MULTIDRUG RESISTANCE IN PAEDIATRIC SOLID TUMOURS:
IDENTIFICATION AND POTENTIAL REVERSAL

A thesis submitted to the University of London in partial fulfilment of the
requirements for the examination of MD.

I confirm that the work included within this manuscript has been approved by the
university for the award of the degree and is (unless otherwise stated) entirely my
own.

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ABSTRACT

Multidrug resistance in paediatric solid tumours; identification and potential reversal.

Multidrug resistance (MDR) is increasingly recognised as an important factor in both refractory and relapsed paediatric solid tumours, although at present most work has been carried out in the leukaemias or adult solid tumours.

The aims of this study were to establish the pleiotropic multidrug resistance status of selected paediatric tumour cell lines, its modulation by non cytotoxic drugs, and to report a clinical study using cyclosporin A to modulate resistance.

The MTT assay was used to assess the chemosensitivity of a panel of ten cell lines (four rhabdomyosarcoma, three neuroblastoma, one T cell leukaemia, and two small cell lung - as controls). Reversal of resistance using verapamil and cyclosporin was investigated and in particular the potential importance of duration and schedule of exposure of cells to modulator was assessed.

Molecular methods were used to document expression of the mdr 1 gene product (immunocytochemistry) and mRNA (RT PCR, northern blot and in situ hybridisation).
A clinical study of patients with relapsed paediatric malignancy is reported using cyclosporin A as a modulating agent combined with etoposide. The effect of cyclosporin on etoposide kinetics was determined.

Those cell lines which displayed a resistant phenotype consistent with MDR also expressed the mdr 1 mRNA. Reversal of drug resistance in these lines was demonstrated with both verapamil and cyclosporin. The timing of administration of modulator does not appear to be of critical importance, though in some cases the duration of exposure has a significant effect upon cytotoxicity.

Although these methods to assess MDR were successfully applied to fresh cell lines, it was not possible to determine the MDR status using fixed tissue from treated and untreated rhabdomyosarcoma specimens.

In children with refractory or relapsed sarcomas use of cyclosporin A as a continuous infusion to modulate etoposide resistance was both tolerable and in some cases resulted in a useful response, in part this response could be due to the altered kinetics of etoposide.
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<tr>
<td>β₂M</td>
<td>β₂ microglobulin</td>
</tr>
<tr>
<td>5FU</td>
<td>5-fluouracil</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>ABMT</td>
<td>Autologous bone marrow transplant</td>
</tr>
<tr>
<td>Actino</td>
<td>Actinomycin D</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukaemia</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>bd</td>
<td>Twice a day</td>
</tr>
<tr>
<td>BDH</td>
<td>British Drugs House</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSEC</td>
<td>Bile salt export carrier</td>
</tr>
<tr>
<td>BSO</td>
<td>L-Buthionine sulfoximine</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CCR</td>
<td>Complete clinical response</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>Cyclo</td>
<td>Cyclophosphamide</td>
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<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
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<tr>
<td>DHRF</td>
<td>Dihydrofolate reductase</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
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<tr>
<td>Dox</td>
<td>Doxorubicin</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>E. Coli</td>
<td>Escherichia coli</td>
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<tr>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>Etop</td>
<td>Etoposide</td>
</tr>
<tr>
<td>FAB</td>
<td>French, American, British</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
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<tr>
<td>G</td>
<td>Guanine</td>
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<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>Gly</td>
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<tr>
<td>GST</td>
<td>Glutathione S transferase</td>
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<td>Guanosine triphosphate</td>
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<td>GvHD</td>
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IHC: Immunohistochemistry
IRS: Intergroup Rhabdomyosarcoma Study
ISH: In situ hybridisation
kb: kilobase pairs
kDa: Kilodalton
LTEC: Leukotreine export carrier
MDR: Multidrug resistance
MDR 1, 2 or 3: Human MDR gene sequence
mdr 1, 2 or 3: Mouse MDR gene sequence
mIBG: meta Iodobenzyl guanidine
MRI: Magnetic resonance imaging
mRNA: messenger RNA
MRP: Multidrug resistance associated protein
MTD: Maximum tolerated dose
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MTX: Methotrexate
NBL: Neuroblastoma
NCI: National Cancer Institute
NHL: Non Hodgkins Lymphoma
OD: Optical Density
Oligos: Oligonucleotides
Osteo: Osteosarcoma
P gp: P glycoprotein
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PFA: Paraformaldehyde
pgp 1, 2 or 3: Hamster MDR gene sequence
PNET: Primitive neuroectodermal tumour
PR: Partial response
PVP: Polyvinyl pyrollidine
RIA: Radioimmunoassay
RMS: Rhabdomyosarcoma
RNA: Ribonucleic acid
RNase: Ribonuclease
rpm: revolutions per minute
RR: Relative resistance
RT: Radiotherapy
RT PCR: Reverse transcription polymerase chain reaction
SDS: Sodium dodecyl sulphate
SR: Sensitisation ratio
SRB: Sulphorhodamine B
SSC: Sodium saline citrate
T: Thymine
T½ α: Distribution half life
T½ β: Elimination half life
TCA: Tricarboxylic acid
tds: Three times a day
TESPA: 3 aminopropyltriethoxysilane
Tm: Melting temperature
Tris: Tris (hydroxymethyl) aminomethane
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<td>Ultrasound scan</td>
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<td>Uridine triphosphate</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VAD</td>
<td>Vincristine, Adriamycin, dexamethasone</td>
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<tr>
<td>Val</td>
<td>Valine</td>
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<tr>
<td>Vdss</td>
<td>Volume of distribution</td>
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<td>VER</td>
<td>Verapamil</td>
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<td>Vinc</td>
<td>Vincristine</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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ACKNOWLEDGEMENTS

I should like to dedicate this work to my parents for their unfailing support.

I am extremely grateful for the advice and encouragement I have received throughout this work from a large number of people.

In particular I would like to thank:-

Ross Pinkerton who has patiently guided me through this project and kept me on course with timely encouragement and assistance.

Kathy Pritchard Jones for her invaluable help in molecular methods, and her ready assistance with expert advice.

Simon Meller and other members of the medical and nursing team for their friendship and patience.

Joan Malings for her assistance and advice in managerial and secretarial matters.

Gina Dick for running the practical side of a clinical study.

Julie Mycroft and the pharmacy staff for assistance and cytotoxics!

Members of the Paediatric Laboratory team in particular Linda King-Underwood and Jane Renshaw.

Members of the Institute of Cancer Research who have helped me, especially those in Molecular Drug Development for allowing me the use of their lab space and assistance in successful tissue culture, in particular Dr R. Knox and Dr. F. Friedlos. Dr L. Kelland for instruction in reading 96 well plates and providing me with cell lines. Dr. J. Peacock for cell lines. Dr P. Monaghan for assistance in dark and bright field photography.

Ray Stucky and all the photography staff for a helpful and efficient service.

Steve Lowis for undertaking etoposide assays at the Cancer Research Unit, Newcastle.

Patrick McCarthy for verapamil assays at Guys Poisons Unit.

Dr P. Twentyman for cell lines, antibody and advice.

Lastly I must thank Paul Bryan for his computer support service (!), but especially for his patience and friendship.
CHAPTER 1
INTRODUCTION
SIZE OF THE PROBLEM

The incidence of childhood cancer in England and Wales is approximately 1 in 1000, resulting in nearly 1200 new cases per year (Parkin et al 1988). Of these almost 60% will be considered cured of their disease 5 years after presentation. The reasons for failing to cure the remaining 40% of children are primarily resistance to available treatment options, though a small number will die of treatment related complications. Malignant disease is far commoner in the adult population with an incidence (1980) of 8 per 1000 per year in S. Thames (Waterhouse et al 1982), and a death rate from cancer of about 4 per 1000 per year.

The commonest malignancies in adults are of epithelial origin, however this is rarely seen in children where cancer of the lymphoreticular and central nervous systems predominate. Carcinomas (especially those arising from the gastrointestinal, respiratory or genitourinary system) are intrinsically more resistant to treatment than most other malignancies, and frequently acquire resistance mechanisms. Cancer in children is generally more chemosensitive than in adults and therefore the proportion who are cured of their disease is higher than that achieved in adults.

The reasons for failure of treatment are various, but are broadly categorised under the term ‘resistance’ (to treatment). Resistance may be intrinsic (de novo or primary) or acquired during therapy. It has been estimated that at least 90% of cancer deaths are due to drug resistance.

The Goldie-Coldman hypothesis (1982, 1983), proposed that failure of chemotherapeutic regimens was due in part to the selection and overgrowth of drug resistant clones that had developed by spontaneous mutation or tumour cell adaptation. Whilst it
is recognised that in general malignant tumours develop from a single clone, this
tumour homogeneity does not persist as the disease progresses. Mutations occur with a
frequency in the order of $1 \times 10^5$ to $10^6$ cell divisions. As a result of this continuing
evolution a tumour may not consist of a population of identical cells at the time of
treatment with chemotherapy. In addition, as the size of a tumour increases the growth
rate slows down due to an inadequate blood supply, with resulting tumour hypoxia.
Cytotoxicity from chemotherapy will preferentially affect cells which are dividing, but
by decreasing tumour bulk (and hence increasing oxygenation to the remaining cells)
non cycling cells are encouraged to undergo division. These cells may be more likely to
be resistant to the chemotherapy agents used initially as they have survived treatment
with those agents, such resistance may have arisen as a chance mutation during cell
division or as a secondary phenomenon directly related to drug exposure. Whilst a
single resistance mechanism may develop within a tumour, it is more likely that several
will be involved in any particular tumour.

Pleiotropic multidrug resistance (or MDR) is one of the most extensively investigated of
these, although as the prevalence of this phenotype is unknown its overall importance in
terms of in vivo resistance to chemotherapy is uncertain.

Other mechanisms of drug resistance to multiple chemotherapy agents include non
pleiotropic MDR (for example due to elevated glutathione-S-transferase or altered
topoisomerase II) and resistance mediated via multidrug resistance associated protein
(MRP). Resistance to single drugs or drug classes is also common and includes
defective drug transport (methotrexate), abnormal drug activation (cytosine
arabinoside), altered hormone receptor number or activity (oestrogen receptor in breast
carcinoma), defective DNA repair (alkylating agents and cisplatin), gene amplification
(amplified DHFR in methotrexate resistant tumours), abnormal drug metabolism (methotrexate), altered target proteins (5 fluorouracil) and intracellular nucleotide pools (6 mercaptopurine).

In paediatric practice many malignant tumours are responsive to initial chemotherapy which results in a long term cure, but if they recur a second remission may be more difficult to obtain. This will vary from tumour to tumour, for example it is more difficult to obtain a second remission in osteosarcoma or Ewings than in leukaemia or lymphoma. Other paediatric tumours are intrinsically resistant to therapy. These are generally much rarer and are often of a tumour type more commonly seen in adults (e.g. hepatocellular carcinoma), or of a more advanced initial stage (e.g. neuroblastoma). There are however several biological features of neuroblastoma (e.g. N MYC amplification, loss of chromosome 1p and ploidy) which are known to be associated with ultimate failure of chemotherapy.
1.2 SCOPE OF THE STUDY

This study examines the pleiotropic multidrug resistance (MDR) status of certain paediatric tumour cell lines and investigates its potential modulation.

a) Sensitivity of neuroblastoma, rhabdomyosarcoma and leukaemia cell lines to cytotoxics (etoposide, doxorubicin and vincristine) is established using the MTT chemosensitivity assay. Reversal of resistance using CSA and verapamil is investigated and in particular the importance of exposure (duration and schedule) of cells to modulator is assessed.

b) Molecular methods are used to document expression of the mdr 1 gene product (immunocytochemistry) and mRNA (RT PCR, Northern blot and in situ hybridisation).

c) A clinical study of patients with relapsed paediatric malignancy is reported using cyclosporin as a modulating agent, in an attempt to increase the chemosensitivity of these tumours.
Pleiotropic or classical multidrug resistance (henceforth called MDR) is the name given to a specific type of cellular resistance to a wide range of structurally and functionally distinct compounds.

It is characterised \textit{in vitro}, by the induction of resistance during exposure to one agent, with simultaneous resistance to many of the other involved drugs, but \textit{in vivo} it may also occur de novo in previously untreated malignancies.

It has now been established that the resistant cell expresses an energy dependant transmembrane protein which functions as an efflux pump. This protein is called P (permeability) glycoprotein, and is coded for by the \textit{mdr 1} gene on chromosome 7 in humans.

Structurally the drugs which are transported by P glycoprotein are dissimilar, though they tend to be hydrophobic compounds derived from natural products.
1.4 HISTORICAL PERSPECTIVES

1.4.1 Historical perspective - Molecular studies on cell lines

In 1968 Kessel et al reported the observation that mice bearing transplantable tumours (intraperitoneal seeding with P388 murine leukaemia cells) selected for induced resistance to daunomycin were also resistant to vinca alkaloids and actinomycin D.

Subsequently, Biedler and Riehm (1970) demonstrated that Chinese hamster cell lines which were maintained in high concentrations of actinomycin D were cross resistant to a number of other cytotoxic agents including (in decreasing order of resistance), mithramycin, vinblastine, vincristine, puromycin, daunomycin, demecolcine and mitomycin C (Table 1.4a).

In 1972 Dano showed (using Ehrlich ascites tumour cells) that resistance could be induced using either vincristine, vinblastine, daunomycin or adriamycin exposure. In each case the cells were also resistant to the other three agents, but always remained sensitive to methotrexate. Resistance developed in this way was stable (in the absence of the selecting agent) for up to 25 weeks. Dano postulated a common mechanism of resistance to these four drugs, though recognised that each cell line could exhibit several resistance mechanisms. He suggested that in view of previous observations (Kessel et al 1967, Biedler and Riehm 1970) demonstrating reduced uptake or reduced retention of daunomycin and adriamycin, a common cellular transport or binding feature was the most likely explanation.

Ling et al (1973) developed colchicine resistant Chinese hamster lines which were then exposed to other cytotoxic drugs. From this work they made some tentative suggestions, including the possibility that altered expression of a single gene was responsible, and
that the product of this gene was likely to be localised in the plasma membrane where it
limited drug accumulation.

Then, in 1976 using cell surface labelling studies Juliano and Ling demonstrated that
several cell lines displaying pleotropic multidrug resistance expressed an increased
amount of a 170 kDa integral membrane protein.

Up until 1979 all the work on MDR had been performed on rodent tumour cell lines.
Owing to the implications these findings might have upon the management of human
malignant disease it was considered vital that this type of resistance was sought in
human cells. A human T cell leukaemia cell line, CCRF CEM (Foley et al 1965) was
selected for resistance to vinblastine (Beck et al 1978), and subsequently shown to
display the pleotropic resistance pattern (Beck 1983).

Following this, cell lines developed from several different human tumours (including
chronic myeloid leukaemia (Tsuruo et al 1983b), epidermal carcinoma (Akiyama et al
1985), uterine sarcoma (Harker and Sikic 1985), multiple myeloma (Dalton et al 1986)
and small cell lung cancer (Twentyman et al 1986b)) were shown to display the
pleotropic resistance pattern, now called multidrug resistance or MDR.

With the development of polyclonal and monoclonal antibodies against P glycoprotein,
Kartner and Ling (1983) and Ling et al (1983) showed that P glycoprotein expression
correlated with induced drug resistance in a variety of rodent and human cell lines.
### Table 1.4a Drugs affected by the multidrug resistant phenotype

<table>
<thead>
<tr>
<th>Class of cytotoxic</th>
<th>Vincristine</th>
<th>Vinblastine</th>
<th>Colchicine</th>
<th>Doxorubicin</th>
<th>Daunorubicin</th>
<th>Mitoxantrone</th>
<th>Mithramycin</th>
<th>Actinomycin D</th>
<th>Puromycin</th>
<th>Etoposide</th>
<th>Teniposide</th>
<th>Mitomycin</th>
<th>Taxol</th>
</tr>
</thead>
</table>

Increased drug resistance was shown to be associated with membrane changes consistent with an energy dependant drug efflux mechanism (Wheeler et al 1982) the efficacy of which correlated with higher levels of resistance.

Whilst the presence of P glycoprotein was recognised as the most consistent marker for MDR, evidence to confirm it as the cause of MDR was slow to accumulate.

The complexity of the MDR phenotype raised the question of whether more than one gene was involved, data to support the role of a single gene or a set of closely linked genes included;

a) The same phenotype was expressed in independent clones of resistant cells selected in a single step with colchicine.

b) Co-expression of MDR phenotype and P gp.

c) Transfer of DNA from resistant to sensitive cells confers resistance (Debenham et al 1982).
In 1985 Ling's group reported the cloning of cDNA encoding P glycoprotein from a highly resistant Chinese hamster line which was then used as a probe on mouse, hamster and human DNA (Riordan et al 1985a). This demonstrated not only that the gene for P gp comes from a highly conserved gene family, but also that in highly resistant cell lines the gene for P gp is amplified. This implies that the basis for the MDR phenotype lies in an increased amount of a normal gene product either due to gene amplification or over expression.

Several groups (Van der Bliek et al 1986, Scotto et al 1986, Endicott et al 1987) showed that more than one gene appeared to be involved by demonstrating in rodent and human cell lines the presence of cDNA transcripts of different lengths, implying different gene classes. This was later clarified with the recognition of a gene family.

There appeared to be a consistent relationship between expression of P gp and level of resistance, but not between gene amplification (homogenously staining regions or copy number) and P gp expression.

Roninson et al (1986) and Shen et al (1986) showed that an amplified region of DNA from resistant Chinese hamster cells was homologous to two human DNA sequences termed MDR 1 and MDR 2, which were later cloned.

One of these sequences (MDR 1) was present in resistant cells but not in their sensitive parent cell lines, and could be detected as a 4.5 Kb mRNA fragment. During development of colchicine resistance in human KB carcinoma cells the MDR 1 mRNA transcript level initially rises without amplification of the corresponding DNA sequence, as colchicine resistance increases both the DNA and mRNA transcript become elevated.
DNA sequences from the human MDR1 and MDR 2 gene were used to probe human chromosomes and demonstrated their localisation on chromosome 7q21.1 (Trent et al 1987, Fojo et al 1986).

It has since been shown that there is a P glycoprotein gene family which consists of two genes in the human (MDR 1 and MDR 3 which is also known as MDR 2) (Chen et al 1986), and three in rodents (these are known as pgp 1,2 and 3 in the hamster (Endicott et al 1987) and mdr 1, 2, and 3 in the mouse (Gros et al 1988)). The second human gene was initially called MDR 3 (rather than MDR 2) by van der Bliek (1988) when they confirmed that this gene differed from mouse mdr 2, on the basis that there would be a human homologue of this mouse gene. In addition at that time there were only two hamster and one mouse multidrug resistance genes.

In the hamster the pgp genes map to chromosome 1q26 and in the mouse to chromosome 5 (Jongsma et al 1987).

The complete cDNA sequence of human MDR 1 (Figure 1.4a) was reported in 1986 (Chen et al). The following year the promoter sequences were identified (Ueda et al 1987) allowing work to begin on regulation of expression of MDR 1.

The gene encoding human P gp (MDR 1) is composed of 27 exons and spans approximately 100 Kb (Chen et al 1990). Whilst it had been proposed that this gene may have arisen from duplication of a more primitive gene (Chen et al 1986), this is probably not the case.

The two human MDR genes are located within 330 bp of each other on chromosome 7q21.1 coincident with the location of the cystic fibrosis gene and met proto-oncogene (Trent and Witkowski 1987). Comparison of the two human MDR genes shows that they are 85% homologous with each other.
Figure 1.4a  Human MDR 1 cDNA sequence

Reproduced from Chen et al 1986

Marked regions indicate sequences used as sense, antisense and internal oligonucleotide primers.
From the cDNA sequence it was deduced that the protein coded by the MDR 1 gene consists of 1280 amino acids and is in two approximately equal (homologous) parts, each containing a hydrophobic and hydrophilic domain. Each hydrophobic domain has six potential transmembrane segments. The predicted structure of this protein was consistent with its proposed function as an efflux pump, and the proteins present in the transmembrane domains were known to form transmembrane channels.

Gerlach et al (1986) recognised that the hydrophilic domains had a striking resemblance to a group of bacterial transport proteins which all contain ATP binding sites believed to be responsible for energy coupling in their corresponding transport systems. These bacterial transport proteins are involved in the uptake (peptides, amino acids and sugars) or export (α haemolysin) of molecules.

The pfmdrl gene in *Plasmodium falciparum* has been implicated in chloroquine resistance, and there is a yeast gene (STE 6) which is 60% homologous with P gp at the amino acid level. STE 6 is the only gene with homology to MDR 1 which has a demonstrable, vital normal function. Disruption of STE 6 inhibits secretion of α-factor which is essential for normal yeast replication (McGrath and Varshavsky 1989).
Evidence to support the hypothesis that P gp causes MDR is now convincing (Ling 1991).

