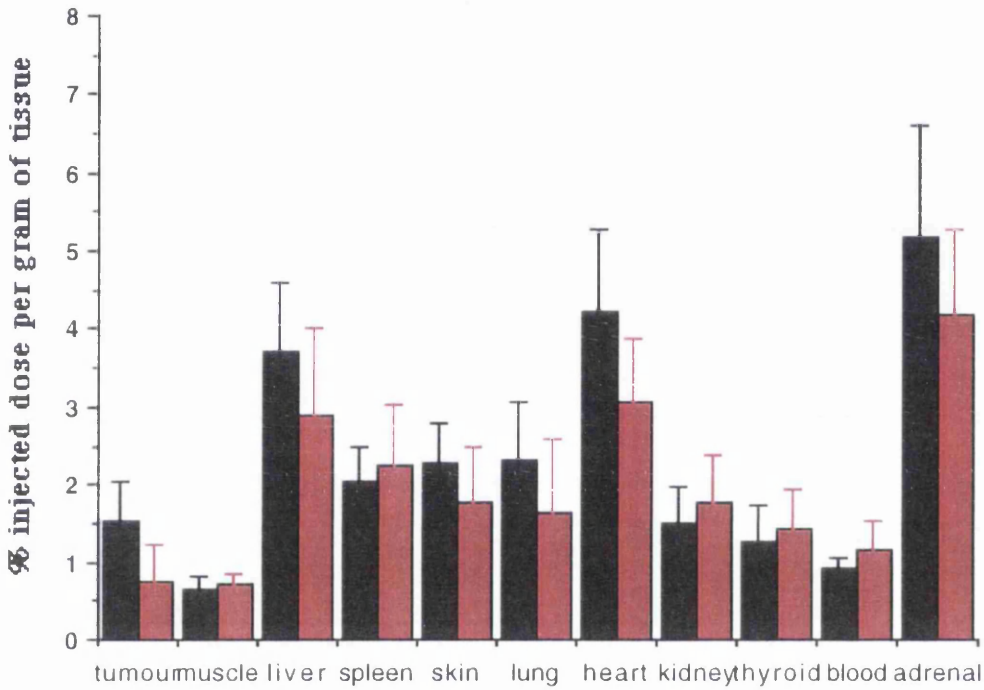
Table 5.2 Biodistribution of no-carrier-added [^{131}I]MIBG in tumour bearing mice expressed as % injected dose per gram of tissue

organ	1 hours	16 hours	24 hours	48 hours	72 hours
tumour	1.54 (0.50)++	2.12 (0.87)+++	2.97 (0.93)+++	0.90 (0.51)++	0.33 (0.14)
muscle	0.63 (0.17)	0.08 (0.04)+	0.07 (0.04)+	0.05 (0.03)	0.02 (0.01)
liver	3.70 (0.88)	1.14 (0.59)	0.65 (0.28)	0.29 (0.11)	0.18 (0.08)
spleen	2.05 (0.42)	0.60 (0.31)	0.64 (0.22)	0.18 (0.08)	0.07 (0.04)
skin	2.27 (0.51)	0.96 (0.41)+	0.79 (0.40)+	0.27 (0.12)	0.12 (0.05)
lung	2.33 (0.75)	0.59 (0.13)	0.47 (0.25)	0.20 (0.07)+	0.08 (0.03)
heart	4.21 (1.08)+	1.92 (0.83)+++	0.95 (0.44)++	0.30 (0.09)++	0.19 (0.06)
kidney	1.49 (0.48)	0.38 (0.11)	0.29 (0.16)	0.18 (0.05)	0.07 (0.02)
thyroid	1.26 (0.46)	0.96 (0.40)	0.81 (0.31)	0.49 (0.27)	0.12 (0.09)
blood	0.93 (0.12)	0.11 (0.04)	0.06 (0.02)	0.04 (0.02)	0.03 (0.01)
adrenals	5.18 (1.44)+	7.71 (3.15)+++	9.06 (3.02)+++	3.08 (0.90)+++	1.16 (0.48)

+ $P < 0.05$ ++ $P < 0.01$ +++ $P < 0.001$

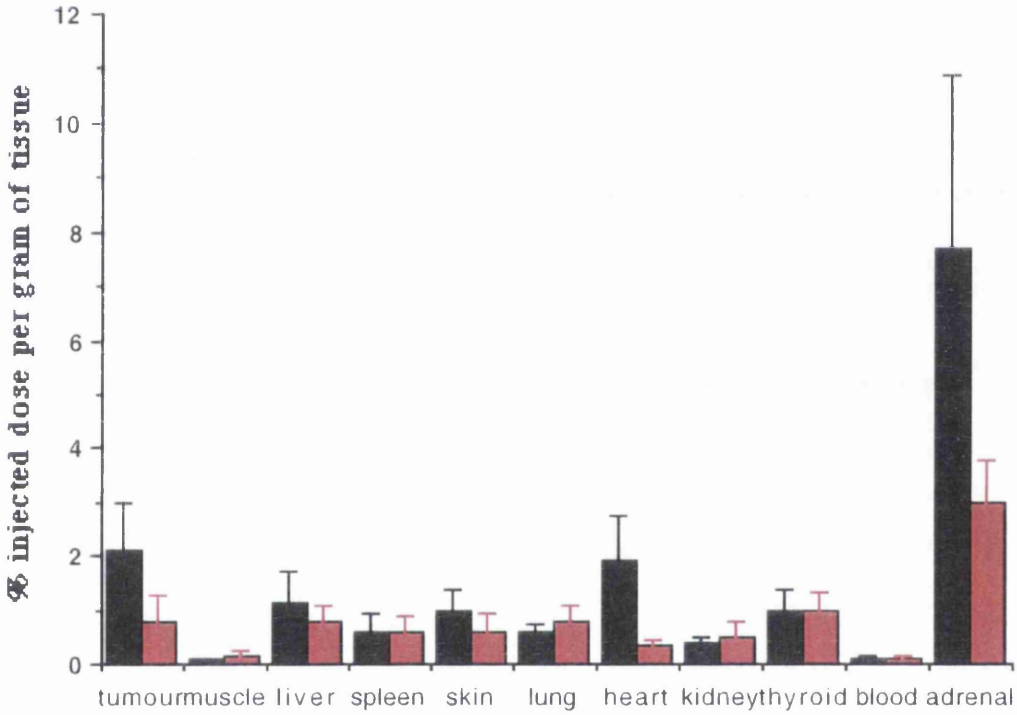
values are the mean (± sd). Data were analysed using the Students *t* - test. Significance of difference between n.c.a [^{131}I]MIBG and commercial ex-[^{131}I]MIBG: + $p < 0.05$; ++ $p < 0.01$; +++ $p < 0.001$

Figure 5.6 Biodistribution of n.c.a[^{131}I]MIBG and ex[^{131}I]MIBG at 1 hour



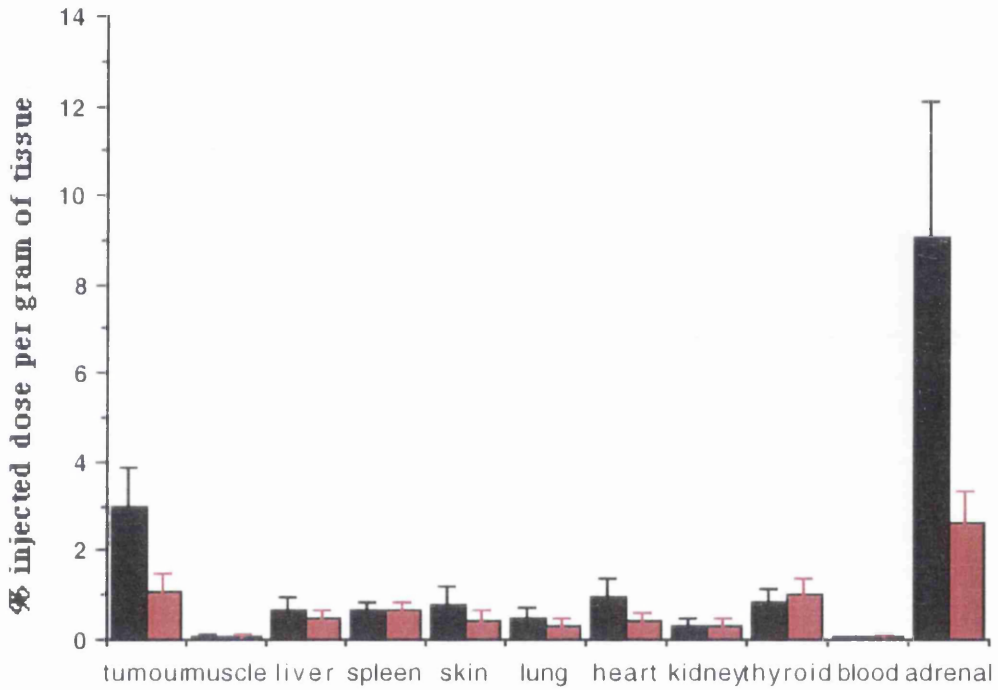
Biodistribution of n.c.a [^{131}I]MIBG (■) and ex- [^{131}I]MIBG (■). Data points represent the means and standard deviations of 7 determinations.

Figure 5.7 Biodistribution of n.c.a[^{131}I]MIBG and ex[^{131}I]MIBG at 16 hours



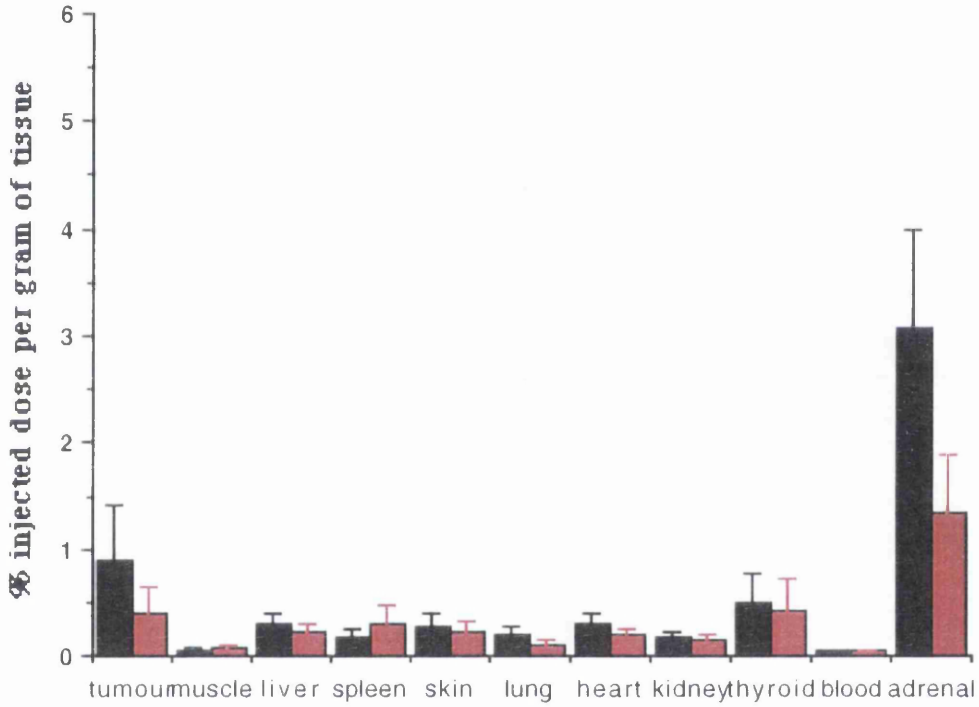
Biodistribution of n.c.a [^{131}I]MIBG (■) and ex- [^{131}I]MIBG (■). Data points represent the means and standard deviations of 7 determinations.

Figure 5.8 Biodistribution of n.c.a[^{131}I]MIBG and ex[^{131}I]MIBG at 24 hours



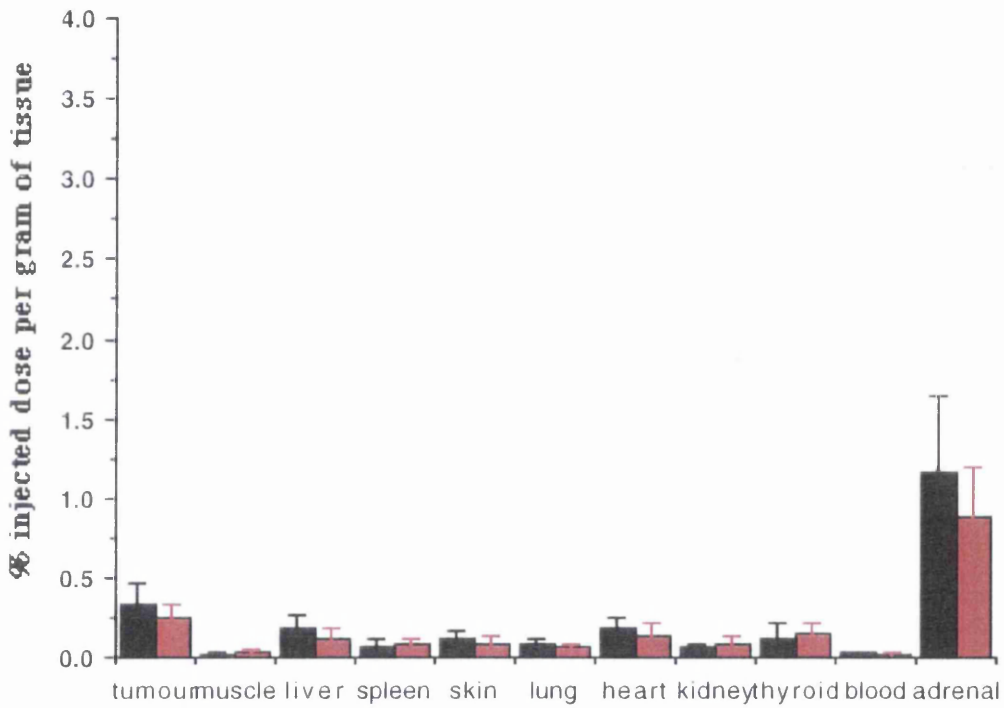
Biodistribution of n.c.a [^{131}I]MIBG (■) and ex- [^{131}I]MIBG (■). Data points represent the means and standard deviations of 7 determinations.

Figure 5.9 Biodistribution of n.c.a[^{131}I]MIBG and ex[^{131}I]MIBG at 48 hours



Biodistribution of n.c.a [^{131}I]MIBG (■) and ex- [^{131}I]MIBG (■). Data points represent the means and standard deviations of 7 determinations.

Figure 5.10 Biodistribution of n.c.a [^{131}I]MIBG and ex-[^{131}I]MIBG at 72 hours



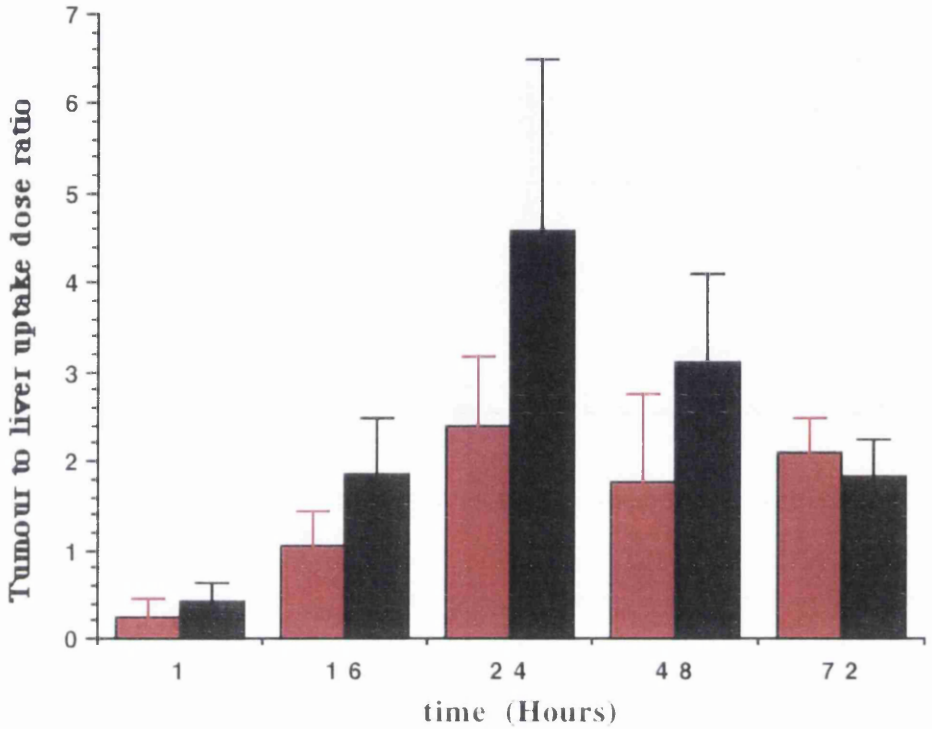
Biodistribution of n.c.a [^{131}I]MIBG (■) and ex- [^{131}I]MIBG (■). Data points represent the means and standard deviations of 7 determinations.

Significantly higher uptake of radioactivity administered in the form of n.c.a MIBG was observed in tumour at all time points except 72 hours ($P < 0.01$ at 1 and 48 hours; $P < 0.001$ at 16 and 24 hours). Substantial increases in uptake

were also observed in the heart ($P < 0.05$ at 1hour; $P < 0.01$ at 24 and 48 hours and $P < 0.001$ at 16 hours) and the adrenal glands ($P < 0.05$ at 1 hour; $P < 0.001$ at 16, 24 and 48 hours). Slightly higher levels were also observed in skin at 16 and 24 hours ($P < 0.05$), and in muscle at 8 and 16 hours ($P < 0.05$).

As described in section 5.1.3 the ratio of target-to-non-target accumulation provides a measure of the therapeutic index of targeted radiotherapy. The ratios between tumour and liver, a representative non-target tissue, were calculated and are shown in Figure 5.11.

Figure 5.11 Tumour to liver radioactivity uptake ratios of n.c.a [^{131}I]MIBG and ex-[^{131}I]MIBG over 72 hours



Tumour to liver uptake ratios at indicated times after the administration of n.c.a [^{131}I]MIBG (■) and ex- [^{131}I]MIBG (■).as means and standard deviations

At all time points except 72 hours the ratio obtained after the administration of n.c.a [^{131}I]MIBG was greater than that obtained with ex-[^{131}I]MIBG.

5.4 Discussion

5.4.1 Stability of n.c.a [^{131}I]MIBG

Dissociation of ^{131}I from MIBG must be limited to ensure that maximum radiation reaches the tumour and to prevent uptake of free radioactive iodine by the thyroid gland. The effects of storage temperature and radioactive concentration on deiodination were determined. Stability was significantly improved by freezing, in agreement with published data on the stability of exchange preparations of MIBG (Wafelman *et al.*, 1994). It is thought that the predominant mechanism affecting stability of radiopharmaceuticals in solution is secondary radiolysis (Spinks and Woods, 1990). That is a large proportion of the decay energy generates free radicals which can attack labelled molecules and cause decomposition. Free radical generation can be minimised by cooling or by the addition of free radical scavengers such as ascorbic acid and gentisic acid, and the preservative benzyl alcohol (Wafelman *et al.*, 1994). Addition of such compounds could be a potential means of limiting deiodination of n.c.a [^{131}I]MIBG.

The second factor investigated was the effect of radioactivity concentration. In all of the *in vitro* and *in vivo* studies described here the concentration of the n.c.a material was 20.7MBq / ml. However if n.c.a MIBG is to be used clinically then the administered concentrations will be significantly higher. The the rate of deiodination was therefore compared at concentrations of 25 and 100MBq / ml. At 25MBq / ml, free iodine was negligible up to 24 hours after synthesis, but at 100MBq / ml there was appreciable free iodine within the first 6 hours. This appears to be a direct result of the increased specific acitvity since studies using conventional exchange preparations, which do not have stabilisers added, do not contain significantly different levels of free iodine at radioactive concentrations of 37, 58 and 77 MBq / ml (Wafelmann *et al.*, 1993). These results could have serious implications for the eventual clinical use of the drug since in current clinical practice commercial [^{131}I]MIBG is discarded if free [^{131}I] is greater than 5% at the time of administration By this criterion the n.c.a

material would have to be administered immediately after synthesis or at lower concentrations.

5.4.2 Effect of inhibitors on n.c.a [^{131}I]MIBG uptake in SK-N-BE(2c) cells

Competition studies demonstrated that the uptake of n.c.a [^{131}I]MIBG was reduced in a dose-dependent manner by noradrenaline and inhibitors of noradrenaline transport. These results indicate that n.c.a MIBG enters neuroblastoma cells by virtue of the noradrenaline transporter. This conclusion is confirmed by the order of inhibition potency which was identical to that observed for the inhibition of uptake of noradrenaline into SK-N-SH cells (Richards and Salee, 1986) and in HeLa cells transfected with noradrenaline transporter cDNA (Pacholczyk *et al.*, 1991). This order is also consistent with the results of low specific activity [^{131}I]MIBG uptake inhibition studies using a range of competitors of varying affinity for the noradrenaline transporter (Lashford *et al.*, 1991).

5.4.3 Biodistribution of n.c.a [^{131}I]MIBG in mice bearing neuroblastoma xenografts

To determine whether n.c.a MIBG offered an advantage in terms of *in vivo* distribution, uptake in mice bearing SK-N-BE(2c) xenografts was compared with that of conventional radiolabelled MIBG prepared by exchange methods.

There was no significant difference in the degree of uptake of radioactivity by the thyroid gland indicating that *in vivo* deiodination was similar for both preparations. The time-dependent distribution profiles observed in most other non-sympathetically innervated tissues were also similar for both preparations: accumulation levels were generally comparable for both types of MIBG and there was a biphasic clearance from normal, non-target sites (ie a rapid initial decline in levels over the first 16 hours followed by a slower decrease over the remaining 56 hours).

In sympathetically innervated tissues, however, there were considerable differences between the two types of MIBG. Both the heart and the adrenal glands took up significantly higher amounts of the n.c.a preparation. This is consistent with previous biodistribution studies in non-tumour bearing mice (Vaidyanathan *et al.*, 1993), although the amounts noted in the latter study were much higher (3.39 % ID / g tissue for heart and 24.14 % ID / g for the adrenals). High uptake in these organs, particularly the heart, could be problematic for therapeutic application of n.c.a material. Agents which could selectively block such uptake would therefore be useful. The granular uptake inhibitor reserpine has been shown to reduce cardiac and adrenal uptake of MIBG in dogs (Weiland *et al.*, 1981; Shapiro *et al.*, 1984), while numerous *in vitro* studies have shown that it has no effect on levels of MIBG in neuroblastoma cells (Smets *et al.*, 1989; Lashford *et al.*, 1991; Montaldo *et al.*, 1991). However its use clinically is limited because of serious and irreversible side effects. A related compound with similar mode of action but less severe side effects is tetrabenazine. *In vivo* studies confirmed that in non-tumour bearing mice treated with 40 mg / kg of tetrabenazine, cardiac uptake of n.c.a [^{123}I]MIBG was significantly reduced and lower levels were observed in the adrenal gland (Vaidyanathan *et al.*, 1994). Further experiments are needed to determine whether this effect can be repeated in tumour bearing mice, at concentrations of the inhibitor which do not adversely effect tumour uptake.

Another potential means of protecting non-target organs could be to administer unlabelled MIBG prior to injection of [^{131}I]MIBG. The concept of predosing is based on the pharmacokinetic behaviour of MIBG *in vivo* : studies in both animals and human patients have shown that MIBG undergoes rapid clearance from the plasma into tissues (Ehinger *et al*, 1987; Lashford *et al*, 1988) and it has been suggested that this extensive translocation could be the result of non-specific tissue binding. It is postulated that these non-specific sites could be blocked by predosing with unlabelled MIBG molecules, reducing non-target uptake. *In vivo* studies using nude mice bearing neuroblastoma xenografts suggested that elevated doses of unlabelled MIBG, administered prior to radiolabelled [^{131}I]MIBG (specific activity 1.5 GBq / mg) , were an effective

way of reducing uptake of radioactive MIBG in normal tissues. Crucially this normal tissue advantage could be obtained without adversely affecting tumour uptake (Rutgers *et al* 1994). Preliminary experiments to determine whether pre-dosing could protect non-target tissues from n.c.a [^{131}I]MIBG demonstrated that uptake was lowered in several non-target sites including the heart. However this advantage was offset by significant decreases in tumour uptake.

The most encouraging results of the present studies are the improved levels of tumour uptake. At all time points, except 72 hours, tumour accumulation was significantly higher with n.c.a-[^{131}I]MIBG than ex-[^{131}I]MIBG. At 24 hours, the point at which maximal tumour accumulation occurs, this represented a 3-fold enhancement in uptake of the radiopharmaceutical. Consequently the target to non-target ratio was also improved: n.c.a [^{131}I]MIBG produced a 2-fold increase in the tumour to liver ratio. These results are consistent with the hypothesis that maximal discrimination between tumour and normal uptake is achieved by administering high specific activity MIBG.

Using the data obtained from the murine biodistribution studies it is possible to estimate the therapeutic advantage resulting from the use of n.c.a [^{131}I]MIBG instead of ex-[^{131}I]MIBG in human neuroblastoma patients. The ratio of absorbed radiation dose per unit of injected activity for tumour and normal organs between n.c.a [^{131}I]MIBG and ex-[^{131}I]MIBG was calculated. The results indicate that for the same injected activity the predicted tumour absorbed dose is approximately 2.3 fold higher with the n.c.a compared with the exchange preparation (Mairs *et al.*, 1995). The data on the biodistribution of n.c.a [^{131}I]MIBG and ex-[^{131}I]MIBG were obtained using SK-N-BE(2c) neuroblastoma xenografts. This cell line was chosen because it had been previously shown to grow successfully as xenografts in nude mice and because of its known capacity for MIBG accumulation (Gaze *et al.*, 1994).

Further confirmation of the advantage of n.c.a MIBG requires the evaluation of its distribution in other *in vivo* neuroblastoma models. Two such studies have been reported and the findings are compared in Table 5.3

Table 5.3 Biodistribution of n.c.a [^{131}I]MIBG in neuroblastoma xenografts

Reference	Nude mouse strain	Xenograft	Delivery	Tumour uptake % i.d/g*		Tumour liver ratio	
Mairs *et al* 1995	MF-1	SK-N-BE(2c)	i.p**.	ex n.c.a	1.08 2.97	ex n.c.a	2.40 4.56
Vaidyanathan *et al* 1996	BALB c	SK-N-SH	i.p / i.v***.	ex n.c.a	1.80 1.80	ex n.c.a	3.00 4.50
Lavitrano *et al* 1996	CD1	SH-SY5Y	i.v.	ex n.c.a	0.65 1.30	ex n.c.a	1.54 3.93

* percent injected dose per gram of tissue
** intraperitoneal injection
*** intravenous injection

Using xenografts established from the cell line SH-SY-5Y, tumour accumulation of n.c.a MIBG was found to be approximately double that of ex-MIBG at 24 hours resulting in a 2.5 fold increase in the tumour to liver ratio. In contrast with SK-N-SH xenografts there was no significant difference in the tumour accumulation levels with n.c.a and ex-MIBG.

The reasons for the discrepancy with SK-N-SH xenografts are not clear since previous work has demonstrated that both cell lines behave similarly in terms of MIBG kinetics: the *in vitro* uptake of MIBG by this cell line is comparable to that of SK-N-BE(2c) (Mairs *et al.*, 1994) and *in vivo* biodistribution studies with conventional exchange MIBG demonstrated no significant differences in accumulation between SK-N-SH and SK-N-BE(2c) xenografts (Gaze *et al.*, 1994). As Table 5.3 indicates there were a number of differences in the way the experiments were conducted. Differences in the route of administration have been shown to alter the biodistribution of MIBG (Rutgers *et al.*, 1996) and paired experiments injecting ^{131}I labelled drug intravenously and ^{125}I labelled material intraperitoneally did demonstrate slight, though not significant, differences in tumour uptake between the two injection sites (Vaidyanathan *et al.*, 1996).

Another factor which may affect the biodistribution of MIBG is the presence of circulating catecholamines. Elevated levels could saturate uptake sites on the tumour which would obscure any differences between high and low specific activity [^{131}I]MIBG. It is possible that there are significant inter-strain variations in circulating catecholamine levels which could adversely effect MIBG biodistribution. A related consideration is the influence of the xenografts themselves on catecholamine levels. *In vitro* measurements of catecholamine secretion by fluorescence demonstrated that neither SK-N-SH nor SK-N-BE(2c) cells exhibited any appreciable fluorescence (Tomayko *et al.*, 1988). However *in vivo* it has been reported that SK-N-BE(2c) xenografts show low levels of fluorescence while SK-N-SH tumours give intense fluorescence (Vaidyanathan *et al.*, 1996). If SK-N-SH xenografts secrete more

catecholamines this would account for the lack of tumour uptake advantage seen with n.c.a [^{131}I]MIBG in this type of xenograft.

Another possible explanation for the observed differences could be related to the way in which the xenograft model itself is created and maintained. The method used to grow SK-N-BE (2c) xenografts is based on published methodology (Rutgers *et al.*, 1991). After establishing splenic and hepatic tumours on donor mice these are removed and fragments of the excised tumour are inserted subcutaneously in recipient mice. Tumours are maintained by repeated subcutaneous passage. It has however been noted that successive serial passage of tumours does result in decreased expression of the noradrenaline transporter particularly in those tumours established using SK-N-SH cells (personal observation). This will ultimately effect the MIBG uptake ability of xenografts. In the Vaidyanathan study, SK-N-SH tumours were initiated by subcutaneous injection of SK-N-SH cells into donor mice. Subsequent tumours were then minced and the resultant homogenate injected subcutaneously into recipient mice who were used for biodistribution studies when tumours reached a suitable size. The effect this technique has on expression of the transporter is unknown but it is possible that the degree of expression may be adversely affected.

5.4.4 Clinical Implications

From a clinical perspective, the use of n.c.a MIBG has several advantages. Currently the administration of [^{131}I]MIBG to patients is limited by normal tissue toxicity - principally to the bone marrow. In practice this means that the administered dose is limited to a whole body dose of 2Gy (Lashford *et al.*, 1992). Since the majority of this dose comes from [^{131}I]MIBG uptake by normal tissue rather than tumour, any formulation which reduces normal uptake is likely to be beneficial. N.c.a MIBG fulfils this objective:- the higher tumour-to-normal uptake ratio means that for a given radioactivity the proportion reaching the tumour is greater, resulting in a larger radiation dose and therefore an increased likelihood of benefit.

In addition to improving therapy, n.c.a MIBG could also be beneficial in diagnostic scintigraphy. Ideally the diagnostic scan should provide accurate tumour and whole body dose data for a specific patient prior to a planned MIBG therapy so that the appropriate amount of activity can be administered. However in practice this is difficult to achieve due to differences in the biokinetics of tracer and therapy doses (Wafelman *et al.*, 1994). It has been suggested that this divergence arises because of the differences in total MIBG concentration between the low-dose diagnostic and high-dose therapy applications (Tristam *et al.*, 1996). The improved tumour to normal ratios obtained with the n.c.a MIBG may improve the sensitivity of the procedure allowing more accurate and reliable staging of patients with disseminated disease, and improve dosimetry and treatment planning for therapy applications.

One other potential advantage of n.c.a MIBG is related to the side effects associated with therapeutic administration of MIBG. Nausea and vomiting have been recorded in patients receiving therapeutic doses of [^{131}I]MIBG (Shapiro and Fischer, 1985; Sisson *et al.*, 1988; Shapiro *et al.*, 1991). In cancer patients undergoing chemotherapy, noradrenaline has been strongly implicated as the potentiator of delayed nausea and vomiting (Fredrikson *et al.*, 1994). This study found a correlation between post-chemotherapy nausea and the levels of noradrenaline, a finding which is supported by previous studies which show that catecholamines can up-regulate nausea and vomiting (Andrews *et al.*, 1988; Leslie and Reynolds, 1993). Since a therapeutic dose of commercial therapy [^{131}I]MIBG contains 7-14 mg of MIBG it is possible that the nausea and vomiting observed is due to the presence of high concentrations of biogenic amine. As a therapeutic dose of n.c.a [^{131}I]MIBG would only contain 3-6 μg of MIBG these unpleasant side-effects could be minimised. Administration of lower amounts of MIBG is also advantageous in terms of blood pressure: therapeutic-level doses of commercial [^{131}I]MIBG can cause an elevation in blood pressure which means the drug has to be slowly infused over 2 hours. Use of lower amounts of MIBG should decrease these pressor effects (Mairs and Zalutsky, 1995).

5.5 Conclusions

The results of these studies are highly encouraging, suggesting that the use of n.c.a MIBG could offer significant advantages over conventional exchange prepared MIBG. The favourable uptake and biodistribution results presented here are supported by *in vitro* cytotoxicity studies using SK-N-BE(2c) spheroids: inhibition of growth was apparent at 2 MBq / ml of n.c.a [^{131}I]MIBG. However this inhibition decreased in a dose dependent manner as the specific activity of the n.c.a preparation was lowered by the addition of increasing amounts of cold carrier MIBG (Mairs *et al.*, 1995). The therapeutic efficacy of n.c.a [^{131}I]MIBG now needs to be evaluated in suitable *in vivo* model systems to confirm that these benefits lead to enhanced tumour cell kill.

CHAPTER 6

THE EFFECT OF CISPLATIN PRETREATMENT ON MIBG UPTAKE BY NEUROBLASTOMA CELLS

6.1 Introduction

6.1.1 Current clinical status of [^{131}I]MIBG

As described in chapter 3, more than a decade of clinical experience with [^{131}I]MIBG as a single agent has confirmed its usefulness in the treatment of advanced neuroblastoma: in addition to its palliative properties and lack of side effects, prolonged remissions have been reported in a significant number of patients.

Despite these encouraging results, it is doubtful that the use of [^{131}I]MIBG alone will cure patients with widely disseminated disease. Consequently, many centres have adopted the use of MIBG targeted radiotherapy in combination with more firmly established treatment modalities (section 3.7.2). To achieve synergism it is essential that the optimal sequence of these treatments is established.

6.1.2 *In vitro* studies of combined therapy approaches

Unfortunately laboratory studies to determine the effects of therapy combinations are lacking. One published study reported the effect of external beam irradiation on MIBG uptake by neuroblastoma cells *in vitro*. Exposure of cultured SK-N-SH neuroblastoma cells to 5 Gy, 24 hours prior to MIBG exposure, resulted in stimulated MIBG uptake. As irradiation would have selectively depleted proliferating cells and left more differentiated cells, it was suggested that these more mature neuroblasts had a greater capacity for active uptake of MIBG (Smets *et al.*, 1991). This hypothesis is supported by the results of Montaldo and colleagues (1992, 1996), who have demonstrated that a variety of agents which are capable of inducing differentiation in nueroblastoma cells can enhance MIBG uptake ability and expression of the noradrenaline transporter in these cells. However, this phenomenon of enhanced uptake is not repeated *in vivo*. Preirradiation of mice bearing SK-N-

SH neuroblastoma xenografts at doses of 5, 10 and 20 Gy failed to enhance MIBG accumulation by the tumours (Sautter-Bihl and Bihl, 1996). The effect of chemotherapeutic agents on MIBG uptake and retention are largely unknown, although one *in vitro* study has reported significant decreases in MIBG accumulation after exposure to adriamycin (Paffenholz *et al.*, 1989).

6.1.3 Aims of this study

To determine what effect anticancer agents may have on MIBG uptake and retention a series of *in vitro* experiments were undertaken. For these initial studies the chemotherapeutic agent cisplatin was chosen because it is used effectively in the treatment of neuroblastoma (eg De Bernardi *et al.*, 1992; Pearson *et al.*, 1992) and because it may act in synergy with radiotherapy (Douple *et al.*, 1985; Dewit, 1987).

6.2 Materials and Methods

6.2.1 Cell culture

SK-N-BE(2c) cells were used for these studies and were cultured as described in section 5.2.3.

6.2.2 Effect of culture density on MIBG uptake and noradrenaline transporter expression

The influence of cell culture density on the active incorporation of MIBG was determined using SK-N-BE(2c) cells that had not been subjected to treatment with cisplatin. Transcription of the noradrenaline transporter gene by SK-N-BE(2c) cells was assessed by RT-PCR. Cells were seeded at a range of cell numbers, from 0.2 x 10^5 to 1.2 x 10^5, cultured for 5 days and then assayed for MIBG uptake as described in section 6.2.4. A second set of cultures were used for RNA extraction and RT-PCR as described in section 6.2.5.

6.2.3 Clonogenic Assay

The toxicity of the cisplatin concentrations used was determined by clonogenic assay. For this, cells were seeded in 25cm^3 flasks at 2.5 x 10^5 cells per flask. After 2 days, medium was removed and replaced with fresh medium containing the appropriate concentration of cisplatin (David Bull Laboratories, Warwick, UK). After 24 hours, medium was removed and the cells were washed three times with PBS. Fresh medium was added and the cells were incubated for a further 24 hours. Cells were then trypsinized and counted. For each cisplatin concentration three flasks were seeded at 1000 cells per flask. Flasks were equilibrated with 5% CO_2 and then incubated at 37°C. After 14 days, medium was removed and the colonies were fixed and stained with Carbol Fuchsin (R A Lamb, Middlesex). Colonies of more than 50 cells were counted using an automated colony counter (Artek Systems).