1. Increased P gp levels are consistently seen in MDR cell lines. The level of P gp expression correlates with the relative degree of drug resistance.

2. P gp genes are often amplified in MDR cell lines.

3. Transfection of P gp genes or cDNA results in increased expression of P gp and consequent increases in the degree of resistance.

4. Structural features of P gp are characteristic of an energy dependent membrane transport protein.

5. Many drugs involved in the MDR phenotype bind to P gp.

The functional role of P gp remained unknown until Tsuruo's group (Hamada and Tsuruo 1986) were able to show, using the monoclonal antibody MRK 16, that P glycoprotein was involved in the transport of vincristine and actinomycin D in resistant cells, and that incubating cells in MRK 16 altered the drug transport and cytotoxicity (of these drugs) in resistant but not sensitive cells.

In the same year Safa et al (1986) and Cornwell et al (1986a) published evidence that showed P gp was able to bind to cytotoxic drugs (vinca alkaloids), and that this binding could be inhibited in a dose dependant manner by vincristine or verapamil, as had already been suggested by Fojo et al (1985).

P glycoprotein is known to be glycosylated as a post translational event (Juliano and Ling 1976), but it is also phosphorylated (Hamada et al 1987). The phosphate groups are metabolically active, P gp undergoes rapid cycles of phosphorylation and dephosphorylation (Ma et al 1991), suggesting that this may have an important role in
the biological activity of the transporter molecule. The molecular weight estimated from its amino acid sequence is 140 kDa, but by electrophoresis a size of 170 - 180 kDa is predicted. This discrepancy is due to post translational glycosylation and phosphorylation.

Glycosylation is not necessary for P gp to be functional (Endicott and Ling 1989), however alterations in phosphorylation do appear to alter the level of resistance. The phosphorylation sites are a substrate for protein kinase C, and occur on the serine and threonine residues with increased phosphorylation resulting in decreased cellular drug accumulation (Chambers et al 1990, Sato et al 1990). Verapamil also increases the phosphorylation of P gp, however this is associated with increased intracellular drug accumulation (Hamada et al 1987). This apparent discrepancy may be due to differences in the amino acids which are phosphorylated by verapamil when compared to sites phosphorylated by other drugs or mechanisms. It has been demonstrated that the usual phosphorylation sites involve serine residues, but that other sites are also involved.

Small changes in the amino acid sequence are known to have a significant effect upon the function of P gp. Choi et al (1988) showed that a single amino acid substitution (aa 185 Gly to Val) in the cytoplasmic domain results in a three fold increase in the relative resistance to colchicine.

The normal role of P glycoprotein is unknown although proposed roles include detoxification (Pastan and Gottesman 1987), steroid transport (Ueda et al 1992) and even protein secretion (Muesch et al 1990). Expression of P gp has been demonstrated to be associated with a cell volume regulated chloride channel (Gill et al 1992, Valverde et al 1992), which appears to have a discrete and separate function from the transporting action of P gp. Though evidence soon to be published may not support this finding.
Initially it was assumed that P glycoprotein would function as a drug efflux pump by allowing drugs to pass through the central channel or pore created by the twelve transmembrane domains (Figure 1.4b). A generally recognised view, is that drugs passively enter the cell by diffusing through the lipid bilayer. Some bind to the cytoplasmic side of P gp, and are then effluxed by the protein which uses energy from hydrolysis of ATP (Figure 1.4c). The presence of ATP binding sites and demonstration of energy dependence supported this.

The major problem with this model is that P gp has to bind to the drug with a greater affinity than any other intracellular target (in order to prevent the drug acting on that target). As P gp is able to recognise and bind to a large number of unrelated compounds, it is unlikely that it has a very high affinity for any one compound. In addition to this a wide range of structurally diverse molecules are apparently able to compete for binding sites on P glycoprotein.
Figure 1.4c 'Pump' model of P glycoprotein action

a) Drug enters the cell by passive diffusion through the lipid bilayer

b) Intracellular drug binds to P glycoprotein and is actively excreted (effluxed) from the cell through the central channel (or pore) of a P glycoprotein molecule
Alternative models have been proposed. The first of these (Gros et al 1986) is that the drugs enter the cell by diffusing through the cell membrane and in doing so they spend some time within the lipid bilayer. Lateral diffusion would bring them into contact with P gp (especially if it were present in large amounts), the drug could then bind to and be effluxed by P gp without entering the cytoplasm. This model has earned the nickname of 'cell membrane vacuum cleaner' (Figure 1.4d).

Another (though not mutually exclusive) model (Beck 1987a) is that P gp is localised on intracellular membranes (e.g. on proposed intra-cytoplasmic vesicles) as well as the cytoplasmic cell membrane. Thus drug within the cytoplasm could be pumped into cytoplasmic vesicles which are then removed from the cell by exocytosis (Figure 1.4e). This is supported by demonstration of the subcellular localisation of P gp in (poorly characterised) intracellular cytoplasmic structures (Boiocchi and Toffoli 1992). In addition, this group showed that doxorubicin appeared to accumulate in these areas within MDR cells.
Figure 1.4d  'Vacuum cleaner' model of P glycoprotein action

a) Drug intercalates into lipid bilayer. Lateral movement of drug and P glycoprotein occurs within the bilayer, bringing drug and P glycoprotein into contact. Some drug diffuses from the inner leaflet of bilayer into the intracellular compartment.

b) Drug (in lipid bilayer) which comes into contact with P glycoprotein is 'bound' to it and exported from the cell membrane.
Figure 1.4e  Intra-cytoplasmic vesicles as a model for drug efflux via P-glycoprotein

a) Drug diffuses into the cell. Intracellular drug comes into contact with intracellular vesicles which have P-glycoprotein in their vesicular membrane.

b) Vesicles actively pump drug into the intra-vesicular space from the cell's cytoplasm.

c) Vesicles (containing drug) are emptied by exocytosis.
A third suggestion (Higgins and Gottesman 1992) is that drug intercalates or dissolves into the inner layer of the cytoplasmic membrane, from here it could 'flip' into the outer membrane layer and then diffuse into the surrounding extracellular fluid (Figure 1.4f). It is known that the phospholipid molecules which make up the lipid bilayer are able to move rapidly in the plane of the membrane (lateral diffusion) and more slowly from one membrane layer to the other (transverse diffusion or 'flip-flop'). It is also known that many drugs (e.g. doxorubicin) are able to intercalate between phospholipids making up the bilayer, and can be flipped from one side of the membrane to the other. This is thought to occur at a slow rate, unless the rate is increased by a 'flippase', which may have the effect of increasing normal 'flip-flop' in one direction, and so increasing (for example) doxorubicin transport from the inner to the outer lipid bilayer. The drug could then passively diffuse from the outer lipid bilayer into the surrounding extracellular space. This mechanism and may be more compatible with the suggested normal role of P gp as a route for cellular detoxification.

It is generally recognised that P gp is able to efflux drugs from the cell, but in some circumstances it also appears to affect the influx of cytotoxic. Shalinsky et al (1993) used a cell line expressing a transfected MDR 1 gene to demonstrate that influx of vinblastine was reduced in these cells (compared to the non transfected parent cell line), and that vinblastine influx could be increased with verapamil. This observation was felt to be consistent with the proposed ability of P gp to remove drug from the lipid bilayer as it diffuses into the cell.
Figure 1.4f 'Flippase' model of P glycoprotein action

a) Drug is able to intercalate and dissociate from both inner and outer lipid bilayer

b) Drug (and phospholipid) slowly 'flips' between bilayer

c) Drug in contact with P glycoprotein is actively 'flipped' from the inner to the outer bilayer. This occurs at a faster rate than normal 'flipping'.

d) From the outer lipid bilayer drug diffuses into the extracellular space
Figure 1.4f  'Flippase' model of P glycoprotein action

a)  Drug is able to intercalate and dissociate from both inner and outer lipid bilayer

b) Drug (and phospholipid) slowly 'flips' between bilayer

c) Drug in contact with P glycoprotein is actively 'flipped' from the inner to the outer bilayer. This occurs at a faster rate than normal 'flipping'.

d) From the outer lipid bilayer drug diffuses into the extracellular space
Whilst it is now recognised that P gp is coded by MDR 1 in humans and it is this protein which confers the MDR phenotype, the role of human MDR 3 remains unknown. It does code for a protein which is very similar to P gp (Van der Bliek et al 1988) and this is known to be expressed in normal human liver where it may have a role in detoxification, though it does not appear to act as an efflux pump.

Twentyman (Barrand et al 1993) has investigated non P glycoprotein multidrug resistance and demonstrated that cell lines which apparently display classical MDR (from their resistance pattern and drug accumulation studies) but which do not express, MDR 1 mRNA, DNA or P glycoprotein, do express a 190 kDa protein. This is distinct from P gp, and is not significantly affected (relative resistance) by modulators of classical MDR. These cells overexpress the 'multidrug resistance associated protein' (MRP) gene and exhibit decreased drug accumulation due to increased drug efflux. Using buthionine sulfoxime (BSO) to inhibit glutathione synthesis the cytotoxicity of daunorubicin can be increased (Versantvoort et al 1994), suggesting a role for cellular glutathione in drug transport in cells expressing MRP.

Atypical multidrug resistance (at MDR) was recognised following the development of a resistant leukaemia cell line (CCRF CEM) with teniposide (Beck et al 1987b). This line showed cross resistance to a wide variety of natural product cytotoxics (with the exception of Vinca alkaloids), but did not show any evidence of either MDR 1 mRNA or P gp expression. Quantitative and qualitative alterations in topoisomerase II activity has been implicated in this type of resistance.
Increased expression of various isoenzymes of glutathione-S-transferase is often seen in association with MDR giving a further mechanism for resistance in these cells.

In addition to increased P gp expression, other biochemical markers have been reported in multidrug resistant cells. These include a 19 kDa cytosolic protein (Meyers et al 1981, Meyers et al 1985) known initially as V19, but more recently as sorcin. This is coded for by one of the five co-amplified genes present within the amplicon containing the hamster pgp genes, and is expressed in many cells (including human) which express MDR. Sorcin is a cytosolic calcium-binding protein which is homologous to both calpain and calmodulin. There are no data to suggest that sorcin interacts with P gp, although it may have a regulatory role.

Increased epidermal growth factor (EGF) receptor expression (Myers et al 1986) can be demonstrated in resistant cells (compared with their sensitive counterparts). The EGF receptor is also a 170-180 kDa glycosylated membrane protein, which is distinct from P gp. It is thought that increased expression of the EGF receptor reflects a combination of changes in cells exposed to cytotoxic agents (possibly conferring a growth advantage), rather than being linked to P gp expression.
1.4.2 **Historical perspective - Molecular studies on clinical material**

Expression of the human MDR phenotype in normal tissues has been the subject of much investigation in an attempt to elucidate both the usual role of P gp and the significance of its expression in malignant tissues.

Most work involves the detection of protein expression using monoclonal antibody and either immunohistochemistry (IHC) or FACS techniques. Several monoclonal antibodies are now available which have slightly different specificities. The level of staining within a tissue which is considered to confers 'positivity' is variable. For example in Chan's study of neuroblastoma samples (1991) positivity was defined by the detection of P gp in more than 20% of cells by IHC, whereas Cornelissen (1994) chose a positive result as at least 30% of myeloma cells demonstrating P gp, and Verrelle (1991) used a combination of intensity and percentage of cells (more than 75%) to define positivity. Most of these studies consider a positive result to require more than a chosen percentage of cells demonstrating P gp. It is not known if this is a reasonable assumption or if any expression (even at very low levels or patchy) is important in chemoresistance.

The monoclonals most commonly used in these studies are C 219, JSB 1 and MRK 16 (all raised in mouse). Both C 219 and JSB 1 recognise an internal epitope, whilst MRK 16 recognises an extracellular portion of P gp. C 219 has some cross reactivity with MDR 3. Other monoclonals developed in individual institutions are sometimes used. Many studies utilise a panel of monoclonal antibodies, or a combination of IHC and assessment of mRNA expression (most commonly RT PCR, but also in situ
hybridisation). Those studies employing RT PCR or northern blot generally make an attempt at quantitation, either using a series of resistant cell lines or dilutions from a resistant line. Whilst the latter methods of providing controls are adequate within a laboratory they are not generally transferable between laboratories or helpful when trying to compare results in different studies.

If different institutions are asked to assess the MDR status of identical specimens using their most reliable technique there is little concordance between the results. This has been highlighted by the recent Memphis workshop (1994) on MDR detection methods, at which the importance of reproducible controls and standardised methodology were emphasised.
Expression in normal tissue

The first reports of normal tissue expression of human MDR 1 appeared in 1987. Fojo et al (1987) used MDR 1 probes against cDNA prepared from frozen tissue. They compared the level of mRNA expression with that in a series of MDR cell lines (KB-3-1, KB-8-5 and KB-V1). KB-V1 is more than 500 fold resistant to vinblastine than KB-3-1 (sensitive parent line). Using this method they showed that human adrenal, (particularly the adrenal medulla) has a high level of expression, kidney, colon, liver, jejunum and rectum have an intermediate level, and brain, prostate, skin, muscle, bone marrow (and a number of other organs) have a low level of mRNA expression.

Thiebaut et al (1987) used the monoclonal MRK 16 to examine a similar range of tissues. They were able to localise P gp within tissues and showed that in liver it is the biliary canalicular surface of hepatocytes and small biliary ductules which express P gp. MRK 16 is not known to cross react with MDR 3 (which is expressed in the liver) and as MDR 1 mRNA has been demonstrated in liver tissue it is likely that MDR 1 and MDR 3 are both expressed there. In jejunum, colon, kidney and pancreas a similar pattern of expression emerged with P gp occurring on the apical surfaces of cells involved in excretion or secretion.

Additional sites of expression do not easily support a proposed physiological role in excretion. Expression has been demonstrated in the gravid (but not the nongravid) uterus (Arceci et al 1988), in the adrenal medulla (at a far higher level than the cortex) (Sugawara et al 1988), also in the capillary endothelium of brain and testes (Cordon-Cardo et al 1989) but not in capillary endothelium of placenta or kidney.
In 1990 Van der Valk et al examined a range of normal tissues using IHC and three monoclonal antibodies (C 219, JSB 1 and MRK 16). They demonstrated that whilst in some tissues there is concordance between these antibodies in others the results are mixed, this highlights the difficulties encountered when only one or two antibodies are used. They also observed cross reactivity of C 219 with striated muscle (also seen by Thiebaut et al 1989), possibly with a myosin protein and cross reactivity of MRK 16 with smooth muscle and dermal capillaries.

Up until recently the only aspect of the haematopoietic system which had been studied was the lymph nodes (showing no P gp expression) and spleen (showing low level expression) (van der Valk 1990, Cordon-Cardo 1989). In 1991 Chaudhary and Ronninson analysed CD34 positive stem cells in marrow using MRK 16 and UIC 2 (both recognise external epitopes), and showed that this cell population expresses high levels of P gp. P glycoprotein expression has also been subsequently demonstrated in peripheral lymphocytes (Chaudhary et al 1992, Drach et al 1992), CD5 expressing B lymphocytes from tonsillar lymph nodes (Wall et al 19994), and many components (myeloid and lymphoid precursors) of normal bone marrow (Marie et al 1992, Hegewisch-Becker et al 1993).

It has become apparent that expression of P gp is widespread, particularly in tissues and organs with an excretory role (Table 1.4b and Figure 1.4g). There are however some clear exceptions. The expression in haematopoietic cells, the capillary endothelium of the brain and in testes does not appear to be related to an excretory or secretory function. Though in the case of P gp expression in CNS capillary endothelium a role in maintaining the integrity of the blood brain barrier has been proposed. This is supported
by increased CNS drug levels seen in knockout mice who are mdr 1a (-/-) and who are
given ivermectin or vinblastine systemically (Schinkel et al 1994). Another knockout
mouse has been developed but not fully investigated. This has no mdr 1b and is
apparently normal. To date a double knockout mouse (i.e. mdr 1a (-/-) and mdr 1b (-/-))
has not been developed.

In mice mdr 1a and 1b share sites of expression with human MDR 1, similarly mouse
mdr 2 shares sites of expression with human MDR 3 (or MDR 2). A mdr 2 (-/-) mouse
has been developed (Smit et al 1993), and is completely unable to excrete phospholipid
into bile and hence suffers severe liver disease. This leads to the suggestion that MDR 3
(or MDR 2) is involved in phospholipid transport.

In tumour cells MDR 1 expression may be an acquired or inherent trait. Although many
tumours which display primary chemoresistance to the cytotoxic agents transported by
P gp develop from tissues which normally express P gp, this is not necessarily the case.
Table 1.4b  Normal sites of tissue expression

<table>
<thead>
<tr>
<th>Organ</th>
<th>Tissue</th>
<th>JSB 1</th>
<th>C 219</th>
<th>MRK 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>(+)</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Blood vessels</td>
<td>Smooth muscle</td>
<td>(+)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Endothelium</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Digestive Tract</td>
<td>Oral mucosa &amp; Oesophagus (Ep)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>Oesophagus (Ep)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Stomach &amp; Intestine (Ep)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Liver</td>
<td>Parenchyma</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bile Canaliculi &amp; Ductules</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gall Bladder</td>
<td>Epithelium</td>
<td>+</td>
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<td>+</td>
</tr>
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<td></td>
<td>Myoepithelial cells</td>
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<td>(+)</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>Tubules</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Prostate</td>
<td>Epithelium</td>
<td>(+)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Smooth Muscle</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Testis</td>
<td>Spermatogenesis &amp; Sertoli-Leydig</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ovary</td>
<td>Epithelium</td>
<td>+</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td>Stromal cells</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>Breast</td>
<td>Glands</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td>Ducts</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td>Stromata</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brain</td>
<td>Parenchyma</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Vessels</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Peripheral nerve</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Spleen</td>
<td>Pulp (red &amp; white)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Marginal zone</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Skin</td>
<td>Epidermis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dermis capillaries</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Dermis sweat glands</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adrenal</td>
<td>Cortex &amp; Medulla</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thyroid &amp; Pancreas</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Ep = Epithelium  
- = Negative  
+ = Positive  
(+) = Weak or Partial Positive

Immunoperoxidase technique on autopsy or surgery, all snap frozen.

(Taken from van der Valk 1990)
Figure 1.4g  Sites Of Normal P Glycoprotein Expression

- Brain (capillary endothelium)
- Breast (ducts)
- Lung (bronchial epithelium)
- Pancreatic ducts and Gall bladder epithelium
- Adrenal
- Prostate
- Testes
- Salivary glands
- Breast (ducts)
- Pancreatic ducts and Gall bladder epithelium
- Adrenal
- Prostate
- Testes
- Sweat glands
- CD 34 positive stem cells
- Bile canaliculi ductules
- Spleen (marginal zone)
- Kidney (proximal tubules)
- Jejunum
- Colon
- Rectum

# Possible excretory role
Expression in malignant tissue

The assessment of MDR expression in malignant specimens is complicated by the presence of normal tissue or stroma within the tissue specimen. If RNA extraction is to be performed there may be difficulties ensuring that RNA comes only from malignant cells. This is less of a problem in leukaemia where a population of cells which are almost all malignant can easily be examined, if necessary using FACS selection methods. Tumour specimens from solid malignancies also present the problem that tissue fixation for histological examination may make it unsuitable for other experimental procedures, or that the fixation history is unknown.

Expression in Leukaemia

The first report in leukaemia (Ma et al 1987) used C 219 to show expression of P gp in two patients both of whom later died of disease. Subsequently, using an MDR 1 gene probe increased mRNA was demonstrated in 18/40 patients with acute myeloid leukaemia (AML), but no gene amplification (Holmes et al 1989).

In 1990 (Sato et al) examined the relationship between MDR mRNA transcript level (arbitrary units) and induction of remission in AML. They showed in 74 adult patients that whilst there was no relationship between MDR status and age or FAB type, those patients with a high mRNA transcript level both failed to achieve remission and tended to relapse earlier than those with low levels.

Similar results were reported by Kuwazuru et al (1990) using C 219 to assess P gp expression in AML and acute lymphoblastic leukaemia (ALL), and Goasguen et al (1993) using C 219 and JSB 1 in ALL.

However Pieters et al (1994) have suggested that drug resistance in childhood ALL is not predominantly due to MDR as chemosensitivity assays have failed to show a clear
correlation between resistance and P gp expression, suggesting that other resistance mechanisms are more important. This finding is in keeping with other published work on childhood ALL using material from patients at presentation or relapse (Rothenberg et al 1989, Mizuno et al 1991), which has been unable to demonstrate evidence of MDR 1 gene expression or P gp by northern or ISH and IHC respectively.

The necessity of performing more than one method of assessing MDR status was highlighted by Brophy et al (1994) who used four techniques (slot blot, RT PCR, IHC with MRK 16 and ISH) to examine freshly prepared cell lines from children with leukaemia or lymphoma. They obtained complete concordance between the four techniques in 14/36 lines. Overall ISH and RT PCR were most concordant, whilst RNA slot blot was the least. The MDR status was deemed to be positive in 14 cases and negative in 13. In eight patients complete clinical response (CCR) correlated with negative MDR status, of the six other patients who achieved a CCR four were MDR positive and two not assessable due to non-concordance between the results.

iv Expression in Adult solid tumours

Adult solid tumours which have been examined for MDR expression include those arising in breast, stomach, testis and lung, and lymphoma, sarcoma and myeloma.