6.2.4 MIBG uptake studies

Cells were seeded in six-well plates at an initial density of 0.5 x 10^5 cells and cultured for 48 h. Cisplatin was then added at the appropriate concentration and the cells incubated for 24 h. The medium was then removed; the cells were washed with PBS, and 5 ml of fresh medium was added. Cells were assayed for MIBG uptake, before cisplatin exposure, at the point of cisplatin removal, and 24 h after cisplatin removal. MIBG incorporation was measured by incubating the cells for 2 h with 7 kBq of [^{131}I]MIBG (specific activity 45-65 MBq / mg, Dupont Radio-pharmaceuticals, Hertfordshire, UK). Non-specific uptake was measured in the presence of 1.5 μM desmethylimipramine (DMI) (Sigma-Aldrich, Dorset, UK). After incubation, medium was removed, the cells were washed with PBS and radioactivity was extracted using 2 aliquots of 10% (w / v) trichloroacetic acid. The activities of the extracts were then measured in a gamma-well counter. Specific uptake, expressed as cpm per 10^5 cells, was calculated by subtracting values obtained in the presence of DMI from total uptake.

6.2.5 Reverse transcription polymerase chain reaction (RT-PCR) analysis of noradrenaline transporter gene expression

Total RNA was extracted from control and cisplatin-treated cultures before treatment, at the time of cisplatin removal and 24 h after cisplatin removal. RNA extractions were performed using a PUREscript RNA isolation kit (Gentra). RNA concentration of the samples was determined spectrophotometrically at 260 nm. 1 μg of RNA was converted to cDNA using the Clontech 1st-strand cDNA synthesis kit (Cambridge Bioscience). cDNA was then PCR amplified using primers specific for the transporter sequence. The sense primer was 5' - CTGGTGGTGAAGGAGCGCAACGGC-3' and the antisense primer was 5' - ATGTCATGAATCCCGCTGCTCTCG-3' (Montaldo *et al.*, 1992).This amplification generated a 590 bp PCR product. Semiquantitation was achieved by comparison of the target signal with

achieved by comparison of the target signal with thesignal generated by co-amplification of a reference sequence - glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH primers were 5' - GCATTGCTGATGATCTTGAGGC- 3' (sense) and
5' -TCGGAGTCAACG GATTTGG - 3' (antisense). These generated a 390 bp product. Co-amplification of target and reference sequences was performed in 10 x synthesis buffer containing 100 mM Tris-HCl, 1.5 mM $MgCl_2$, 500 mM KCl, pH 8.3, with 10 nmol of dNTPs, 20 pmol of each target primer, 20 pmol of each reference GAPDH primer and 2 units of Taq polymerase. Cycling conditions consisted of 1 min denaturation at 94°C, 1 min annealing at 65 °C and 1 min extension at 72°C for 35 cycles. The PCR products were separated by electrophoresis through 1.6% (w / v) agarose (Flowgen, UK, Kent). These were densitometrically scanned using Quality One Image Analysis software.

6.2.6 MIBG release experiments

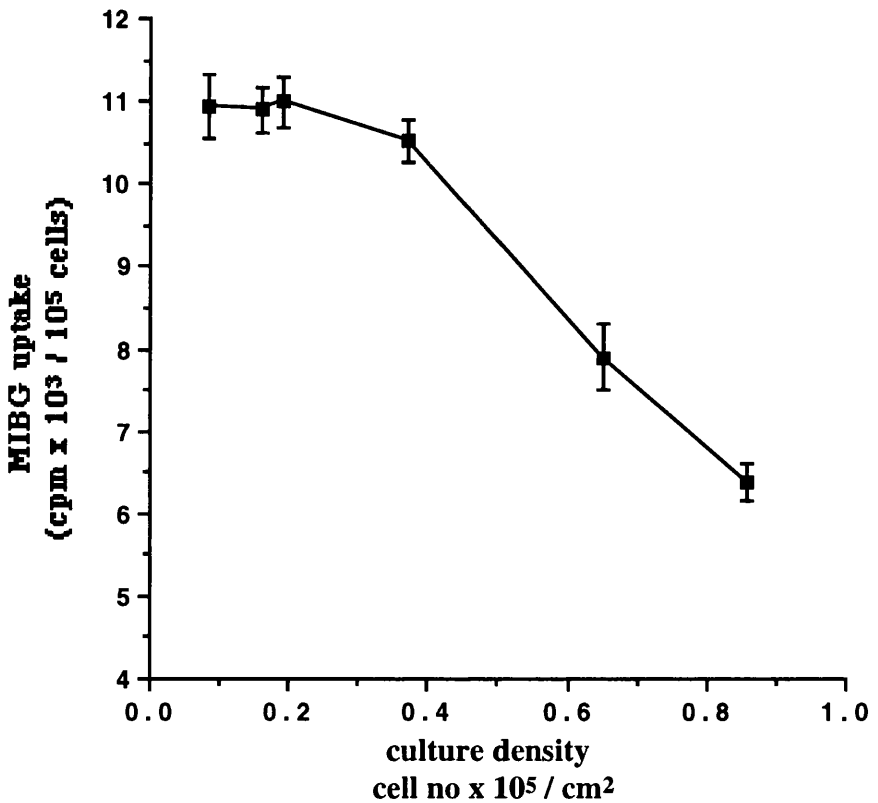
To determine whether cisplatin treatment affected storage capacity of the neuroblastoma cells, experiments were carried out to investigate the kinetics of release of MIBG from control and treated cells. At 48 h after initial cisplatin exposure, cells were incubated with labelled MIBG as described above. The culture medium was then removed and changed for drug-free fresh medium or medium containing 1.5 μm DMI. In a second set of cultures, medium was replaced with drug-free fresh medium or medium containing 2 μM reserpine. At 0, 2, 4 and 6 h after withdrawal of MIBG, the cells were assayed for MIBG retention. Data were analysed using the Students *t* - test.

6.3 Results

6.3.1 Effect of culture density

Initial experiments were undertaken to monitor the effects of culture density on MIBG uptake and noradrenaline transporter expression. These confirmed the previous observations (Montaldo *et al.*, 1992) that at high culture densities (greater than 0.24 x 10^5 cells per cm^2 which is equivalent to 2.5 x 10^5 cells per well), there was a progressive decline in MIBG accumulation (Figure 6.1).

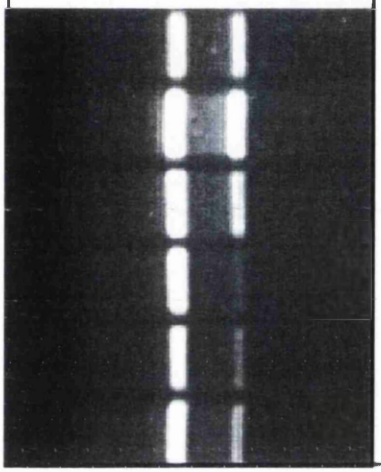
Figure 6.1 The effect of culture density on MIBG uptake by SK-N-BE(2c) cells



Culture density is expressed as the number of cells per cm^2 of culture dish. Data points represent the mean ± sd of three experiments.

RT-PCR analysis indicated that this reduction in uptake could be due to decreased expression of the gene encoding the noradrenaline transporter (Table 6.1).

Table 6.1 Effect of culture density on noradrenaline transporter expression

R T	culture density (cell no x 10^5/cm^2)	target: reference ratio
	0.09	0.96
	0.16	1.01
	0.19	0.82
	0.37	0.45
	0.65	0.32
	0.86	0.25

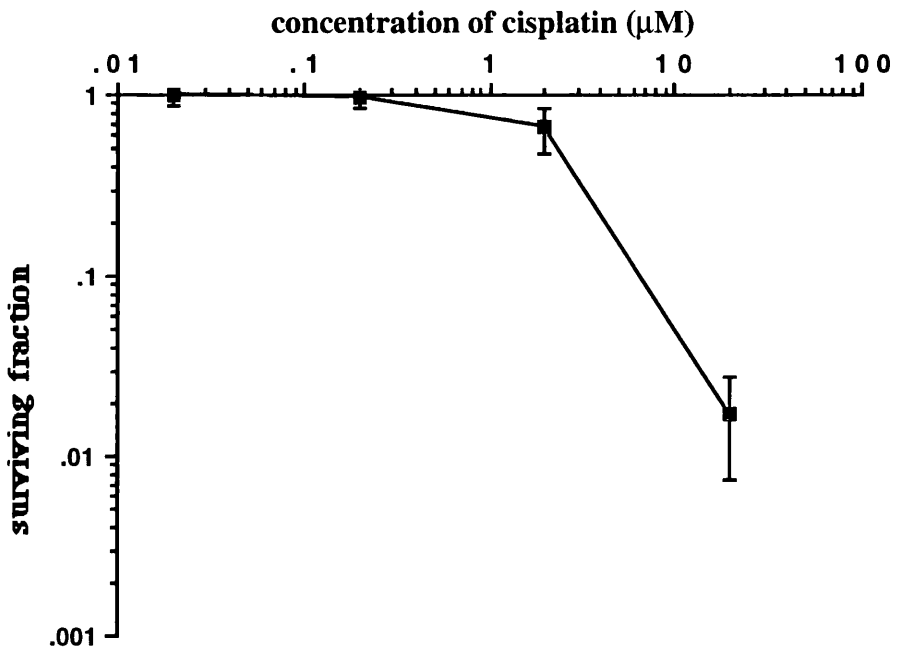
Target to reference ratios were calculated from the intensity of PCR signals measured by scanning of ethidium bromide-stained gels. R = reference sequence (GAPDH); T = target sequence (noradrenaline transporter).

Accordingly, in all cisplatin experiments, cell numbers in control and treated cultures were kept below this figure. Data from wells containing more than 2.5 x 10^5 cells were discarded.

6.3.2 Effect of cisplatin on SK-N-BE(2c) survival

The toxicity of a range of concentrations of cisplatin was determined by clonogenic assay. Figure 6.2 shows that the toxic effects of cisplatin were apparent at concentrations of the drug greater than 0.2 μM.

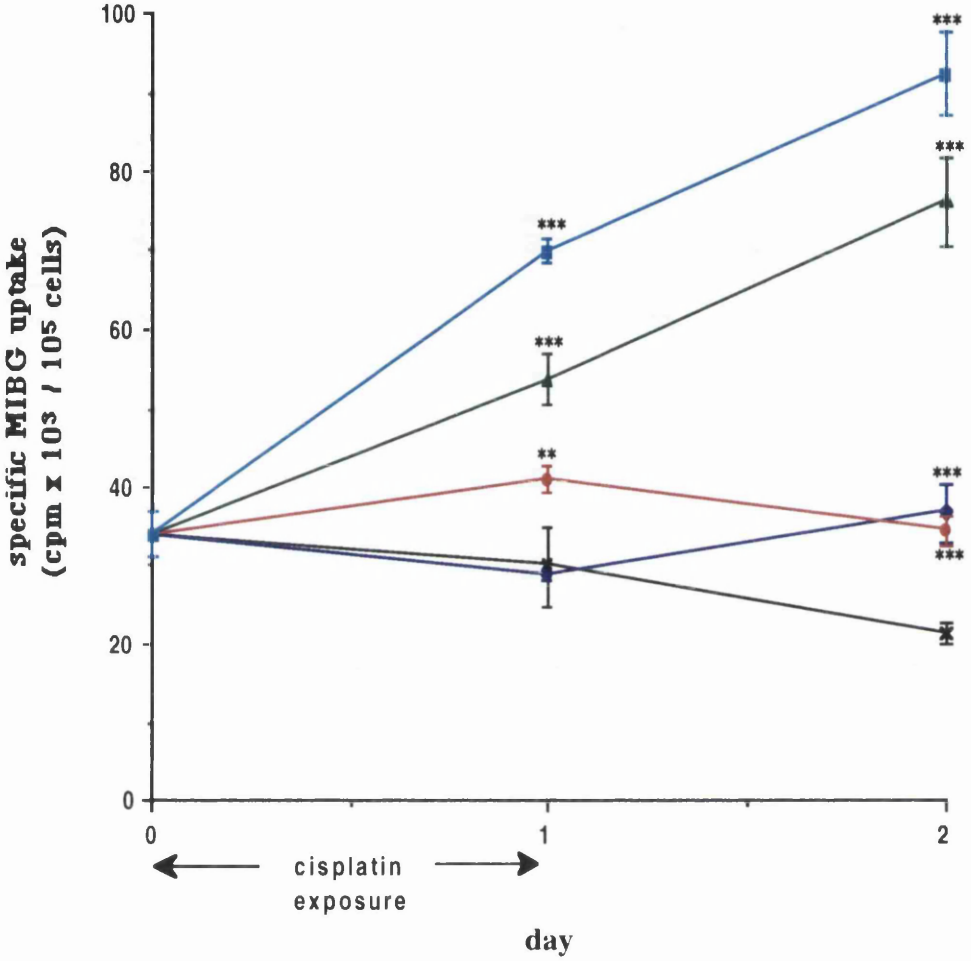
Figure 6.2 The effect of cisplatin on clonogenic survival of SK-N-BE(2c) cells



6.3.3 Effect of cisplatin pretreatment on MIBG uptake

SK-N-BE(2c) cells were incubated with a range of concentrations of cisplatin for 24 h. The ability of the cells to incorporate MIBG was then assessed immediately after drug removal and 24 h after drug removal. The degree of specific uptake was calculated for each cisplatin concentration and compared with that of control cultures. Figure 6.3 shows that cisplatin induced a concentration-dependent stimulation in active incorporation of MIBG.

Figure 6.3 The effect of cisplatin pretreatment on MIBG uptake by SK-N-BE(2c) cells



Cells were incubated with cisplatin (CP) for 24 hours as indicated. MIBG uptake was measured prior to CP treatment (day 0), at CP removal (day 1) and 24 hours later (day 2).

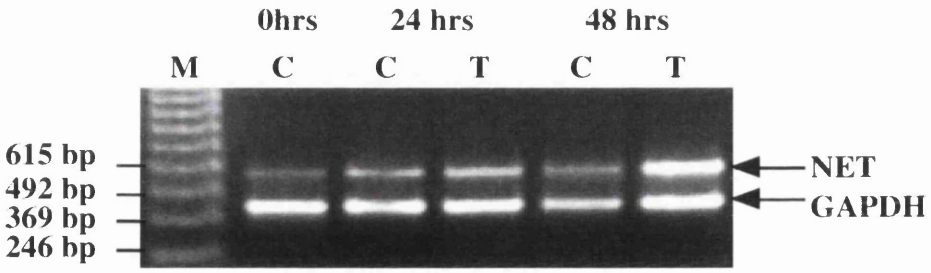
×: control, ◆: 0.02 μM CP, ●: 0.2 μM CP, ▲: 2 μM CP, ■: 20 μM CP. Points represent the means and standard deviations of 3 experiments in triplicate. Double asterisks indicate specific uptake significantly different from control, $P < 0.01$. Triple asterisks indicate specific uptake significantly different from control, $P < 0.001$.

At the point of drug removal, values for MIBG uptake after 0.02, 0.2, 2 and 20 μM cisplatin were 95% (not significantly different from control), 134% ($P < 0.01$), 178% ($P < 0.001$), 232% ($P < 0.001$) of control values respectively. After a further 24 h, this enhancement was even more pronounced with uptake values of 171% ($P < 0.001$), 162% ($P < 0.001$), 355% ($P < 0.001$) and 431% ($P < 0.001$) of controls.

6.3.4 Effect of cisplatin pretreatment on expression of the noradrenaline transporter

Expression of the noradrenaline transporter molecule was examined in control and treated cultures by RT-PCR. The cDNA amplification products obtained after exposure of SK-N-BE(2c) cells to 20 μM cisplatin are shown in Figure 6.4.

Figure 6.4 RT-PCR analysis of noradrenaline transporter expression in cells exposed to 20 μM cisplatin



Expression of noradrenaline transporter (NET) and GAPDH reference determined by co-amplification using the appropriate primers. Expression was assayed prior to cisplatin exposure (0 hours), immediately after cisplatin exposure (24 hours) and 24 hours later (48 hours). M= 123 bp markers.

For each time point and treatment, the ratio of target to reference signal intensity was calculated and expressed as the percent of the control value. These results are summarised in Table 6.2.

Table 6.2 Semi-quantitation of expression of noradrenaline transporter gene in cisplatin treated cells.

Concentration of cisplatin (μM)	Noradrenaline transporter expression (% of control) 24 hours	48 hours
0.02	115 (± 6.2)	89 (± 11.2)
0.20	120 (± 7.5)	94 (± 8.4)
2.00	129 (± 7.9)	134 (± 9.3)
20.00	125 (± 8.2)	165 (± 10.6)

The values presented are ratios of intensity of target sequence to intensity of reference sequence. The results are expressed as the percent of control values.

This data demonstrates that cisplatin induced a dose-dependent stimulation of expression of the transporter molecule. At 0.02 μM and 0.2 μM levels of cisplatin, the enhanced expression was not maintained after removal of the drug. However, at higher concentrations (2.0 and 20 μM), the effect was prolonged and was in fact greater at 48 h than at 24 h after the initiation of exposure to cisplatin. These results indicate that the cisplatin-induced enhancement of MIBG uptake could be due to increased synthesis of new transporter molecules, as opposed to increased activity of existing molecules.

6.3.5 Effect of cisplatin pretreatment on retention of MIBG

To determine whether cisplatin enhanced the ability of SK-N-BE(2c) cells to store catecholamines, experiments were carried out to determine the retention of MIBG. These were performed in the presence of reserpine (which depletes neurosecretory granules) and DMI (which inhibits re-uptake of released drug by the transporter). In control- and cisplatin-treated cells, spontaneous release of MIBG was similar to that in the presence of reserpine (Figure 6.5). In contrast, DMI induced a rapid depletion of MIBG from the cells (Figure 6.6).