In breast carcinoma, P gp expression (using C 219 and MRK 16) appears to correlate with reduced progression free survival (Verelle et al 1991). However there have also been reports of P gp expression in breast stroma (Wishart et al 1990, Verelle et al 1991) which confounds interpretation of these results.

P gp expression in multiple myeloma appears to increase following chemotherapy (Epstein et al 1989, Grogan et al 1993, Cornelissen et al 1994) and correlates with exposure to treatment in vivo when serial bone marrow aspirates are examined.
In gastric (Wallner et al 1992), testicular (Katagiri et al 1993), ovarian and small cell lung cancer (Holzmayer et al 1992) expression of P gp can be demonstrated, but the relevance of this to disease response is unclear. P glycoprotein expression was also shown in a group of untreated lymphomas (Niehans et al 1992), but in this study there was no correlation between P gp expression and either response to treatment or survival.

In a mixed group of adult sarcoma patients, three studies (Gerlach et al 1987, Tom et al 1992, Vergier et al 1993) have shown that whilst some sarcomas appear to exhibit the MDR phenotype (either before or after treatment), there is little consistency between the various methods of assessment either within or between studies.

The observation that invasive carcinoma cells at the leading edges of tumour also express P gp has led to the suggestion that P gp may have a role in local tumour aggressiveness or metastatic ability (Weinstein et al 1991).

**Expression in Paediatric solid tumours**

The solid paediatric tumour most extensively studied to date is neuroblastoma. Other tumours include Ewing's sarcoma, primitive neuroectodermal tumours (PNET), and soft tissue sarcomas.

Using RT PCR, MDR 1 expression was detectable in 6/12 PNET samples (Tishler et al 1992). In situ hybridisation using JSB 1 (Roessner et al 1993) showed that 12/21 Ewing's sarcomas expressed P gp, and this correlated with a poorer response to treatment.

McDowell et al (1993) demonstrated the presence of P gp in 5/24 rhabdomyosarcoma specimens using C 219, and 2/24 using northern blot (low levels only). The few samples which were positive for MDR came from both treated and untreated patients.
Chan et al (1990) reported that 9/30 patients with soft tissue sarcoma (4 at diagnosis, 5 at relapse) expressed P gp as assessed by IHC using C 219. This study used a modified immunoperoxidase method with C219 and C494 to assess the positivity of 62 tumour samples (presentation and relapse where possible). Most of the samples were formalin fixed, paraffin embedded blocks, with a few frozen bone marrow aspirates. Three observers independently evaluated the immunohistochemical staining and expressed the results according to a semiquantitative scale (previously constructed from a series of ovarian cancer cell lines of known vincristine resistance). Expression of P gp was associated with a poor response to both treatment and survival.

Using a similar method with MRK 16 and JSB 1 O'Meras group (O'Mera et al 1992) were unable to demonstrate P gp expression in any of 36 tumour specimens (all fresh frozen at -195°C). Fourteen of these patients had chemoresistant disease, there were 13 neuroblastoma, 12 Wilms, 6 rhabdomyosarcoma, 3 lymphoma, 1 teratoma and 1 Ewing's.

MDR 1 expression in neuroblastoma has been demonstrated using MDR 1 gene probes (Bourhis et al 1989) or IHC with C 219 (Chan et al 1991). In both these papers expression was correlated with a poor outcome. However, Favrot et al (1991) were unable to demonstrate P gp expression (using C 219) in any of 37 neuroblastoma samples. In 9 of these samples P gp was detected in normal cells within the tumour (monocytes, histiocytes or fibroblasts).

In addition to MDR 1 some groups have also assessed N MYC or glutathione-S-transferase (GST) expression (Corrias et al 1990, Goldstein et al 1990, Nakagawara et al 1990, Bourhis et al 1991, Corrias et al 1991). These reports give conflicting results, Goldstein showed no correlation, Nakagawara and Corrias showed an inverse
correlation and Bourhis that N MYC and MDR 1 expression were independent (poor) prognostic factors. Whilst there is an association between immature histology and N MYC gene amplification, there is no such correlation for MDR 1 expression, and indeed the reverse may be the case differentiated ganglioneuroblastoma overexpressing MDR 1.
1.4.3 Historical perspective - Modulation of MDR

The term modulation refers to the observed alteration in chemosensitivity of cells to a drug when given in conjunction with another agent. This is generally considered to result in an increase in cytotoxicity, though in some cases it may result in a selective reduction in toxicity, for example the amelioration of methotrexate toxicity by folinic acid.

In the context of MDR, modulators which increase efficacy can be other cytotoxics, non cytotoxic drugs or compounds or monoclonal antibodies (Table 1.4c). Additional routes by which MDR can be circumvented include using modified cytotoxics (e.g. liposomal doxorubicin) or by exploiting the phenomenon of collateral sensitivity. This occurs in some resistant cells which in the process of developing multidrug resistance become more sensitive than their parent cell line to other drugs, particularly agents which have been used as modulators. For example, verapamil, nifedipine and cremophor EL (Warr et al 1986, Twentyman et al 1986a, Cano-Gauci and Riordan 1987).

Calcium is well recognised as an important intracellular ion with various different (often regulatory) functions. There are several intracellular calcium binding proteins such as calmodulin and sorcin. Calcium is known to have a role in both the intracellular and extracellular transport, regulation of mRNA and in function of both skeletal muscle and microtubules. As vinca alkaloids exert their cytotoxic effect by interfering with the mitotic spindle during cell division Tsuruo's group became interested in the role of calcium in the activity of these drugs. In 1981 (Tsuruo et al 1981, Tsuruo et al 1982) it was demonstrated that the calcium antagonist verapamil and the calmodulin inhibitor trifluoperazine increased the intracellular concentration of vincristine and adriamycin in
both vincristine and adriamycin resistant cell lines. The increased intracellular cytotoxic levels (due to decreased efflux) had the expected effect of increasing cytotoxicity. These findings were subsequently confirmed, Slater et al (1982) in murine lines, and Rogan et al (1984) in human ovarian cell lines.

Since these reports there have been numerous studies demonstrating that various agents are able to potentiate the antitumour activity of several cytotoxic drugs.

Binding of verapamil to P gp was shown by photoaffinity labelling in 1986 (Safa et al 1986, Cornwell et al 1986b) thus linking the observed modulation of the MDR resistance pattern with the known molecular phenotype.

Table 1.4c  Drugs which have demonstrated ability to reverse MDR in vitro

<table>
<thead>
<tr>
<th>Class of drug</th>
<th>Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium channel blocker</td>
<td>Verapamil</td>
</tr>
<tr>
<td></td>
<td>Diltiazem</td>
</tr>
<tr>
<td></td>
<td>Nifedipine</td>
</tr>
<tr>
<td></td>
<td>Nicardipine</td>
</tr>
<tr>
<td></td>
<td>Bepridil</td>
</tr>
<tr>
<td>Other cardiovascular drugs</td>
<td>Dipyrimidole</td>
</tr>
<tr>
<td></td>
<td>Quinidine</td>
</tr>
<tr>
<td>Steroidal agents</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
</tr>
<tr>
<td></td>
<td>Megestrol acetate</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Ceftriaxone</td>
</tr>
<tr>
<td></td>
<td>Erythromycin</td>
</tr>
<tr>
<td>Cyclosporins</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td></td>
<td>PSC 833</td>
</tr>
<tr>
<td>Calmodulin inhibitors</td>
<td>Fluphenazine</td>
</tr>
<tr>
<td></td>
<td>Trifluoperazine</td>
</tr>
<tr>
<td>Antimalarials</td>
<td>Quinine</td>
</tr>
<tr>
<td>Miscellaneous agents</td>
<td>Terfenidine</td>
</tr>
<tr>
<td></td>
<td>Tricyclic antidepressants</td>
</tr>
<tr>
<td></td>
<td>Monoclonal antibodies (MRK 16)</td>
</tr>
</tbody>
</table>
Other calcium antagonists such as diltiazem and nifedipine have been investigated, as have the stereoisomers of verapamil. Owing to the adverse cardiac effects of verapamil, it was hoped that the D isomer (which has fewer cardiac effects) would prove to be as effective as either the L isomer or the racemic mixture (Plumb et al 1990, Hollt et al 1992, Woodcock et al 1993). Several small studies (Satellite Symposium 1st international conference on reversal of MDR) have used oral dexverapamil in dose regimens ranging from 300 mg four times a day to 450 mg four hourly for six days. These regimens were generally well tolerated although in some cases dexamethasone was used to decrease the incidence of hypotension. Cardiovascular side effects seen were similar to those expected with racemic verapamil. Dexverapamil concentrations were measured in some of these studies, with a dose schedule of 450 mg 4 hourly a serum concentration of 4000 ng/ml has been achieved. It appears that oral dexverapamil can be used in this setting as a reversal agent with acceptable toxicity.

The mechanism of action of the calcium antagonists remains uncertain, though it has become clear that it is unrelated to calcium levels or transport (Kessel et al 1984 a & b). As calcium antagonists are known to interact with the plasma membrane, other agents with membrane active properties have been studied. Some of these are also able to reverse MDR, including quinidine, other calmodulin inhibitors (chlorpromazine and thioridazine (Krishan et al 1985, Akiyama et al 1986)), some surfactants (Woodcock et al 1992) and cremophore oil (Schuurhuis et al 1990). Tamoxifen was initially investigated owing to its reported calcium antagonist activity, though this appears to have no bearing upon its action in reversing MDR (Stuart et al 1992). The development of resistance (mediated via expression of MDR 1) in a human breast cancer line (MCF
7) is associated with the loss of oestrogen receptors and elevation of GST levels (Moscow et al 1988).

Liposomal encapsulated doxorubicin is able to circumvent MDR (Thierry et al 1989, Cuvier et al 1992) and the mechanism for this is likely to be the inability of P gp to efflux doxorubicin in this form before it has reached its target.

Cyclosporin A (CSA) was shown to reverse vincristine and daunorubicin resistance in a resistant leukaemia cell line in 1986 (Slater et al 1986). Analogues of CSA have subsequently been investigated (Twentyman et al 1987a, Twentyman et al 1992), and a non immunosuppressive cyclosporin (PSC-833) has now reached clinical trials (Twentyman and Bleehen 1991b, te Boekhorst et al 1992). The modulation seen with CSA is in general more effective than that seen with other agents, however in clinical practice this is associated with a higher incidence of adverse events. The oily vehicle (cremophor) in which both CSA and taxol are suspended has been used in in vitro reversal studies (Watanabe et al 1994, Fjillskog et al 1994). It has been shown to be both cytotoxic and able to modulate the cytotoxicity of other drugs.

There has also been interest in investigating the efficacy of low dose CSA (Twentyman and Wright 1991a), and in determining whether there is synergy between CSA and other MDR reversal agents. CSA and verapamil have been shown to be synergistic in an in vitro model (Larsson and Nygren 1990, Osann et al 1992, Lenhart et al 1991), as are CSA and quinine (Hu et al 1990). Another group have used six modulators together (verapamil, quinidine, promethazine, fluphenazine, amiodarone and tamoxifen) at a sixth of the dose and shown greater modulation than expected (Ayesh et al 1994).

It is difficult to explain the modulating ability of all these drugs by one mechanism. Some have a specific target within cells (calcium antagonists, calmodulin inhibitors and
metabolic inhibitors), others primarily affect the plasma membrane permeability (detergents and lipophilic compounds), and many appear to act in both these ways (CSA, tamoxifen and quinidine). The various modulator classes do not appear to have either equivalent or consistent efficacy, with wide variations seen in vitro in cytotoxicity experiments. In addition modulators (e.g. verapamil) frequently display a preferential ability to modulate one drug over another and their efficacy differs between different tumour cell lines.

Some resistant cell lines showed an increased intracellular accumulation of either vincristine or actinomycin D when exposed to the monoclonal antibody MRK 16 (Hamada and Tsuruo 1986). This led to further work investigating the potential of using monoclonal antibodies as MDR reversal agents. Not all monoclonals raised against P gp are useful in this setting as many antigenic sites are inaccessible in living cells even if the antibodies are introduced directly into the cytoplasm. Moreover, some monoclonals bind to the antigenic site without inactivating it.

Hamada and Tsuruo were able to demonstrate that MRK 16 binds to P gp expressed on resistant cells. The effects of this binding were to increase intracellular vincristine and actinomycin D concentration and cytotoxicity, due to steric hindrance of the pump mechanism at its external surface. Adriamycin was not however affected. Proliferation of the cells was also seen with both MRK 16 and MRK 17 (when cultured without cytotoxics), this effect was not due to cytocidal action and remains to be explained.

Other monoclonals which are able to reverse MDR have since been developed, all those with modulating activity recognise the external epitope of P gp. UIC 2 was as effective as verapamil in an in vitro model (Mechetner and Ronninson 1992), and 4E3 (mouse monoclonal specific for MDR 1 recognised an external epitope) was able to modulate
the cytotoxicity of actinomycin, though not to the same degree as CSA (Arceci et al 1993).

The ability of a panel of newly developed monoclonal antibodies against P gp to enhance intracellular accumulation of vincristine, vinblastine, actinomycin D and doxorubicin was examined by Rittmann-Grauer et al (1992). The most effective monoclonal (HYB 241, IgG1 mouse monoclonal which probably recognises a cell surface epitope) was used in mice with established drug resistant xenografts who then received vinca alkaloids in doses known to be ineffective in this model. A significant inhibition of tumour growth was demonstrated.

A transgenic mouse model overexpressing mdr 1 in bone marrow has been developed by Mickisch et al (1992). This has enabled in vivo study of various MDR reversal strategies. Reversal of resistance (as assessed by myelosupression) was seen when MRK 16 was given with Fab fragments of MRK 16 and cytotoxic drugs (Mickisch et al 1991). If the Fab fragments were not given there was no additional toxicity compared to cytotoxics alone. Nor did combining MRK 16 with verapamil or CSA produce any additional effect. Significant bone marrow toxicity was however demonstrated in the transgenic mice (but not in the normal mice) when MRK 16 was coupled with the pseudomonas exotoxin (MRK-16-PE), suggesting an alternative therapeutic avenue.

The use of antisense oligonucleotides to down regulate expression of MDR 1 has also been explored. In a colon cancer line (Rivoltini et al 1990), inhibition of P gp expression was possible leading to reduction in doxorubicin resistance. However in another study in a breast cancer line (Jaroszewski et al 1990), the oligonucleotides (five different sequences were used) were themselves toxic and less toxic (phosphorothioate) analogues were unable to down regulate P gp expression.
MDR 1 specific ribozymes have been developed (Holm et al 1994). These are small synthetic oligonucleotides which bind to specific complementary regions of mRNA and catalyse cleavage of the RNA strand. This has been shown to decrease the level of MDR 1 mRNA and P gp and to increase daunorubicin toxicity.

Collateral sensitivity was first described in 1976 by Bech-Hansen et al, who showed that resistant Chinese hamster cell lines were more sensitive than their parent lines to some steroids, local anaesthetics and non-ionic detergents. These cells are also more susceptible to physical disruption (Stansted cell disrupter) than their drug sensitive parents, (Riordan and Ling 1979). It has since been established that several of the agents used to modulate MDR by decreasing drug efflux also increase collateral sensitivity (Cano-Gauci and Riordan 1987, Warr et al 1986, Twentyman et al 1986a). This suggests that these compounds have at least two (possibly linked) effects upon MDR cells, a) they decrease cytotoxic drug efflux and b) are in themselves more cytotoxic to these cells (than to their drug sensitive parents).

Some resistant cell lines are sensitive to verapamil alone, this is not due to increased intracellular accumulation of verapamil which may be lower than in sensitive cells. One hypothesis is that verapamil may prevent the efflux of a toxic metabolite, or that binding of this type of drug to P gp significantly detracts from the structural stability of the cell membrane. The toxic level of verapamil (Schmidt et al 1988, Twentyman et al 1986b) when used alone in these resistant cell lines is much higher than usually used in reversing MDR or that achieved clinically. The increased sensitivity of some MDR cells to physical disruption may be related to the large quantities of P gp within the cell membrane, and the effect of local anaesthetics on these cells may be via their ability to affect cell membrane stability.
Multidrug resistant cell lines exposed to verapamil for prolonged periods of time become less resistant to cytotoxics and less sensitive to verapamil. This is associated with decreased expression of P gp, loss of multiple MDR 1 gene copies (Spengler et al 1992), and down regulation of P gp expression at mRNA transcriptional level (Scotto et al 1992).

The phenomenon of collateral sensitivity, whilst interesting, does not immediately offer any avenues for exploitation as the involved drugs are not suitable for clinical use in the amounts required for effectiveness.
1.5 CLINICAL STUDIES

1.5.1 Clinical implications of MDR and its modulation

P gp is now recognised as a common mechanism of resistance, and it is estimated that in the USA up to 450 000 new cases a year may express the MDR 1 gene either at presentation or subsequently (Gottesman and Pastan 1989).

There are various approaches to improve the response in these patients which are gradually being introduced into clinical trials.

1 High dose chemotherapy to increase intracellular drug concentration.
2 Using non cross resistant agents.
3 Altering the function of P gp with modulating agents.
4 Targeting P gp with monoclonal antibodies.
5 Using MDR 1 antisense RNA to inhibit translation of the gene.

Expression of the MDR 1 gene product (which may be at very high levels) in normal tissues and in non malignant cells within tumours must be considered when attempting clinical reversal of MDR. If the modulating agents are used in truly effective amounts, they will affect the normal physiological processes in which P gp plays a part, which may include drug clearance by the liver or kidney thus influencing drug concentration. The modulators may be beneficial in other unexpected ways. Verapamil may significantly alter blood flow, not only to the liver but also (possibly preferentially) to the tumour thus increasing cytotoxic drug delivery, CSA decreases renal clearance of some cytotoxics and can increase AUC.
The adverse effects seen in clinical studies combining a modulator with cytotoxic are due to a combination of three main factors.

1. **Altered pharmacokinetics of the cytotoxic agent.**

   Both calcium antagonists and CSA are now known to alter the pharmacokinetics of some cytotoxics, verapamil with adriamycin or epirubicin (Kerr et al 1986, Mross et al 1993), nifedipine with vincristine (Fedeli et al 1989), cyclosporin with etoposide (Lum et al 1992) or doxorubicin (Bartlett et al 1994). The overall net result of this is to increase the AUC of the drug. In some cases this will be significant and may account for much of the increased tumoricidal effect seen when a cytotoxic agent is combined with a modulator.

   Whilst the mechanism of this increase in AUC may be due to a P gp mediated effect, an alternative has been proposed. Verapamil, CSA, PSC 833, taxol and etoposide are all known substrates for hepatic cytocrome P450 enzymes (in particular the CYP 3A subfamily), and verapamil has been shown to inhibit CSA metabolism (Lindholm and Henricsson 1987). It is possible that the alteration of etoposide pharmacokinetics seen with CSA can be accounted for by inhibition of P450 activity.

   Nausea and vomiting are commonly seen with many regimens of cytotoxics in combination with modulator, and can be due either to the chemotherapy agent or to the modulator used (e.g. CSA). The enhanced nausea observed with etoposide and reversal agents may be due to a combined effect on the blood brain barrier (point 3). Enhanced normal toxicity from the cytotoxic has been observed, for example parasthesia and constipation with vincristine. Other anticipated adverse events may also occur such as an increased severity of myelosupression or mucositis with etoposide.
A direct effect of the modulating agent.

For the purposes of reversing MDR modulator drugs are often used in much higher doses, or for more prolonged periods, than is usually recommended.

Events attributable to verapamil toxicity are largely cardiovascular, with first degree heart block and hypotension being the commonest observed effects. Second and third degree heart block may also occur as may arrhythmias or cardiac failure. Other side effects include constipation, peripheral oedema, headache and reversible changes in liver enzymes (Pennock et al 1991).

The serum concentrations of CSA achieved when this drug is used to modulate MDR are often above 1000 ng/ml, at these levels nausea and vomiting are relatively common, as are hypertensive episodes and renal impairment, less commonly seen effects are dysasthesia, headaches, myalgia and hypomagnesaemia.
An effect of the modulating agent upon Pgp expressed at other sites.

If the concentration of modulator used is sufficient to affect tumour Pgp, it will also affect Pgp expressed normally. This may account for some of the unexpected adverse events which are recorded in clinical reversal studies, for example the hyperbilirubinaemia produced by CSA and quinine.

Bilirubin is a low-affinity substrate for transport by Pgp, however hyperbilirubinaemia is only seen with certain modulators. This may be because some modulators are not present at concentrations high enough to affect biliary Pgp. Or alternatively because hyperbilirubinaemia is related to an effect on the MDR 3 gene product (known to be expressed in the liver), and not all agents used to modulate Pgp affect this protein whose nature remains unknown. Other ATP-dependent carriers in the liver include bile salt export carrier (BSEC) and leukotreine export carrier (LTEC), both of these are affected by CSA (Bohme et al 1993), and may therefore play a part in CSA related hyperbilirubinaemia. The increased nausea associated with etoposide in combination with a reversing agent is often disproportionate to the sum effect expected. It is known that capillary endothelium within the CNS expresses Pgp, and has been postulated that modulation at this site may lead to breakdown of the blood brain barrier allowing increased CNS drug concentrations.

Interestingly, whilst increased myelosuppression is frequently seen with drugs such as etoposide and doxorubicin, delayed recovery is not. As Pgp is known to be expressed on CD34+ stem cells this might have been expected.
1.5.2 In vitro prediction of in vivo effect

Clinical use of modulating agents has generally been directed at achieving a serum concentration equivalent to that required to reverse MDR in vitro. This usually causes significant side effects and in many patients is unattainable. The modulators which have been most extensively studied are verapamil and CSA.

From in vitro studies 2 - 6.6µM (1000 - 3200 ng/L) of verapamil is required to consistently modulate MDR (Tsuruo 1983a, Twentyman 1986b), though clinical experience suggests that 300 - 500 ng/ml is effective in adults and children (Cairo 1989, Salmon 1991). In patients taking verapamil for cardiovascular disease, serum verapamil levels above 100 ng/ml are required, and levels up to 900 ng/ml have been recorded without adverse effects (Reiter 1982).

Clinical trials using verapamil infusions for MDR modulation have achieved peak verapamil levels of 1800 ng/ml (Ozols 1987), at these levels in adults almost all were symptomatic. It may not be necessary to aim for such high concentrations in patients as the in vitro studies are often done on extremely resistant cell lines with amplification of the MDR 1 gene or gross overexpression. Some studies have shown MDR reversal with relatively low concentrations of modulator and verapamil appears to have some effect at serum concentrations below those shown to be useful in vitro (Benson et al 1985, Cairo et al 1989, Dalton et al 1989). This has led to the suggestion that verapamil may itself have some tumoricidal properties. Whilst collateral sensitivity has been seen with verapamil this is at much higher doses than used clinically.

In vitro work using CSA shows modulation with between 100 ng/ml and 10 000 ng/ml (Slater 1986, Twentyman and Wright 1991a) of CSA.
In clinical practice using CSA as an immunosuppressive agent concentrations of between 100 - 500 ng/ml are recommended. Long term CSA concentrations of more than about 500 ng/ml are often associated with renal toxicity, and prolonged exposure to lower CSA concentrations may also be nephrotoxic (Keown et al 1981).

Clinical trials using CSA to modulate MDR have achieved much higher concentrations for a short period (of a few days). Using a continuous intravenous infusion levels of up to 4800 ng/ml have been recorded (Yahanda 1992), and most groups achieve between 1000 - 3000 ng/ml. In most cases a loading dose has been used to ensure adequate serum concentrations prior to chemotherapy, however the importance of this is unclear

The non immunosuppressive CSA analogue PSC 833 is more potent than CSA both *in vitro* and *in vivo* (Boesch et al 1991) using mice bearing MDR-P388 tumours. These chemoresistant tumours are almost always fatal. Following treatment with etoposide and CSA survival time was increased slightly, but this was only significant when PSC 833 was substituted for CSA.
1.5.3  **Clinical studies in adults**

On the whole any beneficial effect of MDR modulation in adult patients has been disappointing. The most encouraging report is of three responses in eight patients (seven with multiple myeloma and one with lymphoma) who progressed on treatment with VAD (continuous infusion vincristine and doxorubicin with oral dexamethasone), and then went on to receive VAD with the addition of a continuous infusion of verapamil (Dalton et al 1989).

**Verapamil**

Verapamil has been widely evaluated as a resistance modifier in adults with a range of malignant diseases. In most cases high dose racemic verapamil has been given by continuous intravenous infusion. There are however a few reports using oral racemic verapamil or D-verapamil.

Both verapamil and its principle metabolite norverapamil are active in modulating MDR in vitro (Merry et al 1989), however norverapamil has fewer cardiovascular effects (Hamann et al 1984). In clinical practice concentrations of up to 3000 ng/ml are possible (Dalton et al 1989), though more realistically, concentrations of 200 - 500 ng/ml can be maintained with little toxicity (Benson et al 1985). However serum levels do not accurately reflect tissue or tumour levels which tend to be higher (Hamann et al 1984).

The first reported phase I study using continuous infusional verapamil to modulate MDR was reported in 1985 (Benson et al 1985), using a loading dose of verapamil (0.02 - 0.1 mg/kg) followed by a maintenance infusion (0.036 - 0.18 mg/kg/hr) for 132 hours. This was administered along with a continuous infusion of vinblastine (1.5 mg/m$^2$/day
for five days) to 17 adults with a variety of advanced relapsed malignant diseases. The observed cardiovascular toxicity consisted of first degree heart block, junctional rhythms and non specific T wave changes. Other toxicity included haematological (neutropenia and thrombocytopenia), and neurological (peripheral neuropathy and myalgia). The steady state verapamil concentrations achieved were between 200 - 400 ng/ml for a continuous infusional dose of 0.12 mg/kg/hr.

An alternative approach (Ozols et al 1987) was to treat each patient (all had advanced ovarian cancer) with a continuous infusion of verapamil in a dose escalating fashion until hypotension or heart block developed. This dose was then maintained for 72 hours, with a 24 hour infusion of adriamycin given on the second day of the verapamil infusion. All patients were electively treated on the intensive care unit, two of the eight patients treated developed atropine responsive complete heart block, and four patients developed congestive heart failure. Delivery of these doses of verapamil resulted in serum verapamil concentrations of up to 2767 ng/ml (about 6 μM), which is the level required to reverse resistance in vitro (Tsuruo et al 1982, Twentyman et al 1986b). There were no objective responses to this regimen, though two patients had stabilisation of their disease.

Cardiovascular toxicity is the most commonly reported dose limiting factor in trials attempting to escalate the administered dose of verapamil (Ozols et al 1987, Miller et al 1991, Salmon et al 1991). Other side effects commonly attributed to verapamil rarely influence the decision to reduce or to stop the infusion.

Whilst it is generally assumed that a higher serum verapamil level is more effective in modulating the MDR phenotype, this is not consistently borne out by the results (Cairo
et al 1989, Dalton et al 1989). For example Cairo (1989) used a continuous infusion of verapamil (0.005 mg/kg/h) and achieved steady state concentrations of 450 ng/ml, 8/11 patients achieved a PR (a mixed group of paediatric tumours). Dalton (1989) used a continuous infusion of verapamil (0.15 mg/kg) in patients with multiple myeloma and achieved a steady state concentration of 200 - 300 ng/ml in two patients who achieved a PR, and 1342 ng/ml in one patient who attained a temporary CR, though this patient received a higher dose of verapamil.

It may be that it is tissue or tumour concentration which is important, or because the amount of verapamil required to modulate MDR in vivo is less than that shown to be necessary in vitro.

Continuous low dose racemic verapamil has been used in patients with anthracycline resistant breast cancer (Belpomme et al 1994). 99 patients were treated, half with vindesine and 5 FU alone and half with additional verapamil. Verapamil was given orally at a dose of 120 mg twice a day for 28 days, this resulted in few side effects when compared with patients not receiving it. The response rate in the chemotherapy alone arm was 5/47 compared with 14/52 in those also receiving verapamil. This suggests a benefit from continuous low dose verapamil, though the mechanism is unclear.

Oral verapamil has been used in adults to try and provide an effective regimen which is less toxic than intravenous verapamil. There is evidence (Hamann et al 1984) that when verapamil is administered orally the hepatic first pass effect differs from that seen with intravenous administration, resulting in lower concentrations of the more cardiotoxic L-isomer and higher concentrations of the D-isomer.

Two of the published studies were randomised, one in small cell lung cancer (SCLC)(Milroy et al 1993), the other in non small cell lung cancer (NSCLC) (Millward
et al 1993), both used the same dose of verapamil (480 mg/day in divided doses) for
five or three days respectively. Neither study had significant problem with
cardiovascular toxicity, though the NSCLC study had an increased rate of neurological
problems (principally constipation and peripheral neuropathy) in the verapamil arm.
The SCLC study (Milroy et al 1993) randomised 226 patients and found no difference
in either response rate or overall survival. The study in NSCLC (Millward et al 1993)
recruited 72 patients, 66 of these were evaluable for response. This study showed a
significant improvement in overall response rate (41% versus 18%), and some
improvement in survival (all patients were dead at 96 weeks in the 'no verapamil' arm,
whilst two were still alive at six months in the verapamil arm).

D-verapamil has been used in an attempt to overcome the cardiovascular side effects
seen with the racemic agent following the observation that both optical isomers of
verapamil are equally effective in vitro as MDR modulators (Plumb et al 1990, Hollt et
1993) suggest that whilst D-verapamil is less cardiotoxic than the racemic mixture a
significant number of patients develop cardiovascular side effects until the dose is
dropped to 800 mg/day or less (resulting in a serum level of about 1000 ng/ml) higher
serum concentrations have been made tolerable however by the addition of
dexamethasone. There is also the observation that when given with an anthracycline, the
resulting myelotoxicity was greater than expected, implying that a pharmacokinetic
interaction occurs between the cytotoxic agent and D-verapamil (and not with racemic
verapamil). Out of ten patients treated on one of these studies (Bissett et al 1991) there
was only one (partial) response.
A more recent study (Bates et al 1994) treated 66 patients (relapsed lymphoma) with a regimen consisting of chemotherapy (etoposide, prednisolone, vincristine cyclophosphamide and doxorubicin) and D-verapamil (dose escalating to a maximum of 1200 mg/day). They found cardiovascular toxicity to be dose limiting though reduced if D-verapamil was given 4 hourly rather than 6 hourly. Twelve of the 62 evaluable patients had a response to this, including two complete responses. MDR 1 expression (PCR method) was quantified in some of the patients pre, peri and post treatment. In some (7/15) there was an increase in MDR 1 following chemotherapy alone, these patients appeared to do better (in terms of response) when D-verapamil was added to chemotherapy.

Whilst a wide range of modulating agents are being investigated in the laboratory, the number of agents used in clinical trials remains limited. Other calcium channel blockers have been evaluated, including diltiazem (Bessho et al 1985), nifedipine (Philip et al 1992) and bepridil (Linn et al 1994), however use of these is also restricted by dose limiting cardiovascular toxicity.

ii Cyclosporin

The modulating agent generating most interest recently is cyclosporin, which has been used in acute leukaemia (Sonneveld et al 1990, List et al 1993), multiple myeloma (Sonneveld et al 1992), and in a collection of relapsed and resistant malignancies (Yahanda et al 1992, Bartlett et al 1994). The regimens used generally consist of a loading dose of up to 6 mg/kg over two hours followed by a continuous infusion of up to 18 mg/kg/day for at least 60 hours. In a series of 42 patients with acute myeloid leukaemia (List et al 1993) there were 29 responses, and in 21 patients with multiple
myeloma (Sonneveld et al 1992) an overall 48% response rate included a more favourable subgroup of patients who expressed MDR 1 and had a 58% response rate. List (1993) treated 42 patients with poor risk AML with cytarabine and daunorubicin. All of these patients received a CSA infusion for 72 hours (i.e. coincident with the daunorubicin infusion). The dose of CSA was escalated from a loading dose of 1.4 to 6 mg/kg and a continuous infusion dose of 1.5 to 20 mg/kg/day. Steady state CSA concentrations of greater than 1500 ng/ml were achieved in all patients receiving more than 16 mg/kg/day continuous infusion. 26 patients achieved a CR or restored chronic phase, 3 a PR giving an overall response rate of 69%.

Patients with VAD refractory multiple myeloma were treated (Sonneveld et al 1992) with identical chemotherapy (VAD) except for the addition of a continuous infusion of CSA (5 - 10 mg/kg/day). All patients who received 7.5 mg/kg/day or more achieved serum CSA concentrations of at least 1000 ng/ml. There were 17 responders, and in 6 of these no MDR 1 positive (C219 by IHC) myeloma cells could be detected.

Concern about the cyclosporin related alteration in etoposide pharmacokinetics has lead to the suggestion that etoposide concentrations need to be measured and the dose adjusted when given concurrently with cyclosporin (Yahanda et al 1992). This would allow a meaningful comparison to be made between the response seen with etoposide given alone, and the response seen with subsequent courses of treatment where etoposide is given with cyclosporin. In this way the additional benefit (in terms of disease response) can be assessed and excess toxicity avoided.

PSC 833 has been used in phase I studies in combination with etoposide, taxol and VAD (vincristine, doxorubicin and dexamethasone) in adult patients with solid tumours and multiple myeloma.
Etoposide or taxol alone were given to patients with resistant tumours (Lum et al 1994, Fisher et al 1994), if there was no response to this oral PSC 833 alone was given (to assess response and pharmacokinetics). Further courses consisted of PSC 833 and cytotoxic (with assessment of response, adverse events and PSC 833 pharmacokinetics). As seen by other groups the dose limiting toxicity of PSC 833 was cerebellar with reduced proprioception and ataxia, this effect was most marked 2 hours after each oral dose and in some cases persisted for weeks. Etoposide doses had to be reduced by more than 50% when given with PSC 833 to achieve the expected degree of myelosuppression. In this (and other) studies a marked difference in the bioavailability of different oral preparations was seen.

Thirty five patients (relapsed solid tumours) have been treated at Cambridge (unpublished meeting report 1994) with intravenous etoposide and PSC 833. Significant increases in etoposide AUC were demonstrated when given with PSC 833 up to a PSC dose of 4 mg/kg/day, above this there was no further change. Severe neurotoxicity was observed in two patients with many more experiencing minor neurological events (paresthesia or dizziness), hyperbilirubinaemia, nausea and vomiting.

In VAD refractory multiple myeloma patients (Sonneveld et al 1994) given VAD with PSC 833, all cytotoxic drugs had to be dose reduced by 50% - 75% (following the first course) on the basis of toxicity and doxorubicin concentrations. Adverse events were similar to those in other studies except for a higher incidence of constipation (attributed to vincristine).
Other agents

Tamoxifen

There is a report on the use of high dose oral tamoxifen (480 or 720 mg/day for six days) with responses in relapsed patients (one each, adenocarcinoma and lymphoma) when given in conjunction with etoposide (Stuart et al 1992). The adverse events associated with this regimen included nausea, vomiting, dizziness and malaise.

Quinine and Quinidine

Quinine (Solary et al 1992) and quinidine (Wishart et al 1993) have both been used as resistance modifiers. Quinine (30 mg/kg/day as a continuous intravenous infusion for five days) was given with mitoxantrone and cytarabine in the treatment of adults with relapsed or refractory acute lymphoblastic leukaemia. There was a response in ten of the 14 patients treated, though most patients went on to receive additional agents depending upon their status (in some cases including a bone marrow transplant). Myelosuppression, nausea and vomiting were common side effects, as was tinnitus and vertigo (10/14), mild hearing loss and a raised serum bilirubin were noted in some patients. Quinidine has been used with epirubicin in a randomised trial of treatment for advanced breast cancer, there was little excess toxicity in the group receiving quinidine, and no additional responses over the placebo group (100 patients in each group).

Trifluoperazine

The calmodulin inhibitor trifluoperazine has been used in a phase I / II trial in the treatment of resistant and relapsed adult malignancy (Miller et al 1988). Oral trifluoperazine (20 - 100 mg/day) was administered for six days together with a 96 hour infusion of adriamycin. The MTD of trifluoperazine was 60 mg/day, with a dose limiting side effect of extrapyramidal toxicity. Out of 36 patients treated seven had a response, these were all in the group who had previously responded to treatment.
Cremophore EL

This has been used in a phase I trial on a mixed group of adult relapsed patients (Rischin et al 1994) as a six hour infusion with bolus doxorubicin. The dose limiting toxicity was neutropenia, two out of 19 patients achieved a partial response.

iv Combinations

Another approach has been to combine two modulating agents (Figuerdo et al 1990), such as oral verapamil (up to 360 mg/day for seven days) and oral tamoxifen (up to 100 mg/day for seven days) in patients with SCLC being treated with vincristine, doxorubicin and etoposide. At these doses there was no significant toxicity attributable to the modulators. The overall response rate was 59%, though this dropped to 40% in the subgroup who had previously been treated, these response rates are said to compare favourably with other studies (which report response rates of 12-21% in this group). Response and toxicity were greater in those patients receiving higher doses of modulators.

The South West Oncology Group have used quinine (400 mg tds) and verapamil (240 mg bd) for six days with chemotherapy (cyclophosphamide, vincristine, doxorubicin and dexamethasone) to treat previously untreated poor prognosis Non Hodgkins Lymphoma patients. This treatment was compared with a historical group receiving a similar protocol. Toxicity in the group receiving verapamil and quinine was far greater than in the control group, 2/73 fatalities compared with 3/255, and 44/73 neutropenic episodes compared with 49/225). There are no data yet on efficacy.
1.5.4 Clinical studies in children

There is very little published work on the clinical modulation of MDR in children and to date only three reversal agents have been used.

1. Diltiazem

In 1985 Bessho et al reported a trial of oral diltiazem (1.69 - 4.83 mg/kg/day for four days) with one bolus dose of vincristine (1.5 mg/m$^2$) given on the second day. This was given on a weekly basis to six children with refractory acute lymphocytic leukaemia (all had previously been treated with multiagent chemotherapy including vincristine). Leukaemic blast cytolysis was seen in four out of the five patients who were able to take oral medication. Two children developed second degree heart block with bradycardia which was reversible on stopping the diltiazem.

2. Verapamil

Continuous intravenous verapamil has been used in children (Cairo et al 1989) in a regimen giving a bolus of vinblastine (2 mg/m$^2$) followed one hour later by a continuous infusion of etoposide (200 mg/m$^2$/day for five days, 1 g/m$^2$/course total). A verapamil infusion was started 24 hours prior to the vinblastine bolus, with a loading dose of 0.15 mg/kg followed by a maintenance infusion of 0.005 mg/kg/min for 144 hours. Eleven courses of this treatment were given to seven children with relapsed malignant disease, (including five leukaemia, one neuroblastoma and one hepatoblastoma). All of the seven children had previously been treated with either etoposide (four) or vincristine (five). Heart block (first or second degree) was seen in five of the eleven courses, and inotropic support was needed in two courses. Steady
the treatment originally received by these children. However, whilst the three children with neuroblastoma who responded to etoposide in combination with verapamil had previously only received $200\text{mg/m}^2$ etoposide, they had failed to respond to this. In addition the three children with rhabdomyosarcoma had relapsed having received etoposide in doses of up to $500\text{mg/m}^2$/course.

In this study serum etoposide concentrations were not evaluated hence the effect of verapamil on the AUC of etoposide cannot be assessed.

3. Cyclosporin

There are three short reports describing the use of cyclosporin as an MDR reversing agent in children.

The Toronto group report the use of cyclosporin in patients with soft tissue sarcoma, neuroblastoma and retinoblastoma with evidence of P gp expression at presentation, (Chan et al 1992a & b). As these were all new cases they are not evaluable for reversal of previously demonstrated resistance to chemotherapy. In these studies a daily three hour cyclosporin infusion has been used for 2 or three days. CSA dose escalation to a daily dose of 33 mg/kg/day has proved tolerable with vincristine and teniposide in patients with retinoblastoma.

Cyclosporin has also been used in two children with progressive metastatic osteosarcoma (Schwartz et al 1992), this study used a continuous CSA infusion of 12 mg/kg/day for three days in conjunction with doxorubicin and cisplatin. One patient had been documented to have P gp negative disease at presentation (IHC with C 219), with strong positivity on relapse, both children had a partial response to the combination of CSA and cisplatin/doxorubicin.
state verapamil levels were above 400 ng/ml. None of the patients achieved a complete response, however all patients (except the child with hepatoblastoma) had a partial response to one or both courses. Subsequently all patients developed progressive disease and died.