Figure 6.5 Effect of reserpine on retention of MIBG in cisplatin treated SK-N-BE(2c) cells

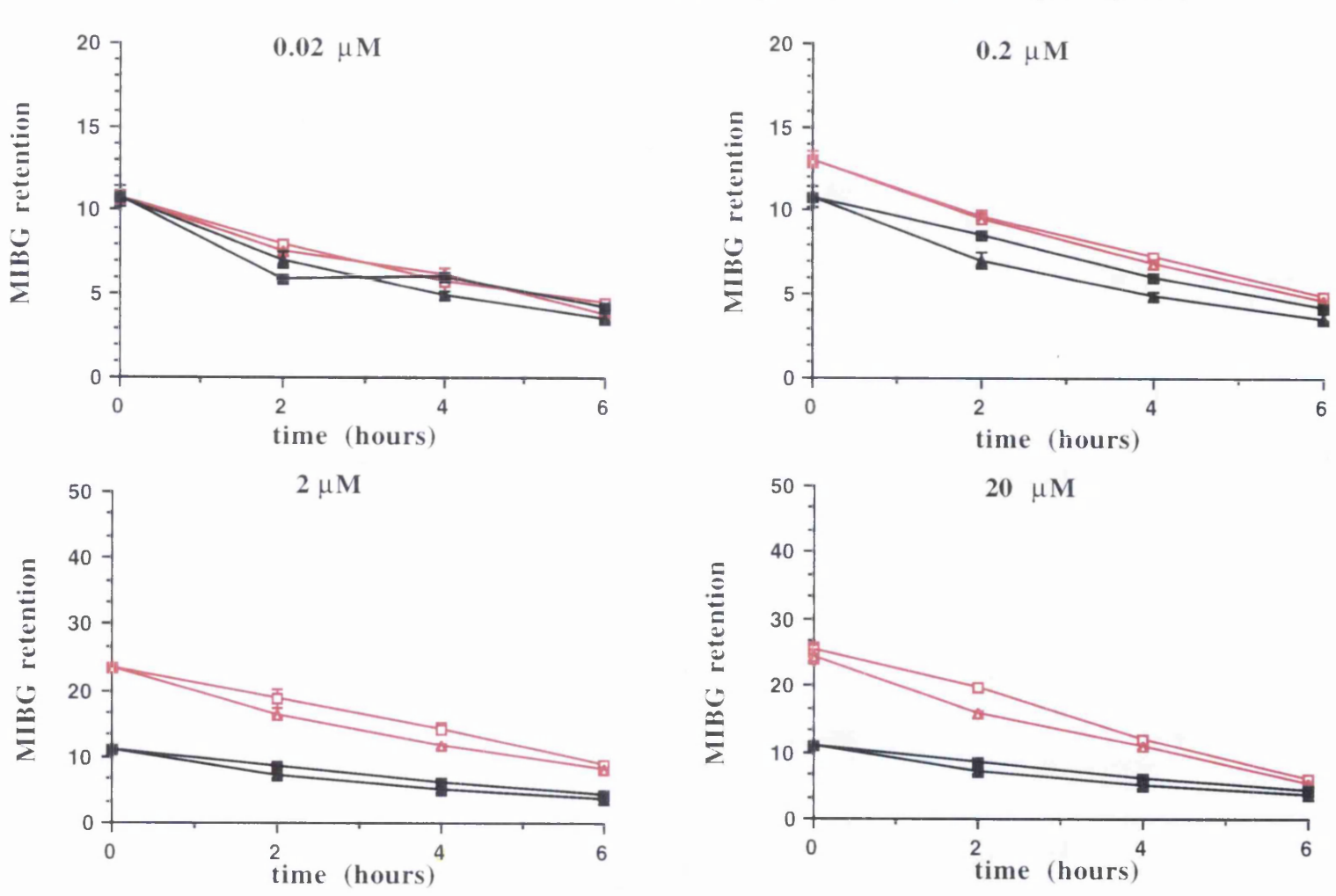
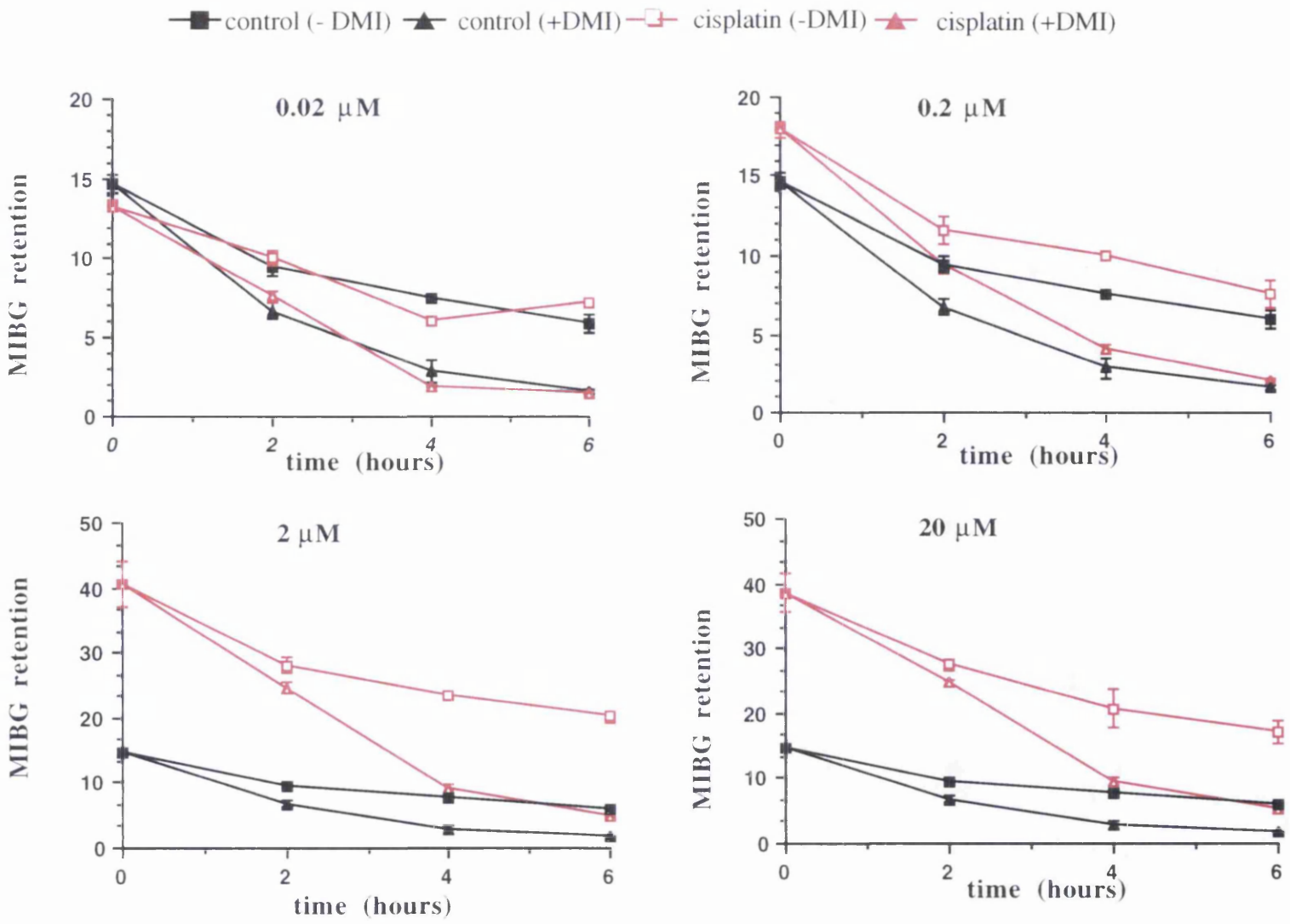


Figure 6.6 Effect of DMI on retention of MIBG in cisplatin treated SK-N-BE(2c) cells



These results show that the cisplatin-treated cells maintain levels of MIBG by continual re-uptake of released drug via the noradrenaline transporter. Cisplatin did not affect the storage capacity of the cells as no statistically significant difference in the retention of radiopharmaceutical was observed in the presence or absence of reserpine ($P > 0.1$).

6.4 Discussion

6.4.1 Effect of culture density

Preliminary experiments were performed to examine the effects of different culture densities on MIBG uptake by untreated SK-N-BE(2c) cells. This ensured that possible differences in MIBG incorporation between control and treated cells were not misinterpreted. The results indicate that MIBG uptake was adversely affected at high culture densities ($> 2.5 \times 10^5$ cells per well). It was therefore crucial that in all subsequent experiments densities were kept below this level. The reasons for this decrease are not clear, although increasing culture confluency has been shown to cause a decline in the number of Na / K ATPase pump sites in a variety of cell types, possibly as a consequence of changes in cell-cell association (Burke *et al.*, 1991).

6.4.2 Toxicity of cisplatin

Clonogenic survival of SK-N-BE(2c) cells was inhibited at concentrations of cisplatin at, or above, 0.2 μM. Cisplatin is thought to cause death by inducing apoptosis: analysis of cells dying as a result of cisplatin treatment reveal the characteristic features of programmed cell death including DNA fragmentation, loss of membrane integrity and cell shrinkage (eg Barry *et al.*, 1990; Sorenson *et al.*, 1990; Maldonado *et al.*, 1995). Supporting evidence comes from studies on cisplatin resistant cell lines which reveal that resistance is associated with a defective apoptotic process (Segal-Benirdjian *et al.*, 1995; Perego *et al.*, 1996). However the situation is complicated by the finding that the mode of death depends on the concentrations of cisplatin used: at supralethal concentrations (100 μM) rapid apoptotic cell death occurred in a murine leukaemic cell line, whereas lower concentrations (1 - 10 μM) caused G2 arrest, followed by slow non-apoptotic death (Ormerod *et al.*, 1994). Independent studies in two neuroblastoma cell lines demonstrated that cisplatin treatment (5 and 6 μM) caused a block in the G2 / M phase of the cell cycle and subsequent apoptosis (Piacentini *et al.*, 1993; Cece *et al..*, 1995).

6.4.3 Effect of cisplatin pretreatment on MIBG uptake and noradrenaline transporter expression

The data indicates that cisplatin pretreatment at therapeutically relevant concentrations causes a significant increase in uptake of MIBG. This appears to be the result of an increased capacity to actively accumulate the drug, since DMI, which specifically prevents reuptake of MIBG by inhibiting noradrenaline transporter function, caused a rapid depletion of MIBG levels in both control and treated cells. This notion is supported by the observation of a cisplatin-provoked (dose-dependent) stimulation of transcription of the noradrenaline transporter gene.

The analysis used to determine noradrenaline transporter gene expression was semi-quantitative : alterations to expression levels of the target noradrenaline transporter sequence were compared to those of a co-amplified internal reference sequence. To be valid it is critical that cycling conditions are optimised so that the amplification efficiency of both products is similar (ie are being produced during the exponential phase of the PCR reaction). In more recent approaches the accuracy and quantitation of PCR based reactions has been improved by preliminary characterisation of the PCR kinetics: Serial cDNA dilutions are made and amplified using appropriate primer sets. The amount of PCR product is then measured and plotted against the input cDNA dilution. By identifying the linear ranges of the amplification subsequent quantification can be achieved by measuring the ratio of total cDNA concentrations of target and reference(Clifford *et al*, 1996).

The reasons for the observed enhancement in noradrenaline transporter expression could be related to the mechanism of action of cisplatin. Previous studies have shown that a variety of agents including ionizing radiation, interferon-γ and phorbol esters can cause an increase in the MIBG uptake ability of neuroblastoma cells *in vitro* (Smets *et al.*, 1991; Montaldo *et al.*, 1992, 1996). In common with these agents, cisplatin causes perturbations of the DNA, which in turn causes upregulation of p53 expression. In addition,

cisplatin has been shown to induce the expression of p53-dependent genes, such as the *CIP1* gene which encodes the cell cycle inhibitor p21 (El-Deiry *et al.*, 1994). It is possible that p53 is involved in the cisplatin induced enhancement of the cellular accumulation of MIBG: the noradrenaline transporter gene may contain a p53 consensus sequence which is transcriptionally transactivated by p53 after cisplatin treatment. Confirmation of this hypothesis awaits sequence analysis of noradrenaline transporter gene regulatory elements.

Alternatively, treatment with cisplatin may induce the SK-N-BE(2c) cells to differentiate. A range of cytotoxic agents including epirubicin and tiazofurin (Rocchi *et al.*, 1987; Pillwein *et al.*, 1993), and gamma irradiation (Rocchi *et al.*, 1993), have been shown to induce biochemical, as well as morphological, evidence of differentiation of neuroblastoma cells *in vitro*. Cisplatin itself has been reported to induce neurite outgrowth at concentrations of 0.4 - 13.2 μM (Konnings *et al.*, 1994). These doses are within the range of plasma concentrations achieved during therapy (Dominici *et al.*, 1989). Induction of differentiation by a range of differentiative stimuli, including γ-interferon, tumour necrosisis factor and all-trans retinoic acid, are known to enhance MIBG uptake by increasing noradrenaline transporter expression in neuroblastoma cell lines (Montaldo *et al.*, 1992, 1996).

6.4.4 Therapeutic Implications

The predominant mechanism by which ^{131}I-labelled radiopharmaceuticals achieve cell kill is through β-particle cross-fire irradiation (see section 2.4.3 and Figure 2.2): areas of the tumour which do not take up ^{131}I- labelled drug are irradiated by adjacent targeted regions. Administration of cisplatin prior to [^{131}I]MIBG may maximise the effectiveness of radiation cross-fire. Even cells destined to die as a result of ciplatin treatment will contribute to this effect by virtue of their increased uptake of the radiopharmaceutical. Such an effect

would increase tumour cell kill by increasing the amount of β-decay particle energy deposited in tumour sites.

The combination of cisplatin and [^{131}I]MIBG has been investigated in relapsed neuroblastoma patients with progressive disease (Mastrangelo *et al.*, 1995). In a regimen involving the administration of cisplatin, followed 24 hours later by [^{131}I]MIBG, a response rate of 67% was obtained. The rationale for combining these two agents was based on the established synergism between radiation and cisplatin when the agents are given closely followed by one another (Douple *et al.*, 1985; Dewit 1987). In light of the *in vitro* findings presented here, it is possible that the impressive response rate is the result of cisplatin-induced enhancement of MIBG uptake by tumour sites.

It has been convincingly argued that the most effective use of [^{131}I]MIBG is "up-front" as the initial treatment in newly diagnosed patients (Gaze and Wheldon, 1996). The rationale for this is based on the special features which apply to targeted radiotherapy (heterogeneity of uptake and the particle emission characteristics of the targeted radionuclide - see section 2.4). The authors maintain that early administration of [^{131}I]MIBG maximises uptake and dose absorption in metabolically viable tumours of all sizes. The resultant tumour regression will leave smaller tumours containing fewer clonogenic cells and an increased growth fraction in surviving repopulating cells - conditions which are more favourable for chemotherapy. The observation that an initial dose of cisplatin can enhance MIBG accumulation would appear to run counter to these arguments, favouring administration of cytotoxic therapy first. Nevertheless, it may be that a single priming dose of cisplatin is sufficient to stimulate noradrenaline transporter expression without causing extensive cell kill and tumour regression. In a possible therapeutic approach, a single cisplatin dose could be administered at first presentation to stimulate MIBG uptake, followed by [^{131}I]MIBG to treat macroscopic tumours and subclinical metastases, and more intensive combination treatments to sterilise cells surviving earlier treatment (Armour *et al.*, 1997).

6.5 Conclusions

The discovery that pretreatment of neuroblastoma cells with cisplatin leads to enhanced uptake of MIBG has important implications for the design and scheduling of combination regimens to treat progressive neuroblastoma. Appropriate scheduling of cisplatin and [^{131}I]MIBG could be exploited to increase tumour accumulation of MIBG which should lead to increased cell kill by maximising radiation cross-fire from ^{131}I disintegration.

CHAPTER 7

THE RADIOTOXICITY OF BETA- AND AUGER ELECTRON-EMITTING CONJUGATES OF BENZYLGUANIDINE TO NEUROBLASTOMA CELLS AND SPHEROIDS

7.1 Introduction

7.1.1 Disadvantages of ^{131}I labelled MIBG

Although many patients show beneficial responses to [^{131}I]MIBG treatment, a significant number subsequently relapse from previously undetected tumour sites (Sisson *et al.*, 1989). This suggests that microtumours below the limit of clinical detectability have survived [^{131}I]MIBG therapy. A possible explanation for the relative sparing of micrometastases is related to the microdosimetry of ^{131}I targeted radiation (section 2.4.2). ^{131}I emits β particles with a mean path length of 800 μm. Therefore, in addition to targeted cells, neighbouring cells will be irradiated by crossfire (Figure 2.2). In large tumours this may be advantageous as underdosing due to heterogeneity of uptake will be offset to some extent. However, as tumour size decreases, the fraction of energy absorbed by the tumour becomes progressively smaller and more of the energy is deposited outside the target (Humm, 1986). Mathematical modelling studies predict that, for ^{131}I, the optimal diameter range for curability is 2.6 to 5.0 mm (Wheldon *et al.*, 1991; O'Donoghue *et al.*, 1995). Tumours below this size are operationally resistant to ^{131}I β emissions since the fraction of energy they absorb is greatly reduced. Experimental evidence supporting these predictions has been provided by *in vitro* investigations using large and small spheroids (Gaze *et al.*, 1992; Weber *et al.*, 1996). In addition to underdosing of small tumour deposits, long range β emissions may also damage surrounding normal tissues. A large proportion of the patients treated with [^{131}I]MIBG suffer significant haematological toxicity which may be partially due to radiation cross fire to haemopoetic cells from MIBG-targeted neuroblastoma cells infiltrating the marrow (Gelfand, 1993) (section 3.7.2).

7.1.2 Alternative radiolabels

Because of these limitations of ^{131}I, alternative radiohaloconjugates of benzylguanidine have been proposed. Short range emitters with therapeutic potential include ^{123}I, ^{125}I and ^{211}At.

^{123}I and ^{125}I decay by electron capture and internal conversion. These processes result in the emission of low-energy "Auger" electrons, which have ultra-short range (~ 10 nm) and are densely ionising. Auger electron emitters deposit highly localised energy, resulting in severe damage to molecular structures in the immediate vicinity of the decay site. This has important consequences for the treatment of micrometastases. Firstly, the efficacy of Auger electron emitters will be unaffected by the same size constraints as ^{131}I, with the result that the energy they deposit will be more fully absorbed in small tumour volumes. In addition, crossfire to adjacent non-target sites will be negligible resulting in the sparing of surrounding normal tissues. However, crossfire will not be available to offset heterogeneity of radionuclide distribution.

[^{125}I]MIBG has been shown to be toxic to neuroblastoma cells *in vitro* (Bruchelt *et al.*, 1988; Guerreau *et al.*, 1990; Senekowitsch *et al.*, 1992). In contrast, *in vivo* data from mice bearing microscopic disease demonstrated no difference in tumour survival between control and [^{125}I]MIBG treated animals (Rutgers *et al.*, 1994). Despite these conflicting laboratory findings [^{125}I]MIBG has been used to treat stage 3 and 4 neuroblastoma patients with bone marrow involvement with encouraging results (Sisson *et al.*, 1990, 1991, 1996; Hoefnagel *et al.*, 1991).

7.1.3 Aims of this study

The advent of chemical syntheses that produce n.c.a radiohalogenated benzylguanidines (Vaidyanathan *et al.*, 1993) facilitates the evaluation of the potential of short-lived radionuclides such as ^{123}I for neuroblastoma therapy. A comprehensive *in vitro* study was undertaken using neuroblastoma cell monolayers and multicellular spheroids to examine the toxicity of 3 different n.c.a preparations of benzylguanidine: [^{123}I]MIBG, [^{125}I]MIBG and [^{131}I]MIBG.

7.2 Materials and Methods

7.2.1 Cell Culture

SK-N-BE (2c) cells were cultured as described in section 5.2.3.

The MCF-7 cell line was used as a negative control. This is a human breast cell line derived from the pleural effusion of a breast carcinoma patient (Soule *et al.*, 1973). These were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum, penicillin/streptomycin (100 I.U / ml), fungizone (2 μg / ml) and glutamine (200 mM) (all Gibco, Paisley, Scotland). Cells were grown in a 5% CO_2 atmosphere at 37°C.