Twenty patients with relapsed paediatric malignancy (8 neuroblastoma, 5 rhabdomyosarcoma, 3 Ewing's sarcoma, and one each Hodgkin's, osteosarcoma, Wilms tumour and ALL, age range 10 months to 43 years, median 6½ years), have been treated with a continuous infusion of verapamil at the Royal Marsden Hospital. A continuous infusion of verapamil was used, starting with a two hour loading dose of 0.1 mg/kg followed by a 72 hour infusion of 0.15 mg/kg/hr. Etoposide was given daily (150 mg/m²/day) in three doses (each over one hour), the first dose 12 hours into the verapamil infusion. Monitoring was simply performed with daily ECG and 2 hourly blood pressure and pulse recordings. Thirty five treatment courses were given, 5 courses required a verapamil dose reduction due to cardiovascular toxicity (bradycardia or first degree heart block). No patient required intensive care or monitoring, and all patients were able to complete the verapamil infusion. A peak serum verapamil concentration of 700 ng/ml was seen in one patient, however most patients achieved and maintained a verapamil concentration of 100-300 ng/ml. There was no correlation between verapamil concentration and either toxicity or response, though all of the patients who required a verapamil dose reduction were over 14 years old. Six patients showed a response to this regimen, 3 with neuroblastoma, one of whom remains in complete clinical remission at more than 30 months following the first course of verapamil. The other three responders had rhabdomyosarcoma, one of these had a good partial response and is alive with disease at more than 30 months following the first course of verapamil. It is recognised that the dose (per course) and schedule of etoposide in this regimen differs from that in
CHAPTER 2.
THE CHEMOSENSITIVITY OF PAEDIATRIC TUMOUR CELL LINES AND MODULATION OF RESISTANCE WITH VERAPAMIL AND CYCLOSPORIN.
2.1 INTRODUCTION

2.1.1 What can be learned from \textit{in vitro} chemosensitivity assays

The principle aims of these assays are to identify new agents which are active against malignant cells and to aid in the investigation of chemosensitivity patterns seen between different tissues. In addition these assays can be used to develop potential therapeutic interventions to circumvent resistance or to improve cytotoxicity.

\textit{In vitro} chemosensitivity testing can be performed on established cell lines and in some cases on fresh tumour material. In the latter case information can be collected which might influence the management of individual patients.

Use of continuous cell lines for \textit{in vitro} drug sensitivity screening was recognised as a useful tool in the early 1980's by Hill et al (1981) who were able to demonstrate the effectiveness of a range of cytotoxics on a neuroblastoma cell line using a colony forming assay. In 1987 the National Cancer Institute (NCI) in America made changes to its drug screening program to include a wide range of human tumour cell lines which were investigated with chemosensitivity assays (Carmichael et al 1987). At the same time they introduced a semiautomated assay which utilised the ability of living cells to reduce a tetrazolium based compound (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to formazan (this has become known as the MTT assay), which was already in use in Japan. Using this approach the NCI was able to screen large numbers of cell lines from different tumour types with new agents as a preliminary step to developing useful drugs.
2.1.2 **Chemosensitivity assays in use**

The importance of using cell lines of human origin was recognised following fears that rodent cell lines might display drug sensitivities which substantially differ from those seen in human lines (Drewinko et al 1980). With the ability to establish cell lines of human tumour origin in the 1970's it was recognised that these would be both more convenient to work with than tumour specimens and would also provide information on a single cell type (cells isolated from tissue samples are rarely all of tumour origin).

Various assays have been developed and are currently in use. The ideal assay would be quantitative, reliable and physiologically relevant. Unfortunately none of the currently available assays meet all these criteria (which should also include a high degree of sensitivity and specificity). Clinically, the sensitivity of an assay is more important than specificity as it allows prediction of those agents which might be useful. Sensitivity however, cannot be predicted as accurately as resistance to an agent.

The population of cells measured differs between assays. It is not always clear how important this is and whilst cell lines are generally thought to consist of a homogenous cell phenotype, they will not all be going through the cell cycle at the same rate. Cells derived from tumour material will contain tumour cells which may not all be identical in addition to cells from surrounding tissues or stroma. Some of the tumour cells will be capable of indefinite division, whereas others may be temporarily or permanently unable to enter the cell cycle.

The simplest assessment of chemosensitivity is to count cell numbers following exposure to an agent. This can be done utilising a vital stain such as trypan blue to ensure only viable cells are counted. This method makes no distinction between malignant and non malignant cells.
The clonogenic assay is considered by many to be a 'gold standard' to which all other assays should be compared. It has been in use for many years and there is a good understanding of its benefits and drawbacks. The principal advantage of this type of assay is that it only counts those cells which have the ability to replicate. However, disadvantages include duration of the test (from a few days to two to three weeks), the requirement for cells to be able to divide in test conditions (often agar), artefact arising from the presence of normal cells and the inability to quantify reduced cell division rate (as evidenced by smaller colonies) which may be seen following exposure to some cytotoxics. In addition cells from clinical specimens are often unable to form colonies under the conditions of the assay.

The DNA incorporation assay is a non clonogenic method in which cells are exposed to labelled thymidine which is incorporated into DNA as cells go through the cell cycle. The amount of incorporation reflects the number of cells undergoing mitosis. Problems with this assay include its inability to distinguish between tumour and non tumour cells, and the importance of accurately timing thymidine exposure.

Another non clonogenic assay is the DiSC (differential staining cytotoxicity) assay. The principle advantage of this labour intensive method is that it can distinguish between malignant and non malignant cells on the basis of morphology. However, in order to achieve this all the cells have to be examined by an experienced operator.

Two commonly used automatable (and rapid) non clonogenic methods are the MTT and SRB (sulforhodamine B) assays. The MTT assay was first described in 1983 by Mosmann, and has since been modified to allow automation and the use of both cell lines and fresh tumour cells. Advantages of this method include the relatively small cell numbers required, lack of observer error in reading results, time required to perform it and the fact that the end point is a measure of the number of viable cells. If the assay is
set up with small numbers of cells it can be assumed that high cell numbers seen at the end of the test are a result of cell proliferation.

The SRB assay measures total cellular protein content as an indicator of cell number. It has similar advantages to the MTT assay but is more easily automatable, as a result of this the NCI is now using this assay in its drug screening program.

The major disadvantage of both these assays is their inability to distinguish between a cytostatic and a cytotoxic effect attributable to the agent under investigation.

The type of assay chosen will depend in part on the previous experience of those who are to perform it, the type of material to be examined and experimental design.

For the purposes of this project a simple reliable method was required which would measure either cell numbers or proliferation. The ability to differentiate between malignant and non malignant cells was not important as cell lines were used. The MTT assay therefore seemed ideal, and enabled variations in drug exposure schedules to be readily studied.
2.1.3 The MTT assay

This assay was first used to measure the proliferative influence of lymphokines, mitogen stimulators and complement mediated lysis (Mossman 1983). It has subsequently been modified as a chemosensitivity assay and is used on both cell lines and tumour specimens.

The basis for this method is that mitochondria in viable cells convert the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to a blue-black formazan product. This is quantified following solubilisation by measuring the optical density of the solution, typically with a microtitre plate spectrophotometer. It is thought that the mitochondrial enzyme succinate dehydrogenase is responsible for this reaction which only takes place in metabolically active cells (Slater et al 1963). In the presence of sufficient MTT the amount of formazan product is proportional to the number of viable cells. The intensity of the optical signal may, however, be diminished by certain methodological factors. For example, Pagliacci et al (1993) recently showed that genestin (a natural isoflavone) induces a G2/M arrest in tumour cells resulting in inhibition of cell proliferation. However this compound also enhanced the conversion of MTT to formazan in viable cells, leading to a gross discrepancy between the estimation of cell numbers from MTT assay and direct observation of cell numbers present in microtitre plates. This highlights the importance of direct observation of cells in culture and the documentation of any direct effect of the study drugs on MTT.

Comparisons have been made between the MTT assay and the clonogenic, SRB and dye exclusion assays showing good comparability in its application both to cell lines and leukaemic blasts (Pieters et al 1989, Perez et al 1993). This method (MTT) is also used in solid tumours, for example, Wilson et al (1990), have used it in ovarian cancer cells
present in specimens of malignant ascites. There is very little published data using *in vitro* chemosensitivity testing on material from solid tumours (except cells collected from malignant effusions or ascites). Of those published none have used the MTT assay on this type of specimen. A large study from Sweden (Fridborg et al 1994) used a proliferation assay which employs a fluorescent label to mark cells with an intact membrane. This group looked at 322 samples from a variety of tumours (less than half were leukaemia), and assessed the activity of cyclosporin A and PSC 833 in modifying the response to doxorubicin or vincristine. They found that there was a modulating effect in leukaemias, childhood tumours (only one was tested) and ovarian tumours. Another study used tritiated thymidine to measure DNA content as a measure of proliferation (Veneroni et al 1994). The chemosensitivity of breast and ovarian tumours was investigated to a variety of cytotoxic agents and this was correlated with P gp expression detected by immunohistochemistry (C 219 antibody). They found that tumours expressing P gp were more likely to be resistant to doxorubicin, and that breast tumours not expressing P gp were more likely to respond (clinically) to vincristine and doxorubicin.
2.1.4  Modulation of MDR in vitro

The effect of agents which influence the chemosensitivity of cells expressing the multidrug resistant phenotype has been demonstrated in a variety of ways. Both short term and prolonged exposure to cytotoxic agents and modulators can be assessed using most assays. The effect of a modulator treatment can be evaluated in cells exposed to a cytotoxic agent. In the MTT assay the IC 50 is the test drug concentration which results in an OD half that of the control, The effect of modulators can then be assessed by comparing the IC 50 with and without those agents.

The sensitisation ratio (SR) of a modulator is the ratio between the IC 50 without and with the agent under investigation Thus a treatment with a high SR in the context of reversing MDR is more effective than one with a low SR.

The SR is a useful way of comparing the efficacy of various treatments within any given model, it is however difficult to directly compare the SR obtained from one model with the SR in another.
2.1.5 The role of scheduling of modulator administration \textit{in vitro}

Both Boiocchi et al (1992) and Cass et al (1989) report evidence (clonogenic assays) suggesting that the continuous presence of a modulator is more effective at reversing resistance than discontinuous exposure. Julia et al (1994) used clonogenic assays to look at combinations of exposure to modulator. They suggest that with a short cytotoxic exposure (one hour), the most effective modulator schedule is peri and post or pre/peri and post cytotoxic exposure.

Toffoli et al (1993) studied the effect of prolonged exposure to verapamil in resistant colon carcinoma lines using clonogenic methods. The addition of verapamil to doxorubicin enhanced the cytotoxicity seen with doxorubicin alone. Increasing duration (doxorubicin and verapamil) of exposure and increasing verapamil concentration further increased toxicity. If verapamil alone was continued after exposure to doxorubicin and verapamil for 8 - 16 hours a significant enhancement of cytotoxicity was seen.

Using a combination of clonogenic, MTT and thymidine incorporation in myeloma and epidermoid carcinoma cell lines, Lang et al (1994) showed that prolonged exposure to vinblastine alone decreased resistance to vinblastine. Exposure to doxorubicin or taxotere had no effect (on resistance to doxorubicin or taxotere respectively), and to etoposide alone significantly increased resistance to subsequent exposure to etoposide. The addition of verapamil or CSA significantly increased cytotoxicity of all these drugs at exposure times of 1 - 72 hours.

These observations may have important implications on the drug schedules chosen in clinical trials, and may aid in the understanding of the mechanism of modulation.
2.2 MATERIALS AND METHODS

2.2.1 Cell lines and culture conditions

All the cell lines used in this study are of human origin.

H69 P and H69 LX 4 were used as standard reference cell lines. H69 P was originally developed at the NCI by Dr D. Carney from a patient with small cell lung cancer who had previously received multiagent chemotherapy (including doxorubicin). H69 LX4 is a derived multidrug resistant line which was developed and kindly provided by Dr Twentyman (Twentyman et al 1986a & b). Both H69 P and H69 LX4 grow as floating cell aggregates and are cultured in standard conditions (below), however the drug resistant line H69 LX4 is maintained in 0.4μg/ml doxorubicin. Twentyman's group (Reeve at al 1989) have extensively investigated these cell lines (and others derived from them) and shown that H69 LX4 is highly resistant with overexpression of P glycoprotein.

CCRF CEM is a T cell leukaemia line developed (Foley et al 1965) from a 3 year old who had received both radiotherapy and multiagent chemotherapy (no anthracyclines). This line grows in standard conditions as a single cell suspension. It is highly sensitive to all chemotherapy agents and has been used by a number of groups who have developed resistant sublines with various chemotherapy agents.

Four rhabdomyosarcoma cell lines were available for investigation, RMS, RD, HS 729T, and HX 170.

RMS (Garvin et al 1986) was established from a malignant effusion which developed in a 14 year old girl with a rhabdomyosarcoma arising in the chest wall. She was initially treated on an Intergroup Rhabdomyosarcoma Study (IRS) protocol (vincristine,
actinomycin and cyclophosphamide), but relapsed 9 months from the end of treatment with disseminated disease including pleural effusion. In this case initial diagnosis proved difficult, however evidence of myogenic differentiation (from tissue culture) and karyotypic analysis (revealing a 2:13 chromosomal translocation) suggested a sarcoma, and at autopsy tissue was obtained which enabled a firm diagnosis of alveolar rhabdomyosarcoma to be made.

HS 729T (also known as ATCC HTB 153), was established from a 74 year old patient with a rhabdomyosarcoma in the leg. This was treated with radiotherapy and amputation. The cell line was established from a post radiotherapy specimen, it is not clear if this patient received chemotherapy.

RD (also known as ATCC CCL 136, TE 32, and 130T) was established from a pelvic embryonal rhabdomyosarcoma arising in a 7 year old patient. No details of treatment are available.

Both HS 729T and RD were obtained from the American Type Culture Collection (ATCC). Details about the patients prior treatment are incomplete.

HX170 was established from a paratesticular embryonal rhabdomyosarcoma recurring in a heavily pre-treated 5 year old (Kelland et al 1989). Chemotherapy had been administered over a two year period and included vincristine, adriamycin and cyclophosphamidene.

All four rhabdomyosarcoma cell lines grow as adherent monolayers and are cultured under standard conditions.

Three neuroblastoma cell lines were available for investigation, two of these originate from the Sloan Kettering Memorial Hospital (SK N BE and SK N SH), and the third from the Royal Marsden Hospital (HX 142).
SK N SH was established from a bone marrow aspiration from a four year old girl with progressive metastatic neuroblastoma (Biedler 1973). At the time the sample was taken she had received treatment with radiotherapy and chemotherapy (which included vincristine, cyclophosphamide, and daunomycin). This line was initially reported as having two distinct cell populations, epithelial and spiny, it is currently thought likely that SK N SH represents cells of neuroepithelial origin.

SK N BE(2) was established from a bone marrow biopsy of a two year old heavily pre-treated patient with disseminated neuroblastoma (Biedler et al 1978).

Both of these lines are mIBG positive and SK N BE(2) is known to amplify N MYC 200 fold (SK N SH only has one copy).

HX 142 is a neuroblastoma cell line developed from a human tumour xenograft involved bone marrow in a mouse (Deacon et al 1985). It is unclear if this tumour had previously been treated with chemotherapy, it was however known to be radiosensitive.

SK N BE(2), SK N SH and HX 142 are all cultured in standard conditions and grow as adherent monolayers.

All cell lines were cultured in 75cm² plastic tissue culture flasks in a humidified incubator at 37°C with 5% CO₂. Standard culture media was used, this was either RPMI 1640, D MEM, or Ham's F12 (see below). All media was supplemented with 10% fetal calf serum (Gibco BRL), penicillin and streptomycin. Cell lines were fed or subcultured every two to three days, adherent lines were trypsinised to remove them from the flask, spun down and resuspended at the required cell density.
Culture medium RPMI 1640 was used for the following cell lines: H69 P, H69 LX4, CCRF CEM.

D MEM was used for: RD, HS 729T, SK N BE, SK N SH, HX 142 and HX 170.

D MEM: Ham's F 12 (1:1) was used for RMS.
2.2.2 **MTT assay**

The MTT assay used in this study is outlined below, it differs slightly from the various reported assays following modifications to improve reproducibility at this institution.

The method is essentially the same for cell lines which grow in suspension and for those which grow as a monolayer, until the final stages.

Cells are harvested from tissue culture flasks, and counted with a haemocytometer and viability stain (trypan blue). Cell numbers are then adjusted (by resuspension in medium) following centrifugation (2000 rpm in Beckman TJ6 centrifuge), to give the required cell density. The cell density used was typically in the order of $10^5$ cells per ml (final well concentration).

Cells were then plated out into 96 well flat bottomed microtitre plates. This was done by adding cells in 100μL aliquots to each of 96 wells. Some investigators have avoided adding cells to the outer rows and columns of wells (putting medium alone into these), this followed experience suggesting cells do not multiply as effectively in these wells possibly as a result of decreased humidity. However in these experiments using a humidified incubator this did not appear to occur.

In all cases the final well volume was 200μL, this generally comprised 100μL cell suspension (in medium) and 100μL drugs.

Drugs were added in aliquots to give the desired final well concentration. All drug dilutions were in medium. Typically modulator was added in 50μL and cytotoxic in a further 50μL aliquot.

Experience showed that if cytotoxics and modulators were added within 6 hours of setting up the microtitre plates the cells were less efficient at proliferating for the remainder of the assay (2.6). To standardise this no drugs were added to the plates until 24 hours after they had been set up. In all cases (unless specified) modulators were
added 1 hour prior to cytotoxics. This arbitrary timing was an attempt to both standardise drug additions and to emulate the clinical situation where an adequate serum concentration of modulator is established prior to cytotoxic administration.

Microtitre plates were incubated for 5 days following addition of cells, at the end of this period MTT was added to all wells. MTT was added in 50μL aliquots from a 2mg/mL solution, plates were then returned to the incubator for four hours. Both the concentration of MTT used and duration of incubation with MTT was assessed for each cell line (see 2.3.5). Whilst some lines convert MTT to formazan more quickly than others, in general the concentration and incubation time chosen provided satisfactory results.

At the end of a 4 hour incubation with MTT medium was removed and the formazan crystals dissolved in DMSO (BDH). Removal of medium at this stage can be quickly and effectively performed by flicking the plates upside down over a sink, if this is done efficiently 10-20μL medium remains. This method was used for all adherent cell lines and for CCRF CEM which becomes moderately adherent under these conditions. The two lines which grow as floating aggregates had to be centrifuged (2000rpm in Beckman TJ6 centrifuge). The medium could then be aspirated with relative ease, though in these cases more medium was left in each well. DMSO (100μL per well) quickly solubilises the formazan, though in some cases gentle agitation is needed.

The plates were read on a microtitre plate spectrophotometer at a wavelength of 540nm. Results were obtained from this as a series of optical density (OD) readings.

In each experiment wells were set up so that four wells would represent each set of conditions (i.e. modulator and cytotoxic). Results which were obviously aberrant were discounted at this stage (the commonest reason for this was isolated infection in a well). The mean OD of four wells was calculated and used to construct a graph of OD against
cytotoxic concentration for each of the possible modulator conditions used (i.e. no modulator, verapamil 10μg/mL, CSA 5μg/mL, and 'double modulator').

The starting OD (i.e. the OD for those wells without cytotoxic) was then described as 100% survival, and all other results (i.e. those wells receiving the same modulator conditions but with various cytotoxic doses) were computed from this.

Graphs of percentage survival against cytotoxic dose used allowed the IC50 to be measured, and then the SR to be calculated.

Percent survival was used throughout to denote the reduction in cell numbers following a treatment. It was recognised that this reduction could either be due to a cytotoxic effect (and hence truly reflect survival) or a cytostatic effect (reflecting decreased capacity to proliferate possibly via a transient cell cycle arrest).
2.2.3 Cytotoxics and other agents

The drugs used to reverse MDR were verapamil (Sigma) and cyclosporin A (pure drug kindly supplied by Sandoz Ltd, UK). Verapamil was dissolved in warm PBS to give a stock concentration of 1mg/mL, this was then further diluted in medium as required. CSA was dissolved in 0.5ml/mg absolute ethanol, then in PBS to give a stock concentration of 1mg/mL, further dilutions were made in medium. Stock solutions of verapamil and CSA were kept at 4°C for up to 10 days and then discarded if not used.

The concentration of modulator used in these experiments was standardised following a series of trials with each cell line in which a dose-response curve was constructed to demonstrate the toxic concentration of both verapamil and CSA in this assay (2.5). Following this a final well concentration of 10μg/mL verapamil and 5μg/mL CSA was chosen, as these were the maximum concentrations tolerated by most cell lines. In all standard experiments a 'double modulator' series was included, this consisted of both verapamil and CSA to give a final well concentration of 10μg/mL and 5μg/mL respectively.

The cytotoxic agents investigated included vincristine (David Bull laboratories), etoposide (Bristol-Myers), doxorubicin (Farmitalia), cisplatin (David Bull laboratories), methotrexate (David Bull laboratories) and cyclophosphamide (kindly supplied by ASTA Medica). The clinical intravenous preparation was used in all cases (except cyclophosphamide), these were simply diluted in medium to give the desired concentration. Cyclophosphamide was supplied as the OH-cyclophosphamide compound, this was dissolved in water to produce the active 4-OH-cyclophosphamide and used immediately.
Cytotoxic agents were stored under the recommended conditions prior to dilution. In all cases these drugs were diluted or reconstituted immediately prior to use, any surplus was then discarded.

A logarithmic dose range of these cytotoxic agents was used in all cases, this was individualised for each drug and cell line to give a dose-response curve which achieved at least the IC50 and preferably the IC80.