7.2.2 No-carrier-added synthesis of radiohaloanalogues of MIBG

Chemicals were purchased from Aldrich Chemical Company (Dorset, UK). HPLC grade solvents were obtained from Rathburn Chemicals (Peebleshire, UK). Carrier free sodium [^{131}I] iodide and sodium [^{125}I] iodide were purchased from Amersham International (Buckinghamshire, UK). Carrier free sodium [^{123}I] iodide was purchased from Cygne (Holland). n.c.a MIBG was synthesised as described in section 5.2.1. As indicated in section 5.2.1 the specific activity of these preparations cannot be calculated exactly due to the limited degree of sensitivity of the UV detector. The three preparations were therefore compared in terms of administered radioactivity per ml of each MIBG conjugate. While this does not give any information about the absorbed dose it allows a convenient means of comparing the relative toxicity of each.

7.2.3 Spheroid Studies

Spheroids were grown by the continuous stirring of 3 x 10^6 SK-N-BE(2c) cells in Techne stirrer flasks. In this study 2 sizes of spheroid, of approximately 240 μm and 400 μm diameter, were used. Small spheroids were ready after 3 - 4 days in the stirrer flask while large spheroids took 6 - 7 days to develop.

For cytotoxicity studies, aliquots of spheroids of the appropriate size were transferred to 20 ml universal containers and suspended in 1 ml of RPMI containing the appropriate activity of radiolabelled drug. After a 2 hour incubation at 37°C the medium was removed and the spheroids washed 3 times with PBS. Spheroids were then transferred to petri dishes and individually pipetted into agar coated wells containing 1ml of RPMI medium. One 24-well plate was used per treatment. These were incubated at 37°C in a 5% CO_2 atmosphere. Growth of the spheroids was monitored over the next 2 to 3 weeks by measurement of their cross sectional area, using a semi-automated image analysis system coupled via a television camera to an inverted optical microscope. From these measurements the median volume of the spheroids was calculated to allow the construction of spheroid regrowth curves.

7.2.4 Clonogenic Assays

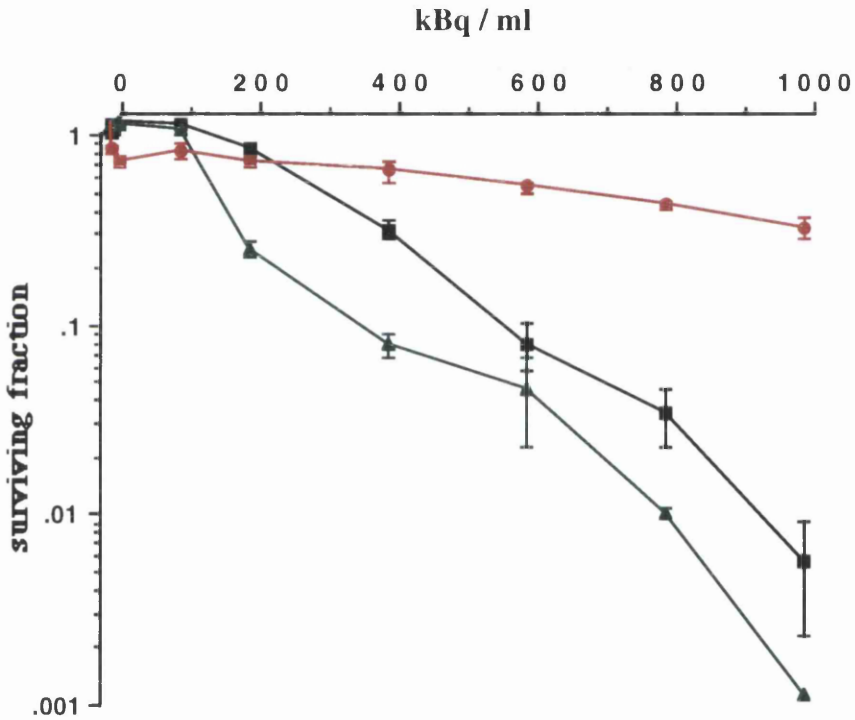
Cells were seeded in 25 cm^2 flasks at 2.5 x 10^5 cells per flask. After 2 days, medium was removed and replaced with fresh medium containing the appropriately labelled benzylguanidine at the desired radioactivity concentration. After incubation for 2 hours, medium was removed and the cells were washed 3 times with PBS. Cells were then trypsinised and counted. For each radioactivity concentration 3 flasks were seeded at 1000 cells per flask. For greater radioactive concentrations (> 400 kBq / ml), additional flasks were seeded at higher cell numbers to compensate for the potential increase in toxicity. Flasks were equilibrated with 5% CO_2 and then incubated at 37°C. After 14 days, medium was removed and the colonies were fixed and stained with Carbol Fuchsin (R A Lamb, Middlesex). Colonies of more than 50 cells were counted using an automated colony counter (Artek Systems Corporation).

7.3 Results

7.3.1. Effect of [^{123}I]-, [^{125}I]- and [^{131}I]-MIBG on SK-N-BE(2c) neuroblastoma monolayers.

Clonogenic assays of neuroblastoma cell monolayers demonstrated that the two Auger emitting conjugates were more toxic to neuroblastoma cells than ^{131}I labelled drug (Figure 7.1).

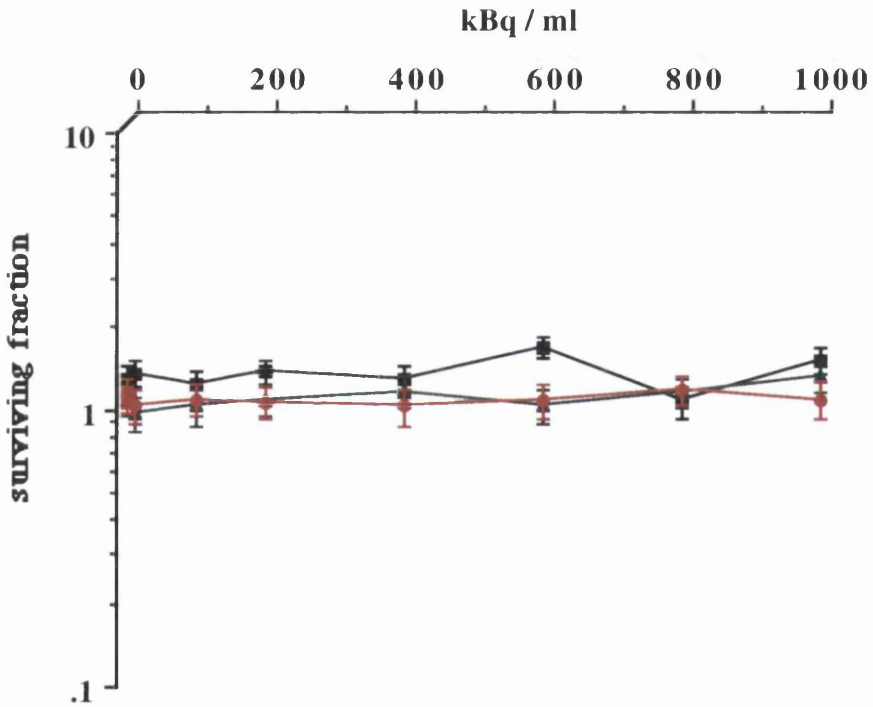
Figure 7.1 Clonogenic survival of SK-N-BE(2c) monolayers after [^{123}I]-, [^{125}I]- and [^{131}I]MIBG treatment.



Means +/- standard deviations of 3 observations.
●: [^{131}I]MIBG, ■: [^{123}I]MIBG, ▲: [^{125}I]MIBG

Even at radioactive concentrations of 1000 kBq / ml the surviving fraction for ^{131}I labelled material was only 0.4. In contrast, using [^{123}I]MIBG, a 1 log cell kill was achieved at a concentration of 600 kBq / ml. [^{125}I]MIBG was even more potent than [^{123}I]MIBG :1 log cell kill was apparent after exposure to approximately 300 kBq / ml of drug. To control for the effects of non-specific irradiation by the isotopes used, monolayers were incubated with identical activities of unconjugated radioactive sodium iodides for 2 hours. As Figure 7.2 shows, over this incubation period none of these was toxic.

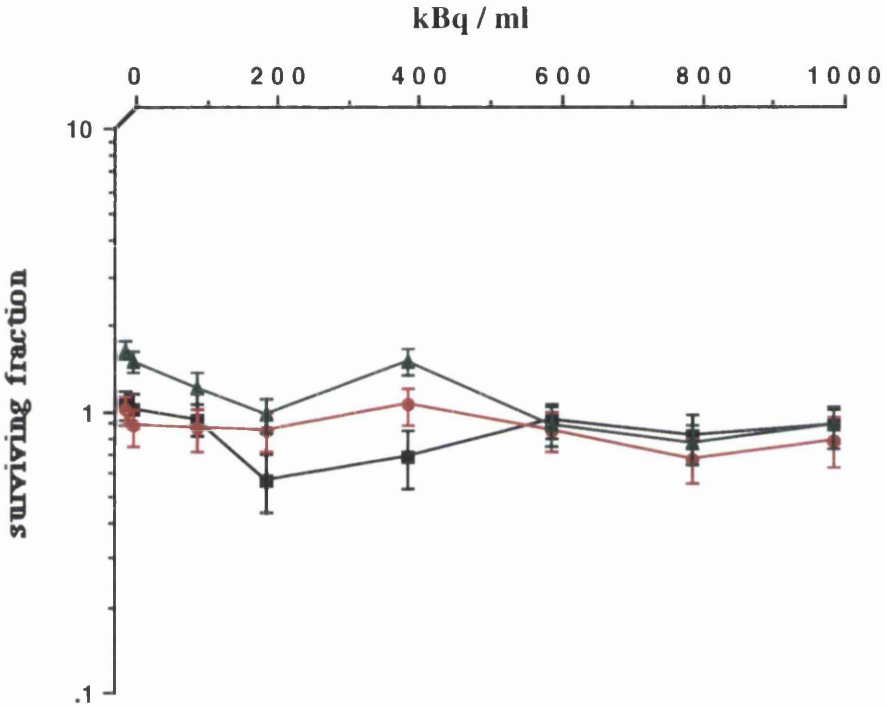
Figure 7.2 The effect of non-specific irradiation on clonogenic survival of SK-N-BE(2c) monolayers.



Means +/- standard deviations of 3 observations..
●: [^{131}I]NaI, ■: [^{123}I]NaI, ▲: [^{125}I]NaI

In addition, to demonstrate that the observed toxicity was the result of specific uptake of the labelled drug, the cell line MCF-7 was used as a negative control. This cell line does not express the noradrenaline transporter and therefore has no capacity for active uptake of MIBG (Mairs *et al.*, 1994). Again, none of the species tested caused inhibition of colony formation (Figure 7.3).

Figure 7.3 Clonogenic survival of MCF-7 monolayers after [^{123}I]-, [^{125}I]- and [^{131}I]MIBG treatment



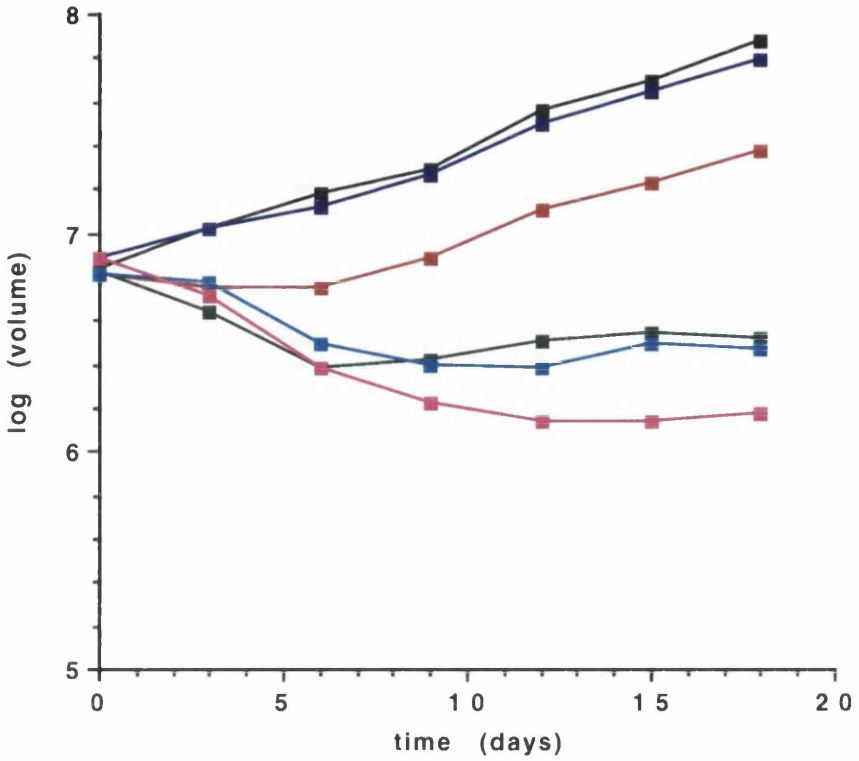
Means +/- standard deviations of 3 observations.
●: [^{131}I]MIBG, ■: [^{123}I]MIBG, ▲: [^{125}I]MIBG

It is concluded that the observed toxicity to neuroblastoma cells was due to the specific incoporation of labelled MIBG.

7.3.2 Effect of [^{123}I]-, [^{125}I]- and [^{131}I]-MIBG on small (240 μm) neuroblastoma spheroids

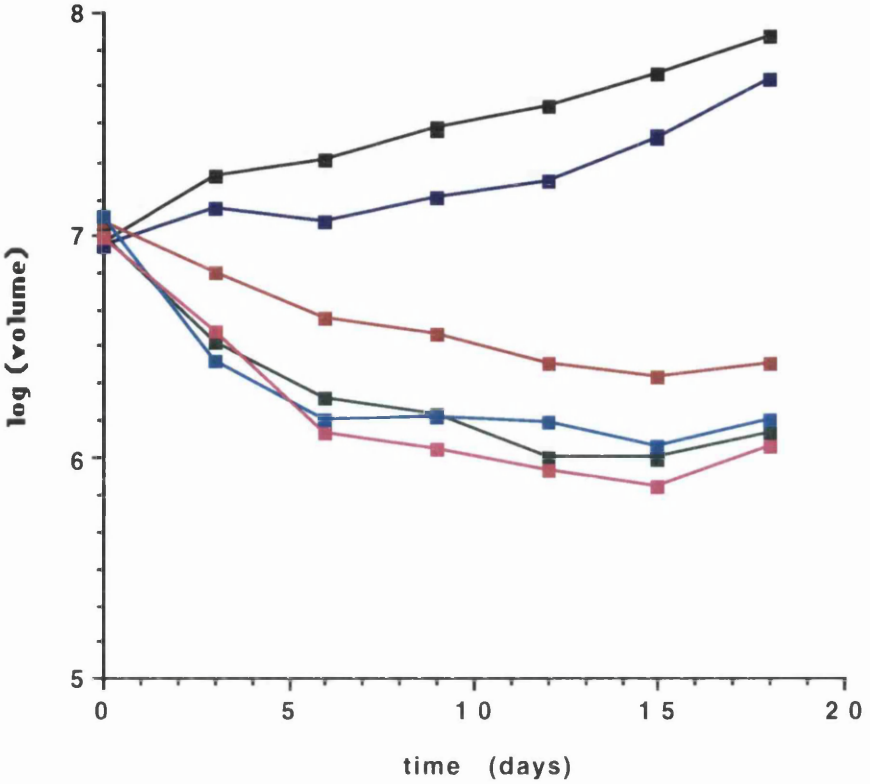
The relative effectiveness of the radiolabelled MIBG analogues was also determined using small (240 μm diameter) spheroids. A result similar to that obtained with cell monolayers was observed. Both [^{123}I]MIBG and [^{125}I]MIBG inhibited spheroid growth in a dose dependent manner (Figure 7.4A + 7.4B).

Figure 7.4A Growth curves of 240 μm diameter SK-N-BE(2c) spheroids after treament with [^{123}I]MIBG



The ordinate is the common logarithim of the spheroid volume in units of $(\mu m)^3$ calculated from measured cross-sectional area. Each point represents the median log volume of 24 measurements. ■: control, ■: 0.1MBq / ml.■: 1MBq / ml, ■: 2MBq/ ml, ■: 3MBq / ml, ■: 4MBq / ml.

Figure 7.4B Growth curves of 240μm diameter SK-N-BE(2c) spheroids after treatment with [^{125}I]MIBG

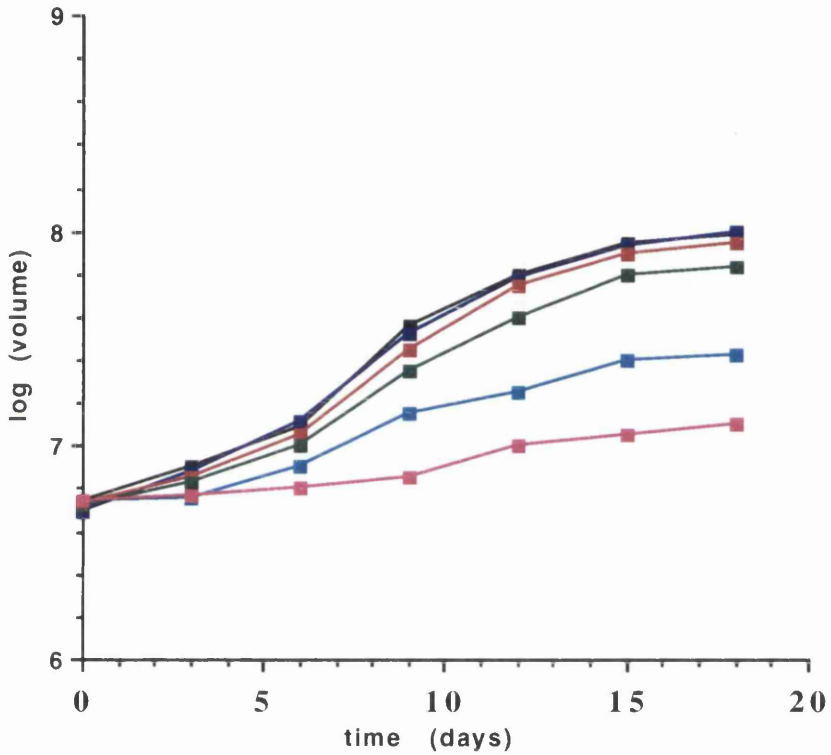


The ordinate is the common logarithim of the spheroid volume in units of $(\mu m)^3$ calculated from measured cross-sectional area. Each point represents the median log volume of 24 measurements. ■: control, ■: 0.1MBq / ml.■: 1MBq / ml, ■: 2MBq/ ml, ■: 3MBq / ml, ■: 4MBq / ml.

[^{125}I]MIBG was the more effective radiopharmaceutical when compared on a radioactivity per ml basis: growth inhibition, defined as a failure to regrow after 20 days, was apparent at 0.1 MBq / ml of [^{125}I]MIBG whereas 1MBq / ml of [$^{1\,2\,3}$I]MIBG was required to achieve a similar effect.