MTT (Sigma) was dissolved in PBS to give a solution of 2mg/mL (i.e. 0.4µg MTT added to each well), as this compound is poorly soluble particulate insoluble MTT was filtered out with a 0.45µM filter. MTT solution was either used fresh or stored in the dark at 4°C for up to 10 days.

Formazan (Sigma) was dissolved in DMSO (BDH) to give the required concentration.

Verapamil concentration was measured by high performance liquid chromatography (HPLC) with fluorescence detection by Dr P.T. McCarthy at Guy's Hospital Poisons Unit.
2.3 STANDARDISATION OF MTT ASSAY VARIABLES.

2.3.1 Sources of error

In order to minimise the experimental error within this assay in each experiment a minimum of four wells of the microtitre plate received identical treatment. When the results were analysed optical density (OD) readings within 10% of the mean were considered to be acceptable. If any of the OD readings were more than 10% from the mean after excluding any obviously aberrant wells (i.e. infected or no cells), then those results were discarded. In practice those experiments with OD results more than 10% from the mean for one treatment had many other sets of wells with this degree of difference. In these cases the entire experiment was discarded. This appeared to occur more frequently when cells were used which were not in an exponential growth phase (in culture) prior to use.

In addition to this if the wells which received no modulator or cytotoxic did not have a high enough proliferation rate (i.e. OD greater than 0.5) results from that plate were discarded. An OD of 0.5 was chosen arbitrarily as a cut off, the minimum OD recorded from any wells was 0.06, with OD less than 0.12 generally reflecting the absence of any viable cells in that well.

Results from this assay never attain an OD of zero due to a constant small OD associated with both the plastic microtitre plate, the DMSO and cell debris remaining in the wells. Therefore when results are expressed as a percentage of the control (no cytotoxic) wells the lowest OD (i.e. 0.12) will typically give a 'survival' percentage of 10 - 20%, even if none of the cells were viable by trypan blue exclusion.

The major sources of inaccuracy were pipetting errors and variation in volume measured by the pipette. This resulted in slight differences in final well volumes and hence drug
concentrations between wells, and perhaps more importantly resulted in differences in the number of cells added to each well.

Cells were added to each microtitre plate as a single cell suspension which was made at the required cell density and placed in a 40ml trough. 10mls of suspension was added to each microtitre plate with an eight channel pipette.

Drug dilutions were performed immediately prior to use in at least 10ml volumes to avoid error associated with evaporation of dilutant or from diluting smaller volumes.

Finally the plates were inverted over a sink and flicked to remove medium and MTT. The addition of 100µL DMSO then solubilised formazan crystals. As illustrated later (figure 2.3f), the small volume of medium remaining in each well after flicking does have an effect on the intensity of colour developing and hence upon the final result.

A spectrophotometer (Titertek multiscan MCC/340 mark II) was used to read a series of identical wells containing either medium alone or formazan alone (0.03mg/ml or 0.1mg/ml). The reproducibility of these readings was very good at both the wavelength used (540nm) to read the 96 well plates, and at the wavelength of maximum formazan absorbance (490nm) (table 2.3a).

<table>
<thead>
<tr>
<th></th>
<th>Optical Density at 540nm</th>
<th>Optical Density at 490nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture medium (RPMI)*</td>
<td>0.072 +/- 0.004</td>
<td>0.075 +/- 0.003</td>
</tr>
<tr>
<td>Culture medium (DMEM)*</td>
<td>0.202 +/- 0.011</td>
<td>0.127 +/- 0.004</td>
</tr>
<tr>
<td>Formazan 0.03mg/ml</td>
<td>0.2 +/- 0.015</td>
<td>0.239 +/- 0.016</td>
</tr>
<tr>
<td>Formazan 0.1mg/ml</td>
<td>0.481 +/- 0.036</td>
<td>0.608 +/- 0.042</td>
</tr>
</tbody>
</table>

Results show mean (n=8) +/- 1 SD

* This medium was preincubated with metabolically active cells in culture, no cells were included in the medium used.
It was however difficult to decide if the variations seen were a result of differences in the liquid added to the wells or in the measurement of OD by the spectrophotometer. Fig 2.3a shows the OD readings for a plate (540 nm) where all wells had had 200 µL of fresh D MEM added. As illustrated the median OD is 0.744 with a range of 0.643 to 0.846, under these circumstances one might have hoped for less variation!

Figure 2.3a  Optical Density readings for one plate (96 wells) where 200µl fresh DMEM had been added to each well

<p>| | | | | | | | | | | | | |</p>
<table>
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<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.684</td>
<td>0.666</td>
<td>0.730</td>
<td>0.735</td>
<td>0.721</td>
<td>0.723</td>
<td>0.727</td>
<td>0.741</td>
<td>0.729</td>
<td>0.729</td>
<td>0.723</td>
<td>0.689</td>
<td></td>
</tr>
<tr>
<td>0.687</td>
<td>0.720</td>
<td>0.735</td>
<td>0.706</td>
<td>0.752</td>
<td>0.829</td>
<td>0.736</td>
<td>0.825</td>
<td>0.756</td>
<td>0.765</td>
<td>0.730</td>
<td>0.741</td>
<td></td>
</tr>
<tr>
<td>0.707</td>
<td>0.712</td>
<td>0.728</td>
<td>0.715</td>
<td>0.760</td>
<td>0.727</td>
<td>0.802</td>
<td>0.750</td>
<td>0.763</td>
<td>0.735</td>
<td>0.704</td>
<td>0.743</td>
<td></td>
</tr>
<tr>
<td>0.711</td>
<td>0.711</td>
<td>0.740</td>
<td>0.643</td>
<td>0.744</td>
<td>0.758</td>
<td>0.791</td>
<td>0.742</td>
<td>0.758</td>
<td>0.751</td>
<td>0.736</td>
<td>0.757</td>
<td></td>
</tr>
<tr>
<td>0.709</td>
<td>0.714</td>
<td>0.727</td>
<td>0.675</td>
<td>0.732</td>
<td>0.771</td>
<td>0.724</td>
<td>0.808</td>
<td>0.751</td>
<td>0.846</td>
<td>0.733</td>
<td>0.746</td>
<td></td>
</tr>
<tr>
<td>0.713</td>
<td>0.738</td>
<td>0.729</td>
<td>0.651</td>
<td>0.737</td>
<td>0.737</td>
<td>0.749</td>
<td>0.748</td>
<td>0.749</td>
<td>0.752</td>
<td>0.792</td>
<td>0.741</td>
<td></td>
</tr>
<tr>
<td>0.702</td>
<td>0.695</td>
<td>0.735</td>
<td>0.718</td>
<td>0.738</td>
<td>0.741</td>
<td>0.782</td>
<td>0.742</td>
<td>0.776</td>
<td>0.735</td>
<td>0.800</td>
<td>0.741</td>
<td></td>
</tr>
<tr>
<td>0.698</td>
<td>0.687</td>
<td>0.709</td>
<td>0.737</td>
<td>0.730</td>
<td>0.735</td>
<td>0.725</td>
<td>0.735</td>
<td>0.733</td>
<td>0.729</td>
<td>0.725</td>
<td>0.736</td>
<td></td>
</tr>
</tbody>
</table>
2.3.2 Verapamil concentration variation during assay

In order to assess both the accuracy of drug dilution and pipetting, and possible metabolism of drug over the duration of the assay, a series of drug concentration assays were performed. Verapamil was chosen to be analysed with a method previously used for analysing clinical samples.

Verapamil concentrations were measured in freshly prepared solutions of known concentration, and in medium which had been incubated in 96 well plates during a standard MTT assay. The assay used measures total (protein bound and unbound) verapamil, which was shown to remain at a constant concentration throughout the assay (table 2.3b).

<table>
<thead>
<tr>
<th>Verapamil concentration (μg/ml)</th>
<th>920</th>
<th>1040</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly prepared verapamil (1mg/ml) in PBS</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>1μg/ml verapamil solution (above) diluted to 20μg/ml in RPMI</td>
<td>9.05 +/- 0.38</td>
<td>9.67 +/- 0.41</td>
</tr>
<tr>
<td>Verapamil concentration in medium aspirated from wells after:-</td>
<td>9.65 +/- 1.3</td>
<td>9.75 +/- 1.1</td>
</tr>
<tr>
<td>24 hours*</td>
<td>11.87 +/- 1.3</td>
<td>11.27 +/- 1.97</td>
</tr>
<tr>
<td>47 hours*</td>
<td>9.65 +/- 1.3</td>
<td>9.75 +/- 1.1</td>
</tr>
<tr>
<td>72 hours*</td>
<td>11.87 +/- 1.3</td>
<td>11.27 +/- 1.97</td>
</tr>
</tbody>
</table>

* mean of 4 assays +/- 1 SD

Verapamil is 90% protein bound in humans (Hamann et al 1984), this binding is independent of verapamil concentration over the range 50-1500ng/ml. In man 70% of the verapamil is excreted in the urine having been metabolised in the liver. Cells in culture will presumably be unable to metabolise verapamil in any way, and so the concentration should remain constant. In the culture system used in this study protein binding of some degree will invariably occur as all medium is supplemented with 10% fetal calf serum.
2.3.3 **Optimal conditions for measuring the optical density of formazan**

Potential factors influencing the optical density of converted formazan other than cell growth were:

1. The wavelength at which OD was read.
2. The OD of remaining medium.
3. The OD of residual cytotoxics.

In order to ensure that the microtitre plates were examined under optimal reproducible conditions, a series of readings were performed to investigate the effect of the wavelength used to read optical density on the result.

Solutions were placed in cuvettes in a Beckman DU-7 spectrophotometer and absorbance spectra were measured. Using a range of formazan concentrations dissolved in DMSO (Fig 2.3b) it can be seen that the peak absorbance occurs at 500 nm for all formazan concentrations. The colour (human naked eye) of formazan at these concentrations and of MTT are illustrated in figure 2.3c. These results were then compared with readings made using two of the solutions (formazan 0.03mg/ml and 0.1mg/ml) placed in 96 well plates and read on a plate reader at 540nm and 492nm, (it was not possible to read the plates at 500nm).

**Table 2.3c**  
Optical Density of formazan at two wavelengths

<table>
<thead>
<tr>
<th>Formazan concentrate</th>
<th>OD at 540nm</th>
<th>OD at 492nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formazan 0.03 mg/ml</td>
<td>0.200 +/- 0.015</td>
<td>0.239 +/- 0.016</td>
</tr>
<tr>
<td>Formazan 0.1 mg/ml</td>
<td>0.481 +/- 0.036</td>
<td>0.608 +/- 0.042</td>
</tr>
</tbody>
</table>

These results (table 2.3c) again show that the OD of pure formazan is higher at 492nm than 540nm, and give an indication of the variability in these readings. The OD readings on solutions of formazan placed in cuvettes (in 1ml volumes) was consistently higher
than the readings of formazan placed in 96 well plates (in a maximum of 200μL volumes). A clue as to part of the reason for this may lie in the observation that doubling the volume of formazan in each well of the 96 well plates from 100μL to 200μL will consistently double the recorded OD (table 2.3d).

<table>
<thead>
<tr>
<th>Volume in well</th>
<th>0.005mg/ml Formazan</th>
<th>0.01mg/ml Formazan</th>
<th>0.03mg/ml Formazan</th>
<th>0.05mg/ml Formazan</th>
<th>0.1mg/ml Formazan</th>
</tr>
</thead>
<tbody>
<tr>
<td>100μL</td>
<td>0.08</td>
<td>0.09</td>
<td>0.15</td>
<td>0.2</td>
<td>0.33</td>
</tr>
<tr>
<td>200μL</td>
<td>0.15</td>
<td>0.16</td>
<td>0.24</td>
<td>0.37</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Before concluding which wavelength should be used to measure these optical densities a further series of experiments was performed to assess the effects of medium and drugs which might remain in the wells after flicking off the excess medium.
Figure 2.3b  Absorbance spectra of formazan

Figure 2.3c  Colour of formazan as seen by the naked eye
2.3.4 Effect of medium and cytotoxics on optical density of formazan

As mentioned previously the volume remaining in a well after flicking off excess medium is 10-20μL. A series of optical density measurements were therefore performed to assess the effect that this had on the final OD of formazan when dissolved in DMSO. This series was read both in microtitre plates and in cuvettes.

These results are shown in figures 2.3d-f.

From these graphs it can be seen that the OD of medium alone is appreciable, figures 2.3d-e. 'Used' medium was aspirated from cells in culture just prior to a medium change, and hence the colour indicator added to both D MEM and RPMI had altered from red to yellow/orange (with a pH shift due to metabolic activity of cells). With the addition of small amounts of medium to the formazan solution a visible colour change occurred with increased intensity of red/purple, this is seen more obviously at a wavelength of 540nm than at 490nm (figure 2.3f). Once more than 10% medium has been added there is a decline in the colour intensity which is similar with both RPMI and D MEM, and more marked at 540nm than 490nm.

Thus it appears that whilst pure formazan has a maximal OD at 490nm when even small quantities of medium are added this peak shifts to over 540nm. Adding these small amounts of medium to formazan in DMSO has a far smaller effect on readings at 490nm than those made at 540nm, suggesting that 490nm might be the best wavelength at which to measure the OD.
Unfortunately, for early experiments it was not possible to use the 490nm filter consistently (technical reasons) so for the sake of continuity all plates were read at 540nm. Bearing in mind the previous discussion, the volume of medium remaining in the wells was kept to a minimum. Other investigators using a similar MTT assay have used either 550nm (Twentyman and Luscombe 1987b), or 490nm (Alley et al 1988) filters with satisfactory results.
Figure 2.3d  Absorbance spectra of DMEM medium

Figure 2.3e  Absorbance spectra of RPMI medium

Figure 2.3f  Absorbance spectra of formazan (0.03mg/ml) in DMSO plus 'used' DMEM (percentage shown)
A further factor influencing the OD readings was the cytotoxic remaining (together with medium) in the wells after flicking. Cytotoxic was added in a logarithmic dose escalation so the highest concentration was $10^4$ times greater than the lowest. From direct observation of the plates this only seemed to be a problem with the cytotoxic agent doxorubicin, and even then only in those cell lines which were highly resistant to it. To examine the absorbance spectra of the two cytotoxics which are coloured (to the naked eye) i.e. doxorubicin and methotrexate, and a third which looks clear (vincristine), drugs were made up in the usual way in 'used' medium. The concentration of methotrexate and vincristine chosen was cytotoxic to all cell lines (except H69 LX4 where these doses were between IC50 and IC80). The higher concentration of doxorubicin used (100µg/ml) was close to the IC50 for unmodulated H69 LX4, the lower concentration (1µg/ml) was cytotoxic (IC50 - IC80) for all other cell lines.

Fig 2.3g shows these absorbance spectra, at the wavelength used in reading plates (540nm) neither low dose doxorubicin, methotrexate or vincristine have a significant OD, however 100µg/ml doxorubicin has a very high OD which peaks around 540nm and would therefore obscure any effect from dissolved formazan.
Figure 2.3g  Absorbance of cytotoxic agents diluted in fresh DMEM medium

Abs
3
15

MTX
1mg/ml

Dox
100µg/ml

VINC
100µg/ml

Dox
1µg/ml

Fresh
DMEM

λ (nm)
2.3.5 Optimisation of MTT conversion

As previously stated MTT powder was dissolved in PBS prior to use to give the required concentration. To standardise the assay the same concentration and incubation time of MTT was used for all cell lines, these were chosen following investigation of each cell line. Each cell line was tested with a concentration range of MTT in a four hour incubation, and with a range of incubation times following the addition of 2mg/ml MTT. These experiments were performed on cells set up at a cell density of $10^4$ cells/well and incubated for 5 days.

The illustrations shown (figures 2.3h-i) give the results of representative lines. The results from H69 LX4 were similar to H69 P; SK N SH and HX 142 to SK N BE; and the other rhabdomyosarcoma lines (RD, HS 729T and HX170) were similar to RMS.

Figure 2.3h demonstrates that incubation with 2mg/ml for 6 hours (or longer) will give a higher OD reading than a shorter incubation, however from the graph it can be seen that much of the conversion of MTT to formazan takes place within the first 2 - 4 hours. A four hour incubation was chosen on the basis of this, though in all cases the plates were examined at about 2 hours into the incubation to confirm that conversion was occurring and to allow for a shorter or longer incubation if required.

Figure 2.3i illustrates the effect of increasing the concentration of MTT added to plates for a four hour incubation. Whilst in some cases there is continuing ability to convert MTT to formazan when more than 2 mg/ml of MTT is present in the well, in most cases this concentration gives near maximal OD readings under these conditions.
Figure 2.3h  Effect of increasing duration of MTT incubation

Figure 2.3i  Effect of increasing concentration of MTT
2.3.6 **Other important considerations when using this assay**

As previously mentioned cells which were not in exponential growth at the time of use in this assay generally gave results which were poorly reproducible, every effort was therefore made to avoid this.

When DMSO is used to solubilise formazan, particularly when medium is present it is important that the plates are read within 1-2 hours of adding DMSO. If more time than this has elapsed further production of formazan occurs which finally results in a uniform colour across the plate, this reaction is complete by 12-24 hours.

A series of experiments were also carried out to confirm that the modulators would remain stable and active for the duration of storage, (up to 10 days). As the cytotoxics were always made up fresh (from intravenous preparations within their 'use by' date) this was not an issue. A comparison of the cytotoxicity of vincristine plus either freshly made or 1 - 4 week old modulator (both verapamil and CSA) demonstrated that there was no difference between them (results not shown).

DMSO was chosen as the solvent for formazan early in the project following a review of experimental methods reported in the literature and discussion with others engaged in this type of assay.
2.4 CELL LINE CHARACTERISTICS IN THE MTT ASSAY.

2.4.1 Introduction and aims

Cell lines were assessed using the MTT assay with a range of cell numbers and assay duration to establish optimal conditions for each line. These studies were necessary to determine whether the proposed plan to use standard conditions with regard to cell density and assay duration would result in any line being evaluated under significantly adverse conditions.

2.4.2 Experimental design

Replicate plates were set up with an increasing cell/well density (from $0.5 \times 10^4$ to $10 \times 10^4$ cells/well) and read after 1 to 9 days.

2.4.3 Results

Graphs representing four cell lines are shown (Figure 2.4a-d), indicating the effect of duration of assay (days) and numbers of cells plated out at the start of the assay on optical density (OD).

Figure 2.4e shows the OD achieved for each line when cells were plated out at $10^4$ cells per well and left for five days. Table 2.4a gives the optimum conditions for each cell line.
Figure 2.4a-d Effect of assay duration on optical density in four cell lines

- **H69 LX4**
  - 10000 cells/well
  - 30000 cells/well
  - 50000 cells/well
  - 70000 cells/well

- **CCRF CEM**
  - 2000 cells/well
  - 6000 cells/well
  - 10000 cells/well

- **SK N BE**
  - 2000 cells/well
  - 6000 cells/well
  - 10000 cells/well
  - 15000 cells/well

- **RD**
  - 500 cells/well
  - 2000 cells/well
  - 6000 cells/well
  - 10000 cells/well
Figure 2.4e Optical density for each cell line under standard and 'optimal' conditions

Table 2.4a Optimum conditions for each cell line

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Duration (days)</th>
<th>Cell numbers (cells/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HX 142</td>
<td>5</td>
<td>$1.5 \times 10^7$</td>
</tr>
<tr>
<td>SK N BE</td>
<td>7</td>
<td>$8 \times 10^4$</td>
</tr>
<tr>
<td>SK N SH</td>
<td>5</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>HX 170</td>
<td>7</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>HS</td>
<td>5</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>RD</td>
<td>5</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>RMS</td>
<td>5</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>CCRF CEM</td>
<td>4</td>
<td>$3 \times 10^4$</td>
</tr>
<tr>
<td>H69 P</td>
<td>2</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>H69 LX4</td>
<td>8</td>
<td>$5 \times 10^4$</td>
</tr>
</tbody>
</table>
2.4.4 Discussion and Conclusions

It is apparent that there are a minimum number of cells which can be placed into each well (96 well microtitre plate) and still proliferate under these conditions. This number differs between cell lines, and is due in part to different doubling times, but may also be dependent upon the concentration of factors (e.g. cytokines) released from cells.

In some cases (particularly those cell lines which proliferate rapidly, i.e. CCRF CEM), the OD declines with time in culture or increasing cell number. OD reflects either cell numbers, metabolic activity of cells or both. This decline may therefore be related to consumption of nutrients from the small volume of medium present in each well or it may reflect cell-cell contact inhibition, both of these would decrease the metabolic activity of the cells. CCRF CEM is a rapidly proliferating line which grows as a single cell suspension under normal conditions, however in 96 well plates it becomes moderately adherent to the plastic, and so may behave more like an adherent line.