[^{131}I]MIBG was again the least effective of the iodinated conjugates: significant growth delay was only apparent at a concentration of 3 MBq / ml (Figure 7.4C).

Figure 7.4C Growth curves of 240μm diameter SK-N-BE(2c) spheroids after treatment with [^{131}I]MIBG

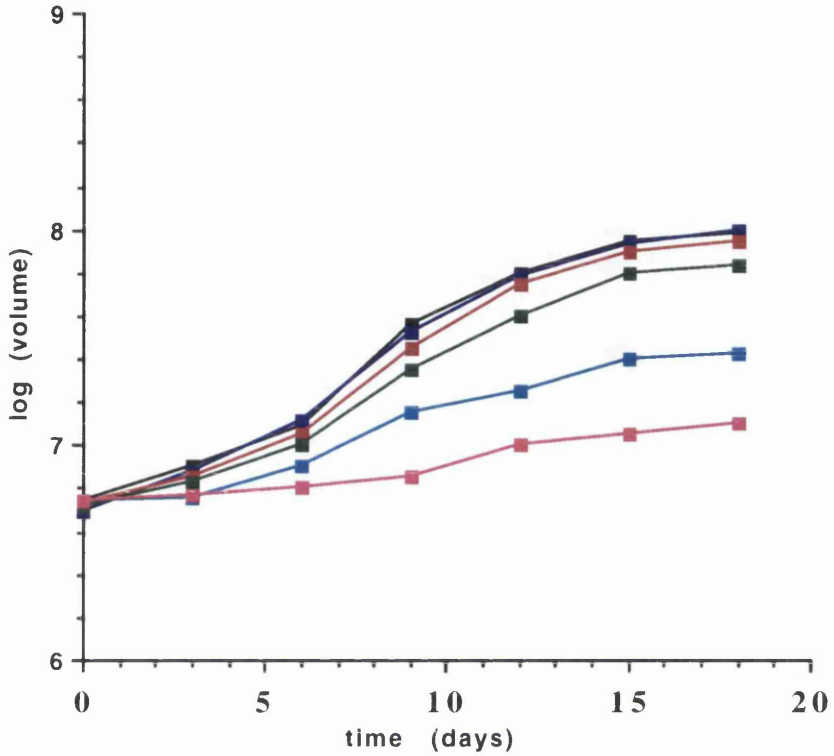


The ordinate is the common logarithim of the spheroid volume in units of $(\mu m)^3$ calculated from measured cross-sectional area. Each point represents the median log volume of 24 measurements. ■: control, ■: 0.1MBq / ml.■: 1MBq / ml, ■: 2MBq/ ml, ■: 3MBq / ml, ■: 4MBq / ml.

7.3.3 Effect of [^{123}I]-, [^{125}I]- and [^{131}I]-MIBG on large (400μm) neuroblastoma spheroids

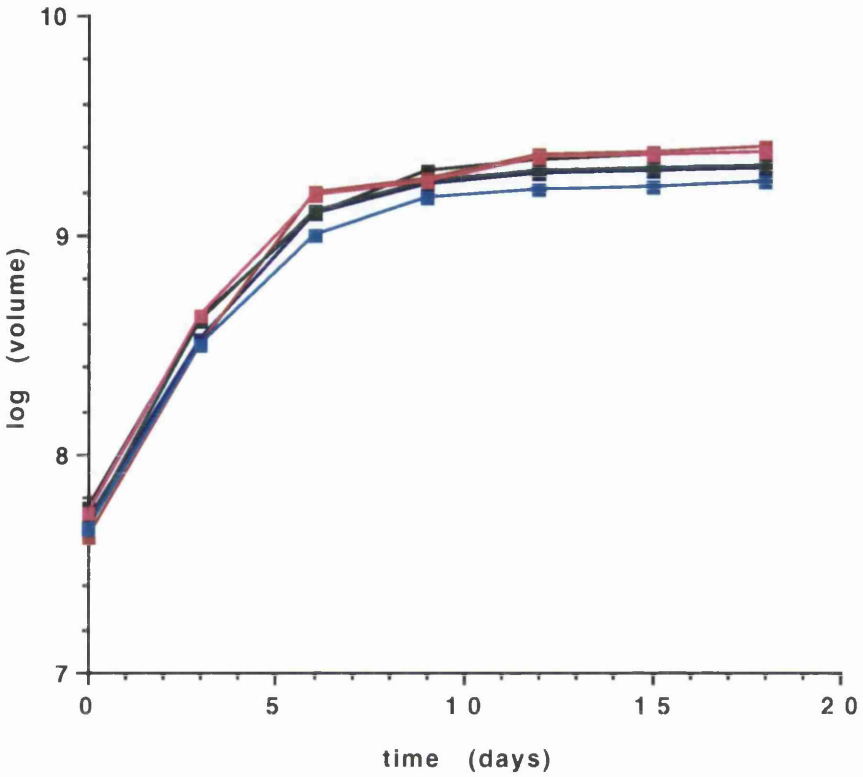
In contrast to the toxic effects of [^{123}I]MIBG and [^{125}I]MIBG on monolayer and small spheroids, ^{123}I and ^{125}I labelled conjugates were completely ineffective in the treatment of large (400μm) spheroids. At the radioactive concentrations tested, no inhibition of growth was observed and all spheroids regrew at rates comparable to controls (Figures 7.5A + 7.5B).

Figure 7.5A Growth curves of 400μm diameter SK-N-BE(2c) spheroids after treatment with [^{123}I]MIBG



The ordinate is the common logarithim of the spheroid volume in units of $(\mu m)^3$ calculated from measured cross-sectional area. Each point represents the median log volume of 24 measurements. ■: control, ■: 0.1MBq / ml.■: 1MBq / ml, ■: 2MBq/ ml, ■: 3MBq / ml, ■: 4MBq / ml.

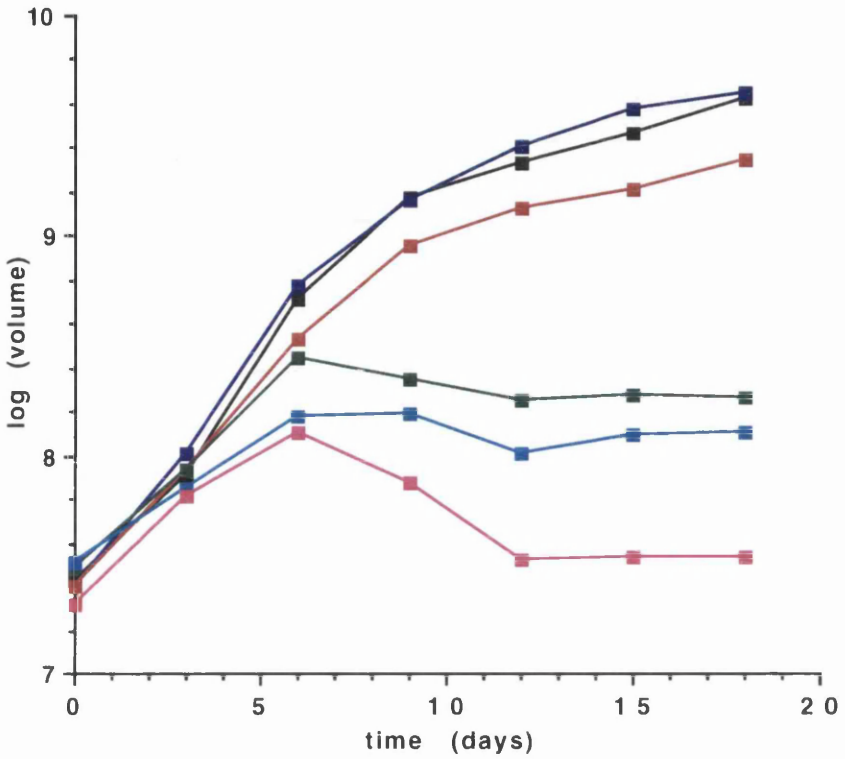
Figure 7.5B Growth curves of 400μm diameter SK-N-BE(2c) spheroids after treatment with [^{125}I]MIBG



The ordinate is the common logarithim of the spheroid volume in units of $(\mu m)^3$ calculated from measured cross-sectional area. Each point represents the median log volume of 24 measurements. ■: control, ■: 0.1MBq / ml.■: 1MBq / ml, ■: 2MBq/ ml, ■: 3MBq / ml, ■: 4MBq / ml.

Conversely [^{131}I]MIBG induced growth inhibition at concentrations greater than 1MBq / ml (Figure 7.5C).

Figure 7.5C Growth curves of 400μm diameter SK-N-BE(2c) spheroids after treatment with [^{131}I]MIBG



The ordinate is the common logarithim of the spheroid volume in units of $(\mu m)^3$ calculated from measured cross-sectional area. Each point represents the median log volume of 24 measurements. ■: control, ■: 0.1MBq / ml.■: 1MBq / ml, ■: 2MBq/ ml, ■: 3MBq / ml, ■: 4MBq / ml.

To control for the effects of non-specific irradiation, spheroids of both sizes were incubated with 4 MBq / ml of the appropriate radioactive sodium iodide. No growth inhibition was observed in any of the spheroids (data not shown).

7.4 Discussion

7.4.1 Relationship between toxicity and target size

These studies have compared the cytotoxic efficacies of beta and Auger electron emitting radioconjugates of benzylguanidine in treating SK-N-BE(2c) cells grown in monolayer and spheroid culture. The results clearly demonstrate a relationship between the physical characteristics of radionuclides and their therapeutic effectiveness in experimental *in vitro* therapy (Table 7.1).

Table 7.1 Relative toxicity of alternative radionuclides conjugated to benzylguanidine

Radiolabel	Radiotoxicity[a]		
	monolayer	small spheroid	large spheroid
^{131}I	+	+	+++
^{123}I / ^{125}I	+++	+++	NE

[a] Number of + signs allow comparison of rank order of inhibitory potency within columns or rows. They do not indicate proportional effectiveness. NE indicates no effect on growth at the concentrations of radioactivity used in this study.

The Auger electron emitting conjugates of MIBG ([^{123}I]MIBG and [^{125}I]MIBG) were capable of killing single cells and small spheroids. However, their toxicity was reduced in larger target volumes. Incomplete drug penetration and lack of crossfire probably account for the absence of growth inhibition in the 400 μm spheroids after exposure to [^{123}I]MIBG or [^{125}I]MIBG. Incomplete penetration of MIBG in neuroblastoma spheroids whose diameter exceeds 400 μm has previously been documented (Mairs *et al.*, 1991). Therefore [^{123}I]MIBG and [^{125}I]MIBG may have been toxic only to the outer, metabolically most active, cell layers. Adjacent untargeted cells would have continued to proliferate since they experienced no crossfire from their targeted neighbours. Consequently, growth would be unaffected.

For the long range β-emitting conjugate ([^{131}I]MIBG) the size dependence of toxicity was opposite to that of [^{123}I]MIBG and [^{125}I]MIBG: i.e. small spheroids were less vulnerable to [^{131}I]MIBG than large ones. This may be due to the dissipation of more β decay energy outside small target volumes as predicted from microdosimetric considerations (Wheldon *et al.*, 1991; O'Donoghue *et al.*, 1995) and in agreement with previous experimental findings (Gaze *et al.*, 1992). This explanation is supported by the results of the data obtained from monolayer studies which indicated that, of the radioiodine isotopes examined, ^{131}I was the least effective inhibitor of colony formation. Due to the planar geometry of cellular monolayers, most of the decay energy would have been deposited above and below the plane of the cells.

These findings are in agreement with the results of a recent study by Weber *et al* (1996), which found [^{125}I]MIBG to be superior to [^{131}I]MIBG in treating small spheroids, but that as spheroid size increased this advantage was lost and [^{131}I]MIBG became the more effective radiopharmaceutical.

7.4.2 Comparative toxicity of radioiodine conjugates

To allow a quantitative comparison of the relative effectiveness of each isotope the administered activity in kBq / ml and in atoms / ml was calculated for each analogue of MIBG (Tables 7.2 + 7.3).

Table 7.2 Comparison of the toxicites of radionuclide conjugates of MIBG in terms of administered radioactivity in kBq / ml

endpoint	activity (kBq/ml) required to achieve endpoint		
	^{123}I	^{125}I	^{131}I
clonogenic survival[a]	480	300	----[b]
spheroid regression[c]	1414	320	3460

[a] surviving fraction of 0.1.

[b] no value is included since ^{131}I failed to achieve the stipulated degree of cell kill.

[c] no spheroid regrowth by 20 days. Only data from the analysis of 240 μm diameter was included.

As Table 7.2 indicates, on a kBq / ml basis, the rank order of toxicity was ^{125}I > ^{123}I > ^{131}I. On conversion of these activities to the number of atoms present per ml, however, ^{123}I rather than ^{125}I was the most effective iodine species (Table 7.3).

Table 7.3 Comparison of the toxicities of radionuclide conjugates of MIBG in terms of number of atoms / ml

endpoint	No of atoms x 10^{10} required to achieve endpoint[a]		
	^{123}I	^{125}I	^{131}I
clonogenic survival	3.5	230	----
spheroid regression	9.68	241	346

[a]The activities presented in Table 7.3 were converted to number of atoms present per ml using the formula: radioactivity (Bq) = λN, where λ = the decay constant and N = number of atoms. Decay constants for each isotope were calculated from $t_{1/2} = \ln 2 / \lambda$ where $t_{1/2}$ is the half life of the isotope.

These calculations highlight the potential of ^{123}I over ^{125}I for targeted radiotherapy. Although it is less efficient in the production of DNA double strand breaks (0.74 compared to 1 per cell per decay for ^{125}I (Makrigiorgos *et al.*, 1992), the number of atoms of ^{123}I present in a given activity is approximately 100 fold fewer. Therefore, significantly lower molar amounts of [^{123}I]MIBG would be required to deliver a dose of targeted radiation. It is predicted that the administration of low concentrations of MIBG should decrease passive uptake relative to active drug uptake, thereby enhancing the therapeutic ratio (Mairs *et al.*, 1995). Furthermore, the number of radioactive atoms administered should be as low as possible to minimise the dose to normal organs which are capable of uptake with long term retention (eg thyroid).

7.4.3 Potential mechanisms by which Auger electron emitter conjugates of benzylguanidine mediate cell death

The observation that Auger electron emitters conjugated to benzylguanidine are capable of killing SK-N-BE(2c) neuroblastoma cells raises important questions about the subcellular location of MIBG and the mechanism of cell death. Several hypotheses could explain these results. MIBG may localise in the nucleus of neuroblastoma cells. Alternatively, particles other than Auger electrons emitted during the decay of ^{123}I and ^{125}I may have sufficient range to penetrate into the nucleus of a targeted cell. A third possibility is that MIBG labelled with Auger electron emitters mediates cell kill through apoptosis triggered by a novel mechanism.

The radiobiological expectation is that the critical cellular target for ionising radiation damage is nuclear DNA. Therefore ultra short range radionuclides, such as Auger electron emitters, should be toxic only if delivered to the nucleus of the target cell (Charltan, 1986). This has been confirmed by *in vitro* studies - using extracellular $Na^{125}I$, cytoplasmic [^{125}I]iododihydrorhodamine and nuclear $^{125}IUdR$ - which demonstrated that significant toxicity was associated only with the nuclear located ^{125}I (Kassis *et al.*, 1987). While subcellular localisation studies have demonstrated that MIBG concentrates mainly in the cytoplasm of neuroblastoma cells (Gaze *et al.*, 1991; Clerc *et al.*, 1993), the fixation procedures employed in these studies may have caused a redistribution of the drug (Smets *et al.*, 1991). The demonstration that significant cell kill can be achieved with ^{125}I or ^{123}I labelled MIBG could represent evidence for a nuclear localisation of MIBG. The amount of drug accumulated at this site may be undetectable by conventional means but nevertheless capable of delivering a toxic dose of radiation to the cell nucleus.

Alternatively, some particles emitted during the decay of ^{123}I and ^{125}I may have sufficient range to reach genomic DNA of a targeted cell despite cytoplasmic or perinuclear localisation. Although the entire Auger and Coster Kronig electron spectra for these radionuclides have not been measured

experimentally, calculations using theoretical transition rates and energies indicate that both isotopes emit some electrons with ranges of the order of the radius of a mammalian cell (Sastry, 1992; Howell, 1992). Assuming a cytoplasmic location for MIBG, it is possible that such emissions would deliver significant doses of radiation to the nucleus. However, this possibility is not supported by the classical experiments of Kassis and colleagues described earlier (Kassis *et al.*, 1987).

A third explanation for the toxicity of Auger electron emitter conjugates of MIBG assumes no nuclear sequestration of the radiopharmaceutical. This challenges the conventional model of radiation-induced cell kill which recognises the cell nucleus as the initial target for the lethal effects of ionising radiation. After radiation exposure cells may undergo cell death in one of two ways. Firstly, cells may experience mitotic death, whereby cells die during cell division. This does not necessarily occur at the first post irradiation mitosis. The cell may struggle through one or two mitoses before the damaged chromosomes cause it to die in attempting to complete cell division. Alternatively, they may undergo programmed cell death (apoptosis). In contrast to mitotic cell death, radiation induced apoptosis generally occurs rapidly and is thought to be a regulated process.

The trigger for the cells to undergo apoptosis after irradiation remains unclear. The initiating signal may be nuclear derived - possibly the double strand break itself. More recent data tentatively suggests that it could also be activated by signals arising at the cell membrane. Experimental evidence to support this comes from studies of the sphingomyelin pathway. This signal transduction pathway mediates the effects of several cytokines including TNF-α. In studies on leukaemic cell lines, it has been shown that binding of TNF-α to its receptor promotes the hydrolysis of sphingomyelin, a membrane phospholipid, to ceramide which acts as a 2nd messenger to trigger a series of events culminating in apoptosis (Obeid *et al.*, 1993; Jarvis *et al.*, 1994). Further work has demonstrated that ionising radiation, like TNF-α, can stimulate this pathway, creating elevated ceramide levels and subsequent apoptosis in bovine

aortic endothelial cells. Irradiation of cell membrane preparations, devoid of nuclei, was also capable of stimulating sphingomyelin hydrolysis suggesting that a direct effect of ionising radiation on the membrane is sufficient to produce ceramide and thereby transduce the apoptotic signals. No nuclear communication is required (Haimovitz-Friedman *et al.*, 1994). Several other studies support the view that alterations at the cell membrane may trigger apoptosis (Langely *et al.,* 1993; Ramafrishnan *et al.*, 1993).

If MIBG is genuinely non-nuclear in location then the toxic effects observed with Auger labelled material could be the result of membrane-signal induced apoptosis. The critical target within the membrane remains unknown. Such a mechanism would occur only in apoptosis-competent cells and could be absent from many long-established cell lines. This would explain the results of classical experiments which demonstrate an obligatory nuclear localisation for cell kill by Auger emitters (Bloomer *et al.*, 1981; Kassis *et al.,* 1987).