In some cell lines (notably H69 LX4), a low OD was observed under standard conditions. In part this reflects the differential ability of different lines to convert MTT to formazan (as shown earlier in Figure 2.3h-i). However, more importantly in this case, a low OD probably truly reflects low cell numbers. H69 LX4 is a slow growing cell line with a longer doubling time than its parent H69 P. This is true both when it is grown with or without doxorubicin. In all experiments using H69 LX4 it had been cultured for 14 days without doxorubicin to ensure there was no potential drug effect remaining, and also to allow the proliferation rate to increase. Longer periods of culture without doxorubicin were not considered advisable as in some cases resistant cell lines lose resistance under these conditions.
In conclusion, a five day assay when cells were plated out at $10^4$ cell per well was satisfactory for H69 P, CCRF CEM, RMS, RD, SK N BE, and SK N SH. A five day assay with higher initial cell density was required for HX 142 and HX 170, for H69 LX4 a seven day assay was needed with at least $5 \times 10^4$ cells per well.
2.5 CELL LINE SENSITIVITY TO MODULATING AGENTS

2.5.1 Introduction and aims

The cytotoxicity of cyclosporin and verapamil were assessed in order to ensure that a relatively non toxic concentration of each was used. It has been previously noted that both of these drugs can be toxic to cells at high concentrations (Schmidt et al 1988, Twentyman et al 1986b). In addition, the effect of ethanol on cell proliferation was assessed as this is the solvent used for CSA. To standardise the assay the same concentrations of verapamil and CSA should be chosen for all lines, to allow direct comparison. Comparable reversal activity might be more relevant but would require for each line with each cytotoxic agent.

2.5.2 Experimental design

Each cell line was plated out at $1 \times 10^4$ cells/well, 24 hours after setting up the 96 well plates a concentration range of verapamil, CSA or ethanol was added across each plate. At the end of five days the plates were read in the usual way.

Verapamil and CSA were made up as previously mentioned, and used in a range from 1-20μg/ml. Ethanol dilutions were made to represent the ethanol concentration found in a 2.5μg/ml, 10μg/ml and 40μg/ml CSA solution. Ethanol is used to dissolve CSA in a ratio of 0.5mls ethanol to 1mg CSA, therefore a 2.5μg/ml CSA solution will contain 1.5μL ethanol per ml.
2.5.3 Results

A summary of the IC 50 for each cell line with verapamil or CSA, and the effect of ethanol is shown in table 2.5a. Figure 2.5a-b illustrate the concentration versus cell survival effect of verapamil and CSA on representative cell lines.

Figure 2.5a Effect of cyclosporin on cell survival

![Graph showing the effect of cyclosporin on cell survival.]

Figure 2.5b Effect of verapamil on cell survival

![Graph showing the effect of verapamil on cell survival.]

---

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Table 2.5a  IC50 for modulators and ethanol

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC 50 Verapamil (µg/ml)</th>
<th>IC 50 Cyclosporin (µg/ml)</th>
<th>Approximate ethanol IC 50*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H69 LX4</td>
<td>&gt; 20</td>
<td>&gt; 20</td>
<td>&gt; 3</td>
</tr>
<tr>
<td>H69 P</td>
<td>&gt; 20</td>
<td>&gt; 20</td>
<td>&gt; 3</td>
</tr>
<tr>
<td>CCRF CEM</td>
<td>15</td>
<td>5</td>
<td>&gt; 3</td>
</tr>
<tr>
<td>RD</td>
<td>12</td>
<td>50</td>
<td>&gt; 3</td>
</tr>
<tr>
<td>RMS</td>
<td>5</td>
<td>10</td>
<td>&gt; 3</td>
</tr>
<tr>
<td>HS</td>
<td>10</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>HX 170</td>
<td>15</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>SK N BE</td>
<td>12</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>SK N SH</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>2</td>
</tr>
<tr>
<td>HX 142</td>
<td>10</td>
<td>15</td>
<td>&gt; 3</td>
</tr>
</tbody>
</table>

* Ethanol in arbitrary units. 1 = equivalent to diluant present in CSA 2.5µg/ml
2 = equivalent to diluant present in CSA 10µg/ml
3 = equivalent to diluant present in CSA 40µg/ml
2.5.4 Discussion and Conclusions

It is clear from table 2.5a that some cell lines are far more sensitive to these agents than others. None of the cell lines were susceptible to ethanol at the concentration used. Sensitivity to modulators did not correlate well with those cell lines which expressed MDR, as evidenced by the fact that H69 LX4 was not affected by relatively high concentrations of either modulator.

CCRF CEM was most sensitive to the cytotoxic effects of CSA as expected owing to its T lymphoblastic origin. It is of note that clinical responses have been reported in T ALL treated with CSA (Laneuville 1992).

To facilitate screening these cell lines for in vitro modulation the maximum concentration of modulator tolerated by most cell lines was chosen, and the inevitable cytotoxicity to some cell lines was accepted. This cytotoxicity did not affect the observed IC 50 in these cell lines, as IC 50 was always calculated with reference to the control containing modulator alone.

The modulator concentrations which were used in most experiments were 10μg/ml verapamil and 5μg/ml CSA, or both these drugs together at these final well concentrations.
2.6 CHEMOSENSITIVITY OF CELL LINES

2.6.1 Introduction and aims

The cell lines used in this study originally came from at least four tumour types, and as such would be expected to display different chemosensitivity patterns. In addition most (if not all) of the patients from whom tissue was taken had received chemotherapy. This \textit{in vivo} exposure of tumour to cytotoxic drugs may have promoted development of resistance to some agents by induction of the MDR phenotype or other resistance mechanisms.

The chemosensitivity (or chemoresistance) pattern of each cell line was established to three cytotoxic agents involved in MDR (vincristine, etoposide and doxorubicin). In four cell lines which later proved to show reversal of resistance with verapamil or cyclosporin, the chemosensitivity (and reversal) pattern to cytotoxics unrelated to MDR (methotrexate, cisplatin, cyclophosphamide) was also established.
**2.6.2 Experimental design**

Each cell line was used under either standard or modified conditions as outlined in previous sections. Cells were plated out for a five day assay, cytotoxic drug was added to the plates 24 hours after plating out. The assay was read using the standard method on day five.

Initially cytotoxic drugs were added within six hours of placing cells in the 96 well plates. In most cases (both when cytotoxic drug or modulating agent were added) poor cell proliferation was observed when these additions were made too early and in order to avoid this problem all drug additions were subsequently made at 24 hours.

Cytotoxic drugs were prepared as previously described, and diluted to give a logarithmic dose range, (i.e. 0.01, 0.1, 1, 10, 100ng/ml). As the final well volume was 200\(\mu\)L in all cases, with 100\(\mu\)L of this made up of cell suspension, a further 100\(\mu\)L of drug was added at double the final desired concentration. If two different drugs were to be added (as in the next section) then each was prepared to give a solution four times the final desired concentration, and 50\(\mu\)L added to each well.

The dose range used for each cell line was modified until the IC 50 and preferably also the IC80 could be demonstrated.
2.6.3 Results

Dose response curves were obtained for each cell line showing the effect of each agent on cell proliferation (or metabolic activity). As can be seen from figures 2.6a-c, percentage survival does not come down to zero with this method. As previously discussed this is due in part to the optical density reading arising from cell debris, DMSO and medium remaining in the wells. However, this effect is exacerbated if the OD of the 'no cytotoxic' well is not very high (e.g. 0.5), because the percent fall from 0.5 to 0.09 (if the well contains no live cells) is less than the percent fall from 1.5 to 0.09.
Figures 2.6a-c Survival curves for cell lines exposed to cytotoxics

Percentage Survival

Vincristine (ng/ml)

Percentage Survival

Etoposide (ng/ml)

Percentage survival

Doxorubicin (ng/ml)
Figures 2.6a-c Survival curves for cell lines exposed to cytotoxics
H69 LX4 is highly resistant to all three cytotoxics. The resistance patterns to etoposide of all lines demonstrated that in addition HX170 and RD (both embryonal rhabdomyosarcoma) were also highly resistant to at least one of the drugs. The highest concentration of etoposide used in these experiments (final well concentration 1,000,000ng/ml or 1mg/ml) was limited by the concentration and viscosity of the intravenous preparation used (20mg/ml, i.e. maximum 5mg/ml final well concentration).

Most of the survival curves show a shoulder followed by a steep dose/response region where percent cell survival falls from 80% to 30%. A few of the cell lines (notably the rhabdomyosarcomas) displayed a gradual decline in percent survival with dose.

Table 2.6a summarises the survival data for each cell line. It is apparent that chemosensitivity varies tremendously between lines, even between those with the same tissue of origin.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>DOX</th>
<th>VCR</th>
<th>ETOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>H69 LX4</td>
<td>SCLC</td>
<td>100,000</td>
<td>2,000</td>
<td>20,000</td>
</tr>
<tr>
<td>H69 P</td>
<td></td>
<td>100</td>
<td>0.6</td>
<td>400</td>
</tr>
<tr>
<td>CCRF CEM</td>
<td>Leuk</td>
<td>6</td>
<td>0.4</td>
<td>300</td>
</tr>
<tr>
<td>RD</td>
<td>Rhabdo</td>
<td>100</td>
<td>6</td>
<td>40,000</td>
</tr>
<tr>
<td>RMS</td>
<td></td>
<td>7</td>
<td>0.3</td>
<td>400</td>
</tr>
<tr>
<td>HX 170</td>
<td></td>
<td>20</td>
<td>70</td>
<td>10,000</td>
</tr>
<tr>
<td>HS</td>
<td></td>
<td>100</td>
<td>0.4</td>
<td>800</td>
</tr>
<tr>
<td>SK N BE</td>
<td>NBL</td>
<td>200</td>
<td>7</td>
<td>400</td>
</tr>
<tr>
<td>SK N SH</td>
<td></td>
<td>15</td>
<td>3</td>
<td>400</td>
</tr>
<tr>
<td>HX 142</td>
<td></td>
<td>3</td>
<td>6</td>
<td>60</td>
</tr>
</tbody>
</table>

SCLC: Small cell lung cancer  
Leuk: Leukaemia  
Rhabdo: Rhabdomyosarcoma  
NBL: Neuroblastoma  

DOX: Doxorubicin  
ETOP: Etoposide  
VCR: Vincristine
Overall the most sensitive cell lines were CCRF CEM and HX 142 (leukaemia and neuroblastoma respectively), and the most resistant were HX 170 and RD (both rhabdomyosarcoma).

The IC50 for one rhabdomyosarcoma and three neuroblastoma cell lines to non MDR drugs are shown in table 2.6b

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MTX</th>
<th>Cyclo</th>
<th>CDDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>HX 170</td>
<td>20,000</td>
<td>3,000</td>
<td>2,000</td>
</tr>
<tr>
<td>SK N BE</td>
<td>70</td>
<td>100</td>
<td>400</td>
</tr>
<tr>
<td>SK N SH</td>
<td>30</td>
<td>300</td>
<td>1,000</td>
</tr>
<tr>
<td>HX 142</td>
<td>90</td>
<td>500</td>
<td>600</td>
</tr>
</tbody>
</table>

MTX Methotrexate  
Cyclo Cyclophosphamide  
CDDP: Cisplatin

Relative resistance (RR) is a measure of the difference in chemosensitivity between a parent and derived cell line, it can be calculated for different drugs.

RR = IC50 resistant line / IC50 sensitive line

The resistant small cell lung cancer line H69 LX4 and its drug sensitive parent (H69 P) are the only cell lines from which relative resistance can be calculated. No other cell line was successfully transformed from sensitive to resistant following exposure to cytotoxics in prolonged culture.
Hence the RR for H69 P and H69 LX4 are:

- RR at IC50 for doxorubicin 1000
- RR at IC50 for vincristine 4000
- RR at IC50 for etoposide 50

This can be compared with results published by Twentyman (1987a) using H69 P and H69 LX4 using the IC80 (measured by counting viable cells) to calculate the resistance factor (RF) which is the same as RR. In this paper RF for doxorubicin was 63-145, and RF for vincristine was 900 - 1700.
2.6.4 Discussion and Conclusions

Using this experimental model it is possible to assess the chemosensitivity of a range of cell lines. Whilst most of the tumours from which these lines were derived had previously been treated with cytotoxics, the chemoresistance pattern was not directly related to tissue of origin or prior treatment.

It has been suggested (Biedler 1993) that prolonged exposure of cells expressing P gp to cytotoxics has the effect of down regulating P gp expression. This may occur over the time course (5 days) of an MTT assay and should be considered in the context of demonstrating MDR modulation in vitro.
2.7 MODULATION OF CHEMORESISTANCE WITH VERAPAMIL OR CYCLOSPORIN A

2.7.1 Introduction and aims

In order to determine which cell lines could have their resistance pattern modulated by verapamil or CSA using this assay, each line was exposed to modulator in addition to cytotoxic agent. The modulator concentration used (chosen following experiments described in 2.5) was far greater than can be achieved in clinical practice. Verapamil serum concentrations of 3200ng/ml (~ 6μM) are possible at the expense of severe cardiovascular toxicity. The verapamil concentration used in these experiments was three times higher than this. CSA concentrations in serum following intravenous infusion can be very high, particularly if a large dose is given over a short time. Under these conditions serum levels in excess of 7500ng/ml are achieved (unpublished data). If CSA is given as a prolonged infusion a sustained concentration of 1000-2000ng/ml is more usually observed. The dose of CSA used in this study was 5μg/ml (5000ng/ml), this would not be tolerated for prolonged periods in vivo.

In addition ethanol alone was used as a potential modulating agent in conjunction with a vincristine dose range in SK N BE, CCRF CEM and H69 LX4.

These relatively high concentrations of modulator were used to ensure that if reversal of resistance was achievable by these agents it should be observed.
2.7.2 Experimental design

As previously described cells were plated out into 96 well plates, the following day modulator was added to each well to give a final well concentration of 5µg/ml CSA and 10µg/ml verapamil. One hour after modulator addition a logarithmic dose range of cytotoxics was added. The plates were then incubated for a further four days before being read using the conversion of MTT to formazan.

2.7.3 Results

Figure 2.7a illustrates the alteration in percentage survival for two cell lines, one known to be MDR 1 negative (H69 P) and the other MDR 1 positive (H69 LX4). Using modulator it is possible to increase the sensitivity of H69 LX4 to a level which approaches that of H69 P. H69 P shows no evidence of enhanced sensitivity using CSA or verapamil. The modulating effect of CSA (5µg/ml) is greater than that of verapamil (10µg/ml).

Figure 2.7a Modulation of H69 P and H69 LX4 vincristine sensitivity by CSA or verapamil
Figures 2.7b-g illustrate the resistance pattern of SK N BE to all the cytotoxics tested, and how this is affected by CSA and verapamil. Figure 2.7b shows the dramatic modulation of vincristine resistance seen in SK N BE with these agents. In this example the IC50 for each treatment can be measured directly from the graph. Gridlines have only been included in this figure for clarity.

**Figures 2.7b-c Modulation of chemoresistance (to vincristine and doxorubicin) in SK N BE cells**

![Graph showing modulation of chemoresistance](image)

- **Vincristine (ng/ml)**: The graph shows the percentage survival of SK N BE cells in response to varying concentrations of vincristine with and without modulators (CSA and Verapamil).
- **Doxorubicin (ng/ml)**: Similarly, the graph demonstrates the percentage survival in response to doxorubicin with the same modulators.

The graphs highlight the effectiveness of CSA and Verapamil in modulating the resistance to both vincristine and doxorubicin, as indicated by the shifts in the IC50 values and the survival curves.
Figures 2.7d-g Lack of modulation of chemoresistance in SK N BE

Percentage survival

Etoposide (ng/ml)

Cyclophosphamide (ng/ml)

Methotrexate (ng/ml)

Cisplatin (ng/ml)

S. 0 1 10 100 1000 10000

-----■—— No modulator
-----♦ — CSA
-----0—— Verapamil

0 1 10 100 1000 10000

-----■—— No modulator
-----♦ — CSA
-----0—— Verapamil

0 1 10 100 1000 10000

-----■—— No modulator
-----♦ — CSA
-----0—— Verapamil

0 1 10 100 1000 10000

-----■—— No modulator
-----♦ — CSA
-----0—— Verapamil

0 1 10 100 1000 10000

-----■—— No modulator
-----♦ — CSA
-----0—— Verapamil

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From figure 2.7b the IC 50 values are:

Vincristine alone 7ng/ml
Vincristine and CSA 0.06ng/ml
Vincristine and verapamil 0.1ng/ml

Using the equation below the SR can be calculated for these treatments.

Sensitisation Ratio (SR) = IC50 without modulator / IC50 with modulator

Therefore:

SR (for vincristine and CSA in cell line SK N BE) = 7 / 0.06 = 116
SR (for vincristine and verapamil in cell line SK N BE) = 7 / 0.1 = 70

Figure 2.7c shows the intermediate degree of reversal of doxorubicin resistance. It is clear from Figures 2.7d-g that etoposide is not modulated and, as expected, the non MDR agents (MTX, cyclophosphamide and cisplatin) remain unaffected by these agents.

The SR for each cell line was calculated with both CSA and verapamil using the doses previously specified unless otherwise stated. These are summarised in table 2.7a-b.

An arbitrary cut off SR value of 5 or less was considered to indicate that no modulation of resistance had occurred. From the tables it can be seen that some of the cell lines do not alter their resistance pattern as a result of exposure to these two modulating agents. Cell lines which show no modulation include H69 P, CCRF CEM, RD, RMS and HS.

None of the cell lines demonstrated modulation of resistance to all three cytotoxics initially tested (vincristine, doxorubicin and etoposide) using these criteria for
modulation. The etoposide resistance of LX4, which has been shown to be highly etoposide resistant (table 2.6c) was not significantly altered by these modulating agents.

Table 2.7a  Sensitisation Ratio (SR) at IC50 for each cell line to three cytotoxics

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>VINC</th>
<th>ETOP</th>
<th>DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CSA</td>
<td>VER</td>
<td>CSA</td>
</tr>
<tr>
<td>H69 P</td>
<td>2.5</td>
<td>3.3</td>
<td>1</td>
</tr>
<tr>
<td>H69 LX4</td>
<td>400</td>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td>CCRF CEM</td>
<td>1.8*</td>
<td>1</td>
<td>2*</td>
</tr>
<tr>
<td>RD</td>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>RMS</td>
<td>1.5</td>
<td>1.25</td>
<td>1</td>
</tr>
<tr>
<td>HS</td>
<td>1</td>
<td>1</td>
<td>0.75</td>
</tr>
<tr>
<td>HX 170</td>
<td>1</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>SK N BE</td>
<td>116</td>
<td>70</td>
<td>1</td>
</tr>
<tr>
<td>SK N SH</td>
<td>75</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>HX 142</td>
<td>100</td>
<td>200</td>
<td>1.5</td>
</tr>
</tbody>
</table>

CSA dose 5μg/ml unless otherwise stated
* CSA 2.5μg/ml as 5μg/ml not tolerated by CCRF CEM
  # When CSA used at 10μg/ml SR = 14
  Verapamil (VER) dose 10μg/ml

VINC = vincristine
ETOP = etoposide
DOX = doxorubicin

Table 2.7b  SR at IC50 using non MDR cytotoxics with CSA or verapamil on those cell lines which apparently modulated with MDR associated drugs

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>MTX</th>
<th>CYCLO</th>
<th>CDDP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CSA</td>
<td>VER</td>
<td>CSA</td>
</tr>
<tr>
<td>HX 170</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>SK N BE</td>
<td>0.5</td>
<td>1.8</td>
<td>0.5</td>
</tr>
<tr>
<td>SK N SH</td>
<td>0.3</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>HX 142</td>
<td>0.1</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

MTX = methotrexate
CYCLO = cyclophosphamide
CDDP = cisplatin
The three neuroblastoma cell lines investigated (SK N BE, SK N SH AND HX 142) were all moderately vincristine resistant (table 2.6c), and in each case this resistance could be substantially reduced.

HX 142 was the most etoposide and doxorubicin sensitive line tested, neither of these sensitivities were affected by CSA or verapamil. Whilst SK N SH was moderately resistant to etoposide and doxorubicin neither of these were reversed. However SK N BE was highly resistant to doxorubicin, a pattern which modulated, but only moderately to etoposide (which was not altered by CSA or verapamil).

The other cell line which displayed reversal of resistance was the rhabdomyosarcoma cell line HX170. Moderate resistance to etoposide was decreased by verapamil but not CSA, however the high level of resistance to vincristine and doxorubicin were not affected.

Those cell lines which were also tested against cytotoxics not implicated in the MDR phenomenon did not show any reversal of resistance using either CSA or verapamil.

Ethanol did not produce modulation of vincristine resistance in the cell lines tested.
2.7.4 Discussion and Conclusions

The results of these experiments demonstrate that the resistance pattern to some cytotoxics can be altered in some cell lines.

It is interesting to note that vincristine was modulated both more often and to a greater extent than either etoposide or doxorubicin. Etoposide was only modulated in one instance (HX170 - with verapamil). This may be due to etoposide resistance being mediated by another mechanism (e.g. topoisomerase II) not involved in vincristine resistance (which is due to a 'pure' MDR mechanism).

The three neuroblastoma lines investigated were both more resistant and easier to modulate than the others. Reversal of resistance does appear to be related to the degree of resistance in these cell lines. A higher level of P gp expression may indicate that this is the main resistance mechanism which is thus amenable to reversal - unlike situations where topoisomerase II is involved.