7.4.4 Implications for targeted radiotherapy with MIBG

Whatever the detailed mechanisms, the demonstration that short range radionuclides conjugated to MIBG are toxic to neuroblastoma cells could have important consequences for the treatment of neuroblastoma patients with disseminated disease. The implication of these studies is that 'combination cocktails' of radiolabelled MIBG conjugates would be the most effective strategy for treating a range of sizes of tumours. The concept of combining two radionuclides, one short and one long range emitter, has been discussed by O'Donoghue *et al* (1995). By choosing the appropriate proportions of activity, it is postulated that the likelihood of tumour cure is kept at a more constant rate throughout the size range, which in principle should extend the range of optimal curability. Of the two Auger electron emitters investigated here, ^{123}I would appear to be the more suitable short range emitter. As a consequence of its short half-life, it is believed to be about 40 times more efficient at producing DNA damage over 8 hours than ^{125}I (O'Donoghue, 1996). Since the biological half life of MIBG is around 37 hours (Ehninger *et al.*, 1987) an ^{123}I

labelled conjugate should inflict much greater damage than its long lived ^{125}I counterpart .

Short range MIBG conjugates could also be useful for the *ex vivo* purging of autologous bone marrow. Many older patients undergo supralethal chemotherapy to eradicate the residual neuroblastoma cells that have escaped conventional treatments. They are then rescued by autologous bone marrow infusion, using marrow harvested while the patient was in remission (Pole *et al..*, 1991; Kremens *et al.*, 1994). With this treatment, a significant number of patients still relapse, suggesting the presence of contaminating neuroblastoma cells in the reinfused marrow (Rill *et al.*, 1992). Auger electron emitter labelled MIBG could be incubated with marrow aspirates to sterilise contaminating neuroblastoma cells, with minimal damage to haemopoetic precursors, prior to reinfusion.

7.5 Conclusion

The experiments reported demonstrate the theoretically expected relationships between radiotoxicity, radionuclide emission characteristics and the geometrical configuration of the target cell kill population, while posing some questions about mechanisms of cell kill by Auger electron emitters. These results suggest that the combined use of short range and long range particle emitting radionuclide conjugates of benzylguanidine could enhance the therapeutic efficacy of targeted radiotherapy in neuroblastoma patients with disseminated disease.

CHAPTER 8

DEVELOPMENT OF A MURINE MODEL OF MICROMETASTATIC NEUROBLASTOMA

8.1 Introduction

8.1.1 Existing *in vivo* models of neuroblastoma

In vitro research provides a controlled environment in which to test new treatments and predict the limitations of novel approaches to therapy. Extrapolation of such results to the treatment of patients is however problematical. It is therefore inevitable that some *in vivo* work is carried out to validate the results of laboratory studies. *In vivo* models must provide a realistic reflection of the disease under investigation.

As discussed in section 3.6.1, several *in vivo* models of human neuroblastoma have been established using a variety of injection sites and with a number of human neuroblastoma cell lines. These approaches resulted in the production of subcutaneous tumours which could be simply maintained by serial passage (Senekowitsch *et al.*, 1989; Rutgers *et al.*, 1991; Gaze *et al.*, 1994). Histological examination of tumour sections revealed that they retained the morphological, biological and biochemical characteristics of the original neuroblastoma tumour. While they have provided useful information about the uptake and biodistribution of MIBG, attempts to perform meaningful therapy experiments have been less successful. An unfavourable characteristic of these *in vivo* models is the failure of the implanted tumours to metastasise. These deficiencies question the reliability and authenticity of such models.

8.1.2 Aims of this study

Widespread dissemination is a characteristic and lethal feature of late stage neuroblastoma which needs to be attacked with new therapy regimens. While various combined approaches are currently under clinical evaluation (see section 3.7.2), they are based largely on the results of *in vitro* findings and theoretical modelling studies. It would be helpful if a more realistic *in vivo* model of metastatic disease was available by which to evaluate novel therapeutic approaches. To address this problem, experiments were undertaken

to develop a reliable and reproducible murine model of micrometastatic neuroblastoma. Nude mice were intrasplenically injected (ISI) with human neuroblastoma cells and then examined for the presence of disease using a PCR based assay. Having developed a reproducible model the therapeutic efficacy of n.c.a [^{131}I]MIBG could be accurately compared with conventional exchange prepared [^{131}I]MIBG. In addition the potential role of alternative short range conjugates of MIBG to treat micrometastatic neuroblastoma could be assessed *in vivo*.

8.2 Materials and Methods

8.2.1 Cell culture

SK-N-BE(2c) cells were cultured as described in section 5.2.3. Clone M3 cells, which were used to obtain murine RNA for control PCR reactions, were established from a cloudman S91 melanoma in a male mouse (Yasumura *et al.*, 1966). These were grown in Hams F10 medium supplemented with 15% fetal calf serum and penicillin / streptomycin (100 IU / ml). All media and supplements were obtained from Gibco (Paisley, UK). Flasks were subcultured every 5-7 days when the monolayers became confluent.

8.2.2 Intrasplenic injection of SK-N-BE(2c) neuroblastoma cells

Initial studies used adult nude mice of the strain MF-1. For subsequent studies infant BALB/c nude mice aged 4 weeks were used. All mice were obtained from Harlan Olack Ltd, Oxfordshire.

Intrasplenic injection of SK-N-BE(2c) cells was carried out as described in section 5.2.5.1. At fixed timepoints after inoculation mice were sacrificed by CO_2 inhalation, dissected and examined for macroscopic evidence of metastatic disease. Tissue samples of liver, spleen, heart, lung, kidney and adrenals were then removed and frozen for subsequent RNA extraction. To obtain bone marrow samples, femurs were excised and the marrow cells flushed through with 1-2mls of PBS.

8.2.3 Reverse transcription polymerase chain reaction reaction (RT-PCR)

Tissues were thawed and minced finely with scissors. RNA extraction was then performed using a PUREscript RNA isolation kit (Gentra). RNA concentration of the samples was determined spectrophotometrically at 260 nm. 1μg of RNA was converted to cDNA using the Clontech 1st-strand cDNA synthesis kit (Cambridge Bioscience). Several primer sets were used all

of which were obtained from Oswell (Southampton, UK). For the human noradrenaline transporter sequence the sense primer was 5'-CTGGTGGTGAAGGAGCGCAACGGC- 3' and the antisense primer was 5' -ATGTCATGAATCCCGCTGCTCTCG -3' (Montaldo *et al.*, 1992). Amplification with these primers generated a 590 bp product. For the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence the sense primer was 5'- TCGGAGTCAACGGATTTGG-3' and the antisense primer was 5'-GCATTGCTGATGATCTTGAGGC-3'. This amplification generated a PCR product of 390bp. For the murine GAPDH sequence the sense primer was 5' - CCATGGAGAAGGCTGGGG - 3' and the antisense primer 5'-CAAAGTTGTCATGGATGACC - 3'. This amplification generated a product of approximately 200bp. Amplification of human and murine sequences was carried out in 10x synthesis buffer containing 100 mM Tris-HCL, 1.5 mM $MgCl_2$, 500 mM KCl, pH 8.3 with 10 nmoles of dNTP (Gibco, Paisley), 20 pmoles of each target primer, 20 pmoles of each GAPDH primer and 2 units of Taq polymerase (Boehringer Mannheim, Sussex). Cycling conditions consisted of a 1min denaturation at 94°C, 1min annealing at 65°C and 1 min extension at 72°C for 35 cycles. The PCR products were separated by electrophoresis through 1.6 % (w / v) agarose (Flowgen, UK, Kent).

8.3 Results

8.3.1 ISI of SK-N-BE(2c) neuroblastoma cells in athymic adult MF-1 mice

Initial experiments were carried out using the standard strain of nude mice used in previous *in vivo* work (section.5.2.5). Groups of 12 mice were inoculated with a range of neuroblastoma cell numbers (10^4, 10^5 and 10^6), sacrificed at 10 weeks after injection and examined for macroscopic evidence of disease on the spleen and liver (Table 8.1).

Table 8.1 Development of tumours in adult MF-1 nude mice after ISI

Tumour cell innoculum	No. mice with splenic tumours	No. mice with macroscopic evidence of metastases
10^4 cells	7 / 12	0 / 12
10^5 cells	7 / 12	0 / 12
10^6 cells	8 / 12	0 / 12

With tumour cell innocula of 10^4 and 10^5 cells, 7 out of the 12 mice (58%) developed tumours on the spleen. With the larger innoculum of 10^6 cells, 8 of the 12 animals (67%) had splenic tumours. Examination of the livers of these animals revealed no visible evidence of metastatic disease.

8.3.2 ISI of SK-N-BE(2c) neuroblastoma cells in infant BALB c mice

Several investigators have postulated that the ability of human tumour cells to metastasise may be affected by the natural killer cell status of the athymic mouse host. As the age and possibly the strain of the athymic mice may influence host natural killer cell activity, a second series of intrasplenic injections were carried out in immature (3-4 weeks) nude mice of the BALB c strain. For these studies a fixed high innoculum of 3 x 10^6 cells was injected and the animals were sacrificed at specific time intervals after injection: either 28 days (group I) or 42 days (group II). Animals were examined at autopsy and scored for the presence of disease on the spleen and liver (Tables 8.2 and 8.3).

Table 8.2 Tumour development in infant BALB c mice after ISI: sacrifice at 28 days

Animal No.	Presence of splenic tumour	Presence of liver metastases
1	+	+
2 (21)*	+	+
3 (23)*	+	+
4 (25)*	+	-
5	+	-
6	-	-
7	+	+
8	-	-
9	+	+
10	-	-
11	+	+
12 (26)*	+	-
	total 9 / 12	total 6 / 12

* numbers in brackets indicate time of death if less than 28 days.

Table 8.3 Tumour development in infant BALB c mice after ISI: sacrifice at 42 days

Animal No.	Presence of splenic tumour	Presence of liver metastases
1 (26)*	+	-
2 (27)*	+	+
3	+	+
4	+	-
5	+	+
6 (32)*	+	+
7	-	-
8 (33)*	+	+
9	-	-
10	+	+
11	+	-
12	+	-
	total 10 / 12	total 6 / 12

* numbers in brackets indicate time of death if less than 42 days.

Infant BALB c nude mice were clearly more susceptible to tumour growth: in group I, 9 / 12 (75%) of the animals had splenic tumours. In group II the figure rose to 10 / 12 (83%). In addition there was macroscopic evidence of metastases: in each group 50% of the animals had visible liver tumour foci.

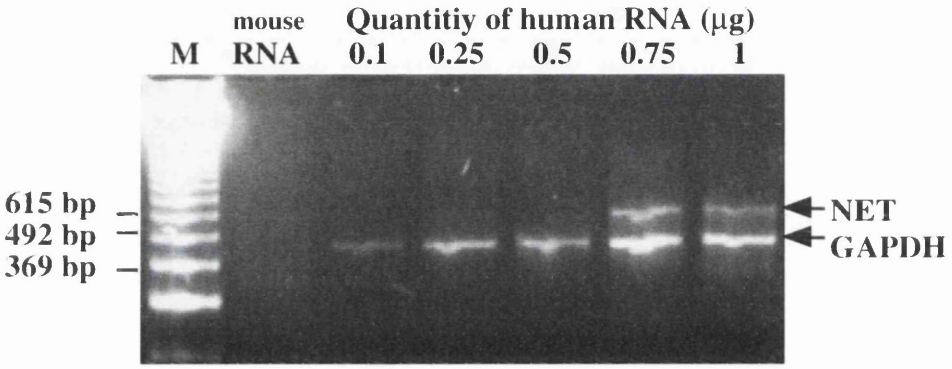
8.3.3 RT-PCR analysis

The use of younger athymic mice of the BALB c strain produced encouraging results, in that half of the animals produced visible signs of disease. To determine whether the animals who appeared disease-free on visual examination might be harbouring microscopic tumours, a more sensitive method was developed to detect metastatic foci of human tumour cells. To assess metastatic tumour burden and response to therapy the assay should be specific and quantifiable. Therefore a modification of the RT-PCR system described in chapter 6 was developed.

8.3.3.1 Species specificity

Preliminary experiments were carried out to confirm that the primers being used were specific for human sequences. RNA from human SK-N-BE(2c) and murine Clone M3 melanoma cells was reverse transcribed and then PCR amplified using primers for the human noradrenaline transporter (NET) and human GAPDH genes (Figure 8.1).

Figure 8.1 Specificity and sensitivity of human noradrenaline transporter and GAPDH primers



RT-PCR reactions were carried out with 0.1 - 1 µg of human RNA or 1 µg of mouse RNA. M = 123 bp markers.

The absence of signal when murine clone M3 RNA was used as the template confirmed that both primer sets were specific for human sequences. The sensitivity of detection was 0.5 µg using the noradrenaline transporter primers and 0.1 µg with the GAPDH primers. Since the limit of detection was 5-fold higher with the GAPDH primers, these were chosen for subsequent studies.

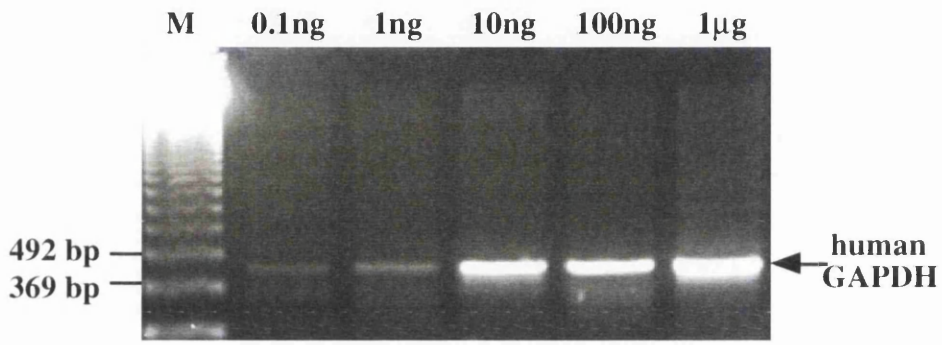
8.3.3.2 Sensitivity of RT-PCR

The ultimate application of this assay would be to detect small amounts of human RNA in a complex background of murine RNA. RT-PCR reactions

were therefore carried out in the presence of murine RNA to ascertain what effect this had on detection sensitivity.

This was determined by mixing a range of amounts of purified human RNA (0.1ng -1 μg) with an excess of murine RNA (1 μg) (Figure 8.2).

Figure 8.2 Sensitivity of detection by RT-PCR of human derived RNA in the presence of murine derived RNA

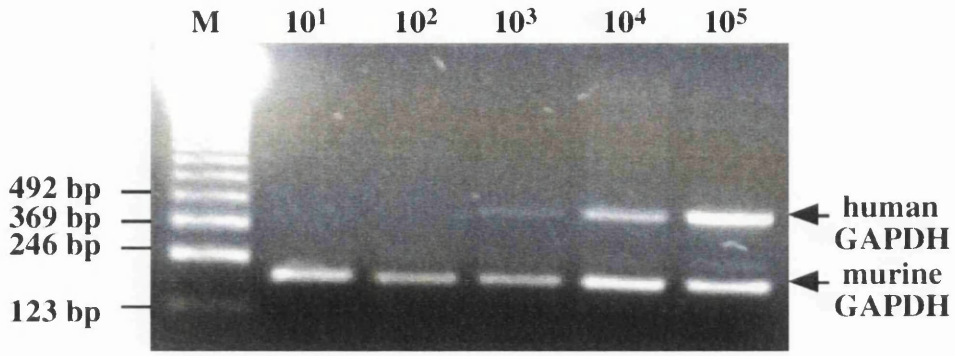


The effect of excess murine RNA on the ability to detect human GAPDH RT-PCR signals. Reactions were carried out with 0.1 - 1 μg of human RNA admixed with 1 μg of mouse RNA. M = 123 bp markers.

In the presence of excess murine RNA, a human GAPDH signal could be detected down to 0.1ng of human RNA, which corresponds to 2-30 cells.

The sensitivity of the method was also determined by mixing increasing numbers of human SK-N-BE(2c) neuroblastoma cells (10 - 100 000) with an excess of murine Clone M3 cells, extracting total RNA from the mixed cell population and then carrying out the RT-PCR reaction (Figure 8.3).

Figure 8.3 Sensitivity of detection by RT-PCR of human derived RNA extracted from a mixed human and mouse cell population



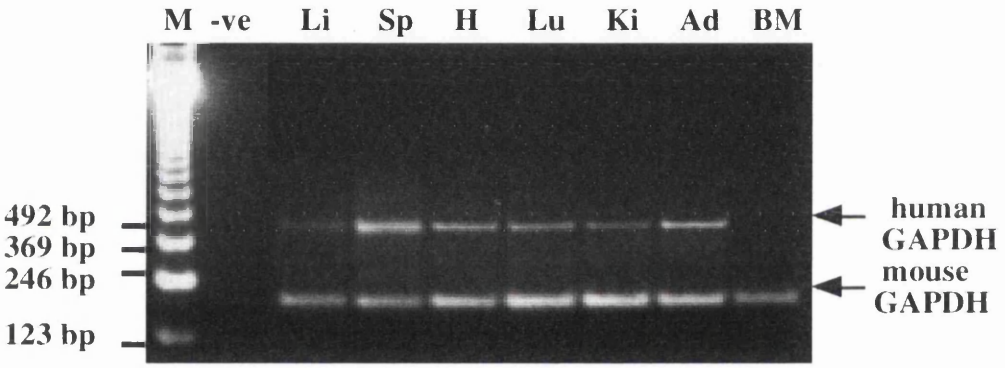
The effect of excess murine cells (10^7) on the ability to detect human GAPDH RT-PCR signals. Numbers above lanes represent the number of human cells present in the cell mixture prior to RNA extraction.

The sensitivity of detection was detrimentally affected when human and murine cell populations were mixed prior to extracting the RNA: the limit of detection of the human GAPDH signal was 1000 neuroblastoma cells.

8.3.3.3 RT-PCR analysis in BALB c mice after ISI

To evaluate the potential for the detection of human neuroblastoma cells in mouse host tissues *in vivo* , the RT-PCR assay was tested on BALB c mice which had been intrasplenically injected with SK-N-BE (2c) cells. RNA was extracted from a variety of tissues, reverse transcribed and PCR amplified using the human-specific GAPDH primers. Amplification was also carried out with mouse-specific GAPDH primers to confirm the integrity of the RNA. This ensured that any lack of signal was attributable to the absence of human cells rather than to general degradation of the RNA sample. A sample gel is shown in Figure 8.4.

Figure 8.4 RT-PCR analysis of a BALB c mouse after ISI



Detection of human cells in a selection of tissues of a BALB c nude mouse after ISI. A negative control containing no RNA was included to ensure that the presence of signal was not due to contamination during the PCR reactions (- ve). M = 123 bp markers, Li = liver, Sp = spleen, H = heart, Lu = lung, Ki = kidney, Ad = adrenals, BM = bone marrow.

PCR signals for the human GAPDH sequence were found in the spleen, liver, heart, lung, kidney and adrenal glands. No human cells were detectable in the bone marrow.

8.4 Discussion

The aims of this study were to develop a realistic *in vivo* model of micrometastatic neuroblastoma. The availability of such a laboratory model would allow the potential of combined therapeutic strategies, designed to treat metastatic neuroblastoma, to be investigated.

As indicated in the introduction (section 8.1.1), existing murine neuroblastoma models do not show evidence of metastasis. This lack of dissemination is a common problem with the growth of human tumour xenografts in nude mice. Regardless of the malignancy of the original tumour in the patient, subsequent implantation in nude mice often fails to produce metastases (Sharkey *et al.*, 1979). A number of factors can influence both the development of metastases and the subsequent ability to detect disseminated disease. The experiences in developing a metastatic neuroblastoma reflect some of these problems and are incorporated in the discussion below.

8.4.1 Selection of appropriate age and strain of athymic mouse

The initial studies relied on the ability of intrasplenically injected cells to spread and produce visible evidence of disease on the spleen and liver. Preliminary experiments were carried out in adult MF-1 mice using a range of tumour cell innocula. These studies revealed that the degree of tumour take was fairly low and demonstrated that there was no real relationship between the size of the innoculum and tumour development: the number of animals with splenic tumours was similar, regardless of the number of cells injected. This could reflect inconsistencies in the injection technique. In addition none of the above animals showed any evidence of metastatic disease on the liver.

The metastatic potential of injected tumour cells is believed to be affected by the natural killer cell status of the host mice. Laboratory studies have revealed that mice who exhibit low levels of NK-mediated cytotoxicity (eg 3-week old syngenic mice, or β-estradiol-treated mice) show increased incidence of

metastases. Conversely, hosts with high NK activity (adult mice and syngenic mice treated with NK-cell-stimulating biological response modifiers) are very resistant to metastases (Hanna, 1982). This might explain the differences observed when the intrasplenic injections were repeated in infant BALB c mice. Comparison of Tables 8.2 + 8.3 with Table 8.1 indicate that BALB c mice were more susceptible to tumour growth both at the initial site of injection and in producing visible liver foci. This increased vulnerability to tumour growth is also reflected in the fact that several animals had to be sacrificed before the appointed time because of haemorrhaging of the primary splenic tumour. It is possible that development of large tumours on the spleen may prevent the development of potential metastases. Several investigators report a higher degree of metastatic spread if the spleen is removed a short time after injection (Laffreniere and Rosenberg, 1986).

8.4.2 Use of RT-PCR to detect micrometastases

A potential problem with the above system is the possibility that metastases do develop but are missed because the techniques used to detect them are not sufficiently sensitive. As in the majority of human xenograft studies, cells were inoculated and the animals observed for macroscopic evidence of metastatic lesions. Such evaluations may not be sensitive enough to detect micrometastases (McKenzie *et al.*, 1991; Shoemaker *et al.*, 1992). To maximise the sensitivity of detection, an assay based on PCR, was incorporated. This enabled the presence or absence of human neuroblastoma cells to be confirmed. Such an approach relies on the use of appropriate primers which are specific for human sequences. Previous studies have utilised the human specific *Alu* repeat sequence which can be used to identify and isolate human DNA from complex backgrounds. Unfortunately because the *Alu* sequences occur frequently in the genome, the signal detected by PCR is a smear rather than a discrete band, which makes quantitation difficult (Nelson *et al.*, 1989). To circumvent this problem primers were required which as well as being human specific, gave rise to a single discrete signal after PCR amplification.

As the primer sets used in the RT-PCR assay in chapter 6 fitted these requirements experiments were undertaken using this system. As Figure 8.1 shows, primers for human noradrenaline transporter and the GAPDH were both specific for human sequences. Attempts to obtain a signal with murine RNA failed. The greater sensitivity observed with the GAPDH sequence probably reflects the higher level of constitutive expression of this housekeeping gene.

In order to assess the susceptibility of the RT-PCR assay to interference by mouse RNA, *in vitro* experiments using mixtures of human- and murine-derived material were performed. When RNA purified from murine clone M3 cells was mixed with human SK-N-BE(2c) RNA, a signal for the human GAPDH could be detected down to 0.1ng of human RNA; estimated to represent around 30 cells. A more realistic assessment of the sensitivity was obtained by using RNA purified from a mixed human and mouse cell population. A detectable signal at 1000 human neuroblastoma cells is probably a more accurate reflection of the true sensitivity of the assay for the *in vivo* context in which it is to be used.

Using RNA from intrasplenically injected BALB c mice, a human GAPDH signal could be detected in a variety of organs. The results demonstrate the feasibility of the assay and suggest that SK-N-BE(2c) cells are widely disseminated after injection. The use of PCR is advantageous since it is direct, sensitive and technically simple to apply. It also avoids detailed histological examination that would otherwise be needed to detect occult micrometastases.

8.5 Conclusions

Attempts to develop a reliable and reproducible model of micrometastatic neuroblastoma in mice have met with partial success. The initial data suggest that RT-PCR could be a viable means of detecting micrometastases. However a number of points need to be clarified:

Does the presence of a positive PCR signal in an organ definitely predict the eventual development of microtumours? Long term survival of the animals after injection would be required to address this question. To ensure this the animals would require a splenectomy to avoid them succumbing to the effects of the primary tumour.

It is likely that tumour cells would be non-randomly distributed throughout a tissue. Therefore, to ensure that the lack of a signal was definitely due to the absence of neuroblastoma cells, RNA would have to be extracted from entire organs.

Quantification of the assay is required to assess the therapeutic effect of treatment(s). One method is to create a standard curve using a range of sample cell numbers. PCR signal strength could be measured by densitometry and plotted against the number of human cells initially present. By measuring the PCR signal strength from tissues of intrasplenically injected mice, the number of tumour cells present could be estimated by reference to the curve. This strategy was successfully used to quantify DNA dot blots from mice bearing melanoma metastases (Shoemaker *et al.*, 1992). In therapeutic studies this has distinct advantages since it means that defined endpoints can be used. Most such studies currently use life span as an endpoint which is problematic due to variability in death patterns between mice and can involve subjecting animals to unnecessary suffering. With a quantifiable RT-PCR approach the metastatic tumour burden in control and treated mice could be compared at pre-determined time intervals.

In summary, the preliminary data presented here demonstrates that, by injecting infant athymic mice with neuroblastoma cells and using RT-PCR to track the fate of inoculated cells, widespread dissemination of tumour cells in mouse tissues can be detected. However, further experiments are required to determine a suitable method of quantifying the assay . Such a model will allow the potential of novel treatment approaches aimed at managing progressive neuroblastoma to be investigated.

CHAPTER 9

FINAL CONCLUSIONS AND PROPOSALS FOR FUTURE WORK

9.1 Aims

The principal objectives of this work, which were set out in chapter 4, were to investigate ways in which the targeted radiotherapy of neuroblastoma with radioiodinated MIBG could be improved. The results presented in this thesis suggest that a number of approaches have the potential to enhance the therapeutic efficacy of MIBG therapy, and raise a number of questions which need to be addressed in future studies. These are discussed below.

9.2 No-carrier-added MIBG

9.2.1 Therapeutic efficacy of n.c.a MIBG

The biodistribution data presented in chapter 5 suggest that n.c.a [^{131}I]MIBG could offer significant benefits both in terms of increased tumour doses and reduced normal tissue uptake. *In vivo* experiments are now required to compare the therapeutic efficacy of n.c.a- and exchange- prepared MIBG to determine whether the observed uptake advantage results in enhanced tumour cell kill. For these experiments to be accurate and informative a realistic murine model of neuroblastoma is needed. The initial data presented in chapter 8 indicate that, with appropriate quantitation, the RT-PCR based system could be used for this purpose.

9.2.2 Clinical use of n.c.a MIBG

The use of a lower total mass dose of MIBG is postulated to enhance the accuracy and sensitivity of neuroendocrine tumour imaging (section 5.4.4). To determine whether this is true clinical evaluation of the scintigraphic potential of n.c.a [^{123}I]MIBG has commenced. Initial investigations are being conducted in adult patients with suspected phaeochromocytoma. Encouraging preliminary results confirm that n.c.a MIBG detects sites of neuroendocrine tumours and is at least as sensitive as its conventional exchange-prepared counterpart.

9.3 Appropriate combination and scheduling of [^{131}I]MIBG therapy

The finding that cisplatin pretreatment of neuroblastoma cells enhances MIBG uptake raises a number of questions which need to be addressed using *in vitro* and *in vivo* models. Experiments are required to determine whether other cytotoxic agents currently in use to treat neuroblastoma are capable of eliciting the same responses as cisplatin. Indeed recent results suggest that the effect is not confined to cisplatin. Meco and colleagues have reported that both cisplatin and adriamycin are capable of stimulating enhanced MIBG uptake in a panel of neuroblastoma cell lines (Meco *et al.*, 1997). Clonogenic and spheroid regrowth delay assays would confirm whether enhanced uptake of MIBG results in a complementary increase in cell kill. It would also be useful to determine whether cytotoxic drug pretreatment is a potential means of stimulating the MIBG uptake ability of neuroblastoma cell lines which are negative for or have low levels of expression of the noradrenaline transporter. This opens the possibility of converting an MIBG negative neuroblastoma into one which is amenable to MIBG therapy.

From a mechanistic standpoint, the roles of differentiation and commitment to apoptosis after cisplatin pretreatment need to be clarified. There are several independent markers of neuroblastoma cell differentiation which could be monitored, including decreased expression of N-myc, increased activity of the enzyme acetylcholinesterase and the measurement of neurite projections. Assessment of apoptosis can be achieved directly by the recognition of cells which exhibit chromatin condensation and nuclear fragmentation (Russell *et al.*, 1995). The potential role of p53 as a transcriptional transactivator of noradrenaline transporter expression could be addressed by comparing cisplatin pretreatment and subsequent MIBG uptake in wild type and p53 mutant neuroblastoma cell lines.

The specificity of this effect needs to be established: ie is cisplatin-induced enhancement specific to neuroblastoma cells or are non-malignant sympathetically innervated tissues such as heart and adrenals also affected?

An equally important consideration is whether cisplatin is capable of modulating MIBG uptake in non-neuronal cells. The most important toxic effect observed with the clinical use of [^{131}I]MIBG is bone marrow depression which may be a result of active accumulation by megakaryocytes via the serotonin transporter (Rutgers *et al.*, 1993) (although this hypothesis is contentious, section 3.7.2). The possibility that uptake in haematopoetic progenitor cells could be enhanced by cisplatin pretreatment needs to addressed.

9.4 Alternative radiohalogens and modified MIBG carrier molecules

9.4.1 Therapeutic potential of [^{211}At]MABG

The experiments described here have confirmed the importance of the cross-fire effect in targeted radiotherapy. As illustrated in Figure 2.2 the radioiodines tested here represent the two extremes of the 'particle range / crossfire' spectrum. That is long range β-particles and ultrashort range Auger electrons. Intermediate in path length are alpha particles which may exhibit a small crossfire effect. Based on this, it has been suggested that another radiohalogen with potential for the treatment of neuroblastoma micrometastases is ^{211}At, an α particle emitter with a range of 50 to 100 μm in tissues, corresponding to 6-10 cell diameters. In addition to cross-fire, an added advantage is the high LET quality of the emitted radiation with its added toxicity. This radiopharmaceutical has been synthesised and its uptake and toxicity have been characterised *in vitro* (Vaidyanathan *et al.*, 1992; Strickland *et al.*, 1994). With respect to kinetics of accumulation and retention MABG was identical to MIBG. However, as expected, MABG was significantly more toxic to neuroblastoma monolayers than MIBG. This high degree of toxicity has also been demonstrated in small spheroids (P Welsh, personal communication), although their efficacy in the treatment of larger cellular aggregates is yet to be evaluated. It would be interesting to determine the size/cure relationship for MABG. The expectation is that the optimal target size for MABG will lie between those of ^{123}I / ^{125}I and ^{131}I. It is probable that

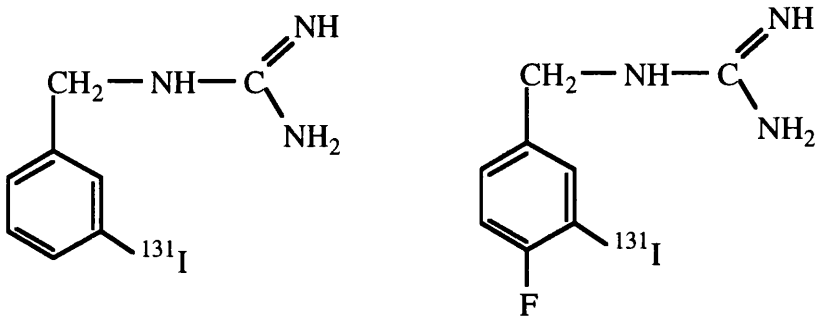
cocktails of all three conjugates would constitute the optimal means of eradicating tumours of a range of sizes.

While ^{211}At is significantly more toxic than any of the radioiodines, considerable work is still needed *in vivo* to confirm its efficacy and, perhaps more crucially, its effects on normal non-target tissues. Recent biodistribution studies in xenografted nude mice, suggest that normal tissue toxicity could be problematic since significantly higher uptake of MABG was observed in normal tissues, especially the heart (Vaidyanathan *et al.*, 1994). Strategies which limit such uptake will need to be developed to minimise radiation dose to non-target organs.

9.4.2 Alternative analogues of iodobenzylguanidine

The development of alternative analogues of MIBG arose out of the search for PET (positron emission tomography) imaging agents for neuroendocrine tumours. PET like SPECT (single photon emission computerised tomography) allows tomographic imaging to be performed but is thought to be advantageous as it provides superior quantitative information. Several PET agents have now been synthesised including [^{76}Br]-meta-bromobenzylguanidine ([^{76}Br]MBBG) (Valette *et al.*, 1993) and [^{124}I]meta-iodobenzylguanidine (Ott *et al.*, 1992). Another potential agent is 4-fluro-3-[^{131}I]iodobenzylguanidine ([^{131}I]FIBG) (Figure 9.1).

Figure 9.1 Chemical structures of [^{131}I]MIBG and [^{131}I]FIBG



[^{131}I]MIBG [^{131}I]FIBG

Laboratory investigations have shown that [^{131}I]FIBG has several advantageous properties. *In vitro* binding studies indicate that uptake and retention of [^{131}I]FIBG was significantly higher than that of MIBG. This enhanced retention is also seen *in vivo,* where approximately 2-fold higher retention was observed in the adrenal glands of normal mice with [^{131}I]FIBG (Vaidyananthan *et al.*, 1997). Prolonged retention of the radiopharmaceutical could have significant benefits for MIBG therapy since it would result in an increased radiation dose to the tumour. It would be useful to assess the potential of this agent with *in vitro* and *in vivo* models of neuroblastoma.

9.5 Investigating mechanisms of cell kill by Auger-electron emitting radionuclides

The most surprising feature of the results presented in chapter 7 is the effectiveness of the Auger electron emitters ^{123}I and ^{125}I in treating monolayer cultures and small spheroids. As discussed, this finding could be the result of membrane-signal induced apoptosis.

This possibility could be investigated using Auger labelled targeting agents for which the subcellular fate is known. Unequivocal DNA-targeting can be achieved using ^{125}I incorporated in IUDR. Membrane targeting can be accomplished by Auger electron emitters conjugated to concanavilin A. These

two agents could then be used to treat a panel of cell lines to determine the role of membrane irradiation in radiation cell killing in apoptosis competent cells.

Russell *et al* (1995) have reported the isolation of a radioresistant neuroblastoma cell line variant which evolved under the selective pressure of repeated irradiation. Comparison of the parent and resistant lines demonstrated that the resistant variant had a decreased propensity to undergo apoptosis after irradiation. The effects of MIBG labelled with alternative Auger electron emitters on clonogenic survival in these two lines could be compared. This would confirm the role of apoptosis in cell death mediated by MIBG labelled with Auger electron emitters. Such experiments should provide a definitive evaluation of the role of apoptosis and of membrane irradiation in cell kill by Auger electron emitters.

9.6 Alternative approaches to establishing a metastatic model of human neuroblastoma

Although the RT-PCR based assay system described in chapter 8 was successful in detecting disseminated neuroblastoma cells, a number of recent reports in the literature on murine metastatic models indicate that several alternative approaches might be worth pursuing.

The selection of an appropriate tumour injection route has proved to be important in achieving tumour cell metastases. Choice of a sub-optimal site may prevent tumour dissemination (Kozzlowski *et al.*, 1984). Attempts to establish neuroblastoma tumours via intra-peritoneal and intravenous routes failed (Gaze, personal communication). As intrasplenic injection had previously been used to generate hepatic metastases (Giavazzi *et al.*, 1986; Laffreniere and Rosenberg, 1986) and to generate splenic neuroblastomas (Rutgers *et al.*, 1991) this injection route was chosen for metastatic studies. However, an increasing number of studies suggest that orthotopic injection is required to assess metastatic potential, in keeping with the theory that the

establishment of metastases depends on the interaction of metastatic cells with their relevant organ environment (Manzotti *et al.*, 1993; Kubota, 1994). Although beyond the scope of this present work, it may be that selection of an orthotopic injection site, in the case of neuroblastoma cells probably the adrenal gland, could be a more effective and reliable means of establishing neuroblastoma metastases.

Another approach would be to screen alternative neuroblastoma cell lines for metastatic potential. Subculturing techniques and *in vitro* assays which measure invasive potential have identified human neuroblastoma cell lines which are more likely to metastasise *in vivo* (Ferrandis *et all.*, 1994). There are also reports of a murine model of metastasis using the mouse neuroblastoma cell line C-1300 (Iwakawa *et al.*, 1994). The disadvantage of using alternative cell lines is the lack of relevant *in vitro* data on them. SK-N-BE(2c) cells were used in these studies because their radiobiology, MIBG uptake ability and response to chemotherapy are all well characterised, a prerequisite to testing therapeutic regimens incorporating these agents in combination. One final possibility would be to use severe combined immune deficient (SCID) mice which have been shown to have marked advantages over nude mice for studying human tumours *in vivo* (Mueller and Reisfield, 1991). Indeed these mice have recently been used to successfully develop a human metastatic neuroblastoma model (Bogenmann, 1996).

9.7 Conclusions

While radioiodinated MIBG is now a firmly established diagnostic tool, its potential as a therapeutic agent is yet to be fully realised. Progress is however being made. For stage IV neuroblastoma patients, regimens which integrate [^{131}I]MIBG with other treatment modalities are yielding promising results. From a research standpoint, ongoing laboratory studies should identify the mechanisms underlying the interactions between [^{131}I]MIBG and other treatment agents. This will establish the optimum sequencing and scheduling of [^{131}I]MIBG, ensuring that maximum benefit is achieved with this targeted radiotherapy agent.

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