A criticism of using this type of assay with prolonged exposure of proliferating cells to cytotoxics is that the assay conditions themselves may alter the expression of P gp or the resistance pattern. It is suggested that P gp expression would be down regulated under these conditions, and hence cells should lose resistance and display less reversal of any resistance. Alternatively, the assay conditions may select out a more highly resistant subset within a cell line, leading to greater observed resistance under these conditions than seen in other experimental systems.
2.8 LOW DOSE MODULATION OF CHEMORESISTANCE

2.8.1 Introduction and aims
As mentioned in the previous section, the concentrations of modulator used to
demonstrate reversal of resistance are far in excess of the serum concentrations which
are achievable in clinical practice. In those cell lines which displayed evidence of
modulation, lower doses of the two reversal agents were used to investigate 'dose
response' effect in modulating MDR.

2.8.2 Experimental design
The only alteration to experimental design used in section 2.6 was in modulator
concentration. The final well concentrations used are indicated below.

2.8.3 Results
For those cell lines which displayed modulation of resistance with a particular cytotoxic
agent, a 'dose response' effect could be seen with increasing modulator concentration.
However lines which had not previously modulated with any agent did not show any
change in chemosensitivity following exposure to different modulator concentrations.
Similarly those lines which only demonstrated modulation with some agents were
unaffected by variation in modulator concentration when used in conjunction with
cytotoxics not previously modulated, but did show a 'dose response' when exposed to
the combination of modulator and previously reversed cytotoxic.
Figure 2.8a-b show the type of 'dose response' seen with a dose range of modulator
(from 0.1μg/ml to 10μg/ml) given to SK N BE cells subsequently exposed to a
logarithmic dose range of vincristine. From these figures it can be seen that even the
lowest concentration of modulator used in this cell line was able to alter vincristine resistance.

Figure 2.8a-b Effect of modulator dose on reversal of vincristine resistance in SK N BE

![Graph](image)

---

Vincristine (ng/ml): 0, 1, 10, 100, 1000

Percentage survival: 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 0

Legend:
- No modulator
- CSA 0.1ug/ml
- CSA 0.5ug/ml
- CSA 1ug/ml
- CSA 5ug/ml
- CSA 10ug/ml
- VER 0.1ug/ml
- VER 0.5ug/ml
- VER 1ug/ml
- VER 5ug/ml
- VER 10ug/ml
Table 2.8a gives the sensitisation ratios (SR) at IC50 vincristine for each cell line tested in this way. Again a 'dose response' effect is seen.

Table 2.8a  SR at IC50 for vincristine treated cell lines

<table>
<thead>
<tr>
<th>CSA (µg/ml)</th>
<th>H69 LX4</th>
<th>SK N SH</th>
<th>SK N BE</th>
<th>HX 142</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>1.6</td>
<td>125</td>
<td>75</td>
</tr>
<tr>
<td>2.5</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>400</td>
<td>70</td>
<td>120</td>
<td>150</td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
<td>100</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Verapamil (µg/ml)</th>
<th>H69 LX4</th>
<th>SK N SH</th>
<th>SK N BE</th>
<th>HX 142</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>12</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>2.5</td>
<td>40</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>ND</td>
<td>25</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>50</td>
<td>75</td>
<td>150</td>
</tr>
</tbody>
</table>

ND: Not done
2.8.4 Discussion and Conclusions

These observations suggest that malignant cells are potentially reversible by a range of modulator concentrations and cell lines which modulate *in vitro* with high doses of reversal agents can also be reversed by much lower concentrations of modulator.

In all cases examined higher modulator concentrations led to greater modulation. How important this might be in clinical practice is difficult to estimate, although it suggests that useful modulation may be achieved *in vivo* without necessarily requiring unpractical toxic drug levels.
2.9 COMBINATIONS OF MODULATORS

2.9.1 Introduction and aims

The effect of combining modulators was examined to determine if this could increase the degree of modulation previously demonstrated.

There is some published work (Ayesh et al 1994) suggesting that in vitro, many different drugs each with modulating activity can be used in combination at a fraction of the known effective dose to produce a superior effect in terms of cytotoxicity.

A study by Hu et al (1990) suggested that CSA and verapamil might be synergistic when used to modulate MDR. However this observation was questioned in subsequent correspondence (Samuels et al 1991). Despite this, a greater effect upon cell survival was demonstrated when these modulating agents were given together (compared with when they were given singly). A similar study by Osann et al (1992) apparently demonstrated synergy of CSA and verapamil by isobologram analysis.

In clinical practice this has been used to both increase the efficacy of modulation and to decrease toxicity attributed to reversal agents by using lower concentrations of each. The use of more than one reversing agent may however, result in difficulties with pharmacokinetics and drug interactions. In some clinical studies two agents have been given together. For example verapamil and quinine, given orally, to modulate resistance to combination chemotherapy in high risk patients with non Hodgkin's lymphoma (Miller at al 1994). This study was stopped early following a significant increase in toxicity, including toxic deaths.
2.9.2 Experimental design

These experiments are essentially the same as in previous sections, with the exception that both modulators were added to the wells to give the same final well volume (200μl) and the final well concentrations as stated.

2.9.3 Results

Exposing cells to more than one modulator simultaneously proved to be more cytotoxic to most cell lines even in the absence of cytotoxic agent. Therefore in many cell lines only limited investigation of double modulator exposure was possible.

Each line was assessed against a very low modulator dose (verapamil 0.1μg/ml and CSA 1μg/ml), an intermediate (verapamil and CSA 2.5μg/ml) and a high dose (verapamil 10μg/ml and CSA 5μg/ml).

CCRF CEM was unable to tolerate a double modulator dose of greater than the lowest level, i.e. these cells did not proliferate under these conditions.

All cell lines displayed reduced proliferative ability in the absence of cytotoxic when double modulator is compared to single agent. For example, the optical density of wells without cytotoxic (i.e. 100% survival) were:

- No modulator = 1.9
- CSA 5μg/ml = 0.7
- Verapamil 10μg/ml = 1.5
- CSA 5μg/ml and verapamil 10μg/ml = 0.55

In many lines this reduction resulted in an OD too low to proceed with analysis from that series (i.e. less than 0.5). This occurred in the following lines; HX170, RMS, HS and RDS.
In those cases with sufficient information the SR at the IC50 for each cytotoxic was computed, the following cell lines did not demonstrate any modulation (SR of 5 or less); CCRF CEM, H69 P, RDS, RMS, SH and HX170.

Table 2.9a summarises the results for vincristine, there was no demonstrable modulation with any other cytotoxic (except doxorubicin with SK N BE).

<table>
<thead>
<tr>
<th></th>
<th>H69 LX4</th>
<th>SK N SH</th>
<th>SK N BE</th>
<th>HX 142</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSA 0.1</td>
<td>0.1</td>
<td>1</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>VER 1</td>
<td>2</td>
<td>12</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>V1 / C0.1</td>
<td>1</td>
<td>500</td>
<td>150</td>
<td>35</td>
</tr>
<tr>
<td>CSA 2.5</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VER 2.5</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V2.5 / C2.5</td>
<td>200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSA 5</td>
<td>400</td>
<td>75</td>
<td>116</td>
<td>100</td>
</tr>
<tr>
<td>VER 10</td>
<td>200</td>
<td>50</td>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td>V10 / C5</td>
<td>5000</td>
<td>200</td>
<td>150</td>
<td>67</td>
</tr>
</tbody>
</table>

These results indicate that in this highly overexpressing line (H69 LX4) there is a clear beneficial effect which appears to be more than additive. The effect with SK N SH is partially additive and no enhancement in SR was seen with the other two lines.
2.9.4 Discussion and Conclusions

The use of combinations of modulators may be helpful where P gp is over expressed.

Taking the case of H69 LX4, a lower dose of two modulators can be used together to achieve the same degree of modulation as with either agent alone. This may allow use of less toxic regimens in clinical practice.
2.10 IMPORTANCE OF TIME AT WHICH MODULATOR ADDED WITH RESPECT TO THE TIME AT WHICH CYTOTOXIC ADDED

2.10.1 Introduction and aims

In clinical practice patients who are treated with a schedule attempting to modulate MDR will generally start to receive the reversing agent before chemotherapy. In many cases the reversing agent is then given continuously for a prolonged period of time. The importance of uninterrupted modulator presence is examined in section 2.12. It is usually assumed that *in vivo* pre-exposure of tumour to modulator is only necessary in order to achieve 'adequate' serum or tissue concentrations prior to exposing tumour to cytotoxic agent. It is unknown, though presumed, that tumour concentrations are more important than serum, nor is it known if the duration of pre-exposure is of importance.
2.10.2 Experimental design

The assay was performed in essentially the same way as previously. Only SK N BE with vincristine was examined, as this provided a relatively trouble free assay. In these experiments cytotoxics were added 48 hours after plating out cells. As modulator addition in the first few hours after setting up the plates had previously been shown to result in extremely poor cell proliferation, no modulator was added until at least 24 hours. Modulator additions are indicated by the time since the plates were set up (which is called T0, i.e. time zero). Vincristine was always added at T48 (i.e. 48 hours after the plates were set up). The plates were always read at T96 (i.e. 96 hours after the plates were set up). Figure 2.10a illustrates the schedule used. Modulator concentrations were kept constant for these experiments, (CSA 5µg/ml and verapamil 10µg/ml). The optical density results were computed into sensitisation ratios (at the IC50) in the usual way.

Figure 2.10a  Diagram to show times at which cytotoxic and modulator were added to plates

<table>
<thead>
<tr>
<th>Plates set up</th>
<th>Vincristine added</th>
</tr>
</thead>
<tbody>
<tr>
<td>T 0</td>
<td>T 48 hours</td>
</tr>
<tr>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

↑  T 24         ↑  T 45         ↑  T 49         ↑  T 72
↑  T 47         ↑  T 51         ↑  hours after plates set up
Times at which modulator added
2.10.3 Results

The IC50 and SR results for modulation of vincristine resistance with CSA and verapamil are presented in table 2.10a.

Table 2.10a Variation in modulation of vincristine resistance in SK N BE cells by verapamil or CSA with time of modulator addition (with respect to time of vincristine addition)

<table>
<thead>
<tr>
<th>Time from set up</th>
<th>Time from VCR addition</th>
<th>No Mod IC50</th>
<th>CSA IC50</th>
<th>SR</th>
<th>VER IC50</th>
<th>SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>T + 24</td>
<td>T - 24</td>
<td>6</td>
<td>2.4</td>
<td>2.5</td>
<td>0.3</td>
<td>20</td>
</tr>
<tr>
<td>T + 45</td>
<td>T - 3</td>
<td>5</td>
<td>0.1</td>
<td>50</td>
<td>0.1</td>
<td>50</td>
</tr>
<tr>
<td>T + 47</td>
<td>T + 1</td>
<td>8</td>
<td>0.05</td>
<td>160</td>
<td>0.1</td>
<td>80</td>
</tr>
<tr>
<td>T + 49</td>
<td>T + 1</td>
<td>3</td>
<td>0.05</td>
<td>60</td>
<td>0.05</td>
<td>60</td>
</tr>
<tr>
<td>T + 51</td>
<td>T + 3</td>
<td>4</td>
<td>0.05</td>
<td>80</td>
<td>0.05</td>
<td>80</td>
</tr>
<tr>
<td>T + 72</td>
<td>T + 24</td>
<td>10</td>
<td>1</td>
<td>10</td>
<td>3</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Times are measured (in hours) from the time at which the plates were set up or vincristine (VCR) added

IC50 of vincristine in ng/ml

In these experiments the 'no modulator' wells had medium added at the equivalent time of modulator addition. Whilst there is variation in the 'no modulator' IC50 (3-10ng/ml) this is not great enough to significantly alter these results.

From the above table it can be seen that the addition of verapamil or CSA at any time to these cells results in modulation of resistance. However, the degree of reversal varies according to time of modulator addition with respect to vincristine addition. Figure 2.10b displays this variation graphically, and shows that the maximum effect is seen when modulator is added to the cells close to the time of cytotoxic addition. Conversely modulator added to wells either one day before or after vincristine had much less effect.

There was variation in the optical density (OD) which resulted in 100% survival, i.e. the 'no vincristine' wells which were used as '100% cell survival' for subsequent calculations
of vincristine toxicity. However in these experiments this OD was always greater than 0.5, as illustrated in table 2.10b.

Table 2.10b  Optical Density (arbitrary units) for the 'no vincristine' well in each series

<table>
<thead>
<tr>
<th>Time</th>
<th>No mod</th>
<th>CSA</th>
<th>VER</th>
</tr>
</thead>
<tbody>
<tr>
<td>T + 24</td>
<td>0.8</td>
<td>0.58</td>
<td>0.6</td>
</tr>
<tr>
<td>T + 45</td>
<td>0.76</td>
<td>0.51</td>
<td>0.72</td>
</tr>
<tr>
<td>T + 47</td>
<td>0.75</td>
<td>0.68</td>
<td>0.68</td>
</tr>
<tr>
<td>T + 49</td>
<td>0.9</td>
<td>0.71</td>
<td>0.58</td>
</tr>
<tr>
<td>T + 51</td>
<td>0.83</td>
<td>0.67</td>
<td>0.74</td>
</tr>
<tr>
<td>T + 72</td>
<td>0.89</td>
<td>0.8</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Time (hours) as in table 2.10a

Figure 2.10b  Sensitisation ratio following different modulator addition times

![Sensitisation ratio chart](chart.png)
2.10.4 Discussion and Conclusions

Some variation is evident in the degree of modulation seen when the time of modulator addition is varied in relation to the time of cytotoxic addition.

It can be concluded that prolonged exposure to modulator prior to addition of cytotoxics is not necessary and may even have an adverse effect - primarily by impairing drug uptake. Exposure to modulator too long after cytotoxic exposure will result in reduced efficacy.

These observations are unlikely to have been due to variation in the concentration of modulator within each well as these agents are not metabolised by cells in culture. However, with increasing time in assay there may be more cells per well (following cell proliferation), which could result in less available modulator per cell. Alternatively drug (cytotoxic or modulator) addition to this type of culture system may be more (or less) toxic at different stages in the exponential growth of a cell population. As no drugs were added to any cells until 24 hours after the plates had been set up, an interval known to be sufficient to allow the cells to plate out and start to proliferate, it is unlikely that addition of modulator at T+24 hours significantly interfered with initiation of cell proliferation.
2.11 EFFECT OF PROLONGED PRE-EXPOSURE OF CELLS TO MODULATOR

2.11.1 Introduction and aims

Following from observations reported by Beidler (1993) that P gp expression can be down regulated if P gp over-expressing cells are exposed to cytotoxics or modulators, the effect of prolonged exposure to verapamil or CSA at the concentrations used in previous experiments was examined. If P gp expression was reduced by prolonged exposure to modulator alone it might be expected that those cells would become less resistant but also less susceptible to modulation.

2.11.2 Experimental design

SK N BE cells were grown in tissue culture flasks as previously described. When the cell line was in exponential growth, flasks were set up with either verapamil (final concentration 10µg/ml) or CSA (final concentration 5µg/ml). These cells were incubated in this way for five days with two medium (and modulator) changes. During this time the cells exposed to verapamil proliferated normally, however those exposed to CSA were very slow to multiply. These cells were harvested in the usual way, and then washed in two changes of medium alone prior to use in the assay. The assay was otherwise performed in the same fashion as described in 2.10.
2.11.3 Results

The initial observation that those cells exposed to CSA were less able to proliferate when compared with those exposed to verapamil or to medium alone, was borne out by their behaviour in this assay.

Table 2.11a OD for CSA pre-treated cells following exposure to CSA and VER without cytotoxic

<table>
<thead>
<tr>
<th>Time*</th>
<th>No mod</th>
<th>CSA</th>
<th>VER</th>
</tr>
</thead>
<tbody>
<tr>
<td>T + 24</td>
<td>0.8</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>T + 45</td>
<td>0.6</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>T + 47</td>
<td>0.5</td>
<td>0.24</td>
<td>0.48</td>
</tr>
<tr>
<td>T + 49</td>
<td>0.55</td>
<td>0.25</td>
<td>0.55</td>
</tr>
<tr>
<td>T + 51</td>
<td>0.51</td>
<td>0.28</td>
<td>0.48</td>
</tr>
<tr>
<td>T + 72</td>
<td>0.7</td>
<td>0.3</td>
<td>0.45</td>
</tr>
</tbody>
</table>

* Time (hours) since plate set up

No mod No further modulator added
CSA Cyclosporin added
VER Verapamil added

Table 2.11b OD for verapamil pre-treated cells following exposure to CSA and VER without cytotoxic drug

<table>
<thead>
<tr>
<th>Time</th>
<th>No mod</th>
<th>CSA</th>
<th>VER</th>
</tr>
</thead>
<tbody>
<tr>
<td>T + 24</td>
<td>1.9</td>
<td>1.3</td>
<td>1.7</td>
</tr>
<tr>
<td>T + 45</td>
<td>1.5</td>
<td>0.95</td>
<td>1.3</td>
</tr>
<tr>
<td>T + 47</td>
<td>2</td>
<td>1.5</td>
<td>1.7</td>
</tr>
<tr>
<td>T + 49</td>
<td>1.97</td>
<td>0.7</td>
<td>1.6</td>
</tr>
<tr>
<td>T + 51</td>
<td>1.5</td>
<td>0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>T + 72</td>
<td>2</td>
<td>0.75</td>
<td>1.75</td>
</tr>
</tbody>
</table>

* Time (hours) since plate set up

No mod No further modulator added
CSA Cyclosporin added
VER Verapamil added

If the 'no modulator' columns of table 2.10b, 2.11a-b are examined, it is apparent that cells pre-exposed to verapamil are able to proliferate in this assay to a greater extent
than either those pre-exposed to medium alone or to CSA. The effect on these cells is further illustrated by the effect of adding modulator to pre-treated cells. Verapamil pre-treated cells remain able to proliferate following modulator addition in this assay, i.e. table 2.11b shows that the 100% OD values remain high (usually more than 1).

However cells not pre-treated appear to be less able to proliferate (100% OD values between 0.51 - 0.8), and those pre-treated with CSA show greatly reduced proliferation (100% OD values between 0.2 - 0.6). It is possible that those cells pre-treated with CSA are in fact unable to proliferate, confirmation of this possibility is not achievable using this assay alone.

A further observation on the effect of pre-treating cells with modulator is that either pre-treatment (CSA or verapamil) then renders the cells more sensitive to the cytotoxic (or cytostatic) effect of CSA than verapamil.

Unfortunately the effect of pre-treatment with CSA resulted in optical density readings which would not have normally been accepted. It was not possible to improve these unless the CSA concentration was dropped to less than 0.1μg/ml, and even then the proliferation was not great. So, for this experiment the OD of the '100% survival wells' was considerably lower in those cells pre-treated with CSA and then receiving CSA as modulator of vincristine resistance.

When the IC50 values of pre-treated (tables 2.11c-d) and non pre-treated cells (table 2.10a) are compared, a few important points emerge.
Table 2.11c  Modulation of CSA pre-treated cells

<table>
<thead>
<tr>
<th>Time*</th>
<th>No Mod</th>
<th>CSA IC50</th>
<th>SR</th>
<th>VER IC50</th>
<th>SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>T + 24</td>
<td>10</td>
<td>0.8</td>
<td>12.5</td>
<td>0.3</td>
<td>30</td>
</tr>
<tr>
<td>T + 45</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>0.4</td>
<td>7.5</td>
</tr>
<tr>
<td>T + 47</td>
<td>6</td>
<td>0.2</td>
<td>30</td>
<td>0.5</td>
<td>12</td>
</tr>
<tr>
<td>T + 49</td>
<td>10</td>
<td>0.4</td>
<td>25</td>
<td>0.2</td>
<td>50</td>
</tr>
<tr>
<td>T + 51</td>
<td>8</td>
<td>0.2</td>
<td>40</td>
<td>0.7</td>
<td>11</td>
</tr>
<tr>
<td>T + 72</td>
<td>10</td>
<td>1</td>
<td>10</td>
<td>0.4</td>
<td>25</td>
</tr>
</tbody>
</table>

* Time (hours) since plate set up
No mod  No modulator added
CSA     Cyclosporin added
VER     Verapamil added

Table 2.11d  Modulation of verapamil pre-treated cells

<table>
<thead>
<tr>
<th>Time*</th>
<th>No Mod</th>
<th>CSA IC50</th>
<th>SR</th>
<th>VER IC50</th>
<th>SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>T + 24</td>
<td>20</td>
<td>3</td>
<td>7</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>T + 45</td>
<td>10</td>
<td>0.2</td>
<td>50</td>
<td>0.3</td>
<td>33</td>
</tr>
<tr>
<td>T + 47</td>
<td>10</td>
<td>0.2</td>
<td>50</td>
<td>0.5</td>
<td>20</td>
</tr>
<tr>
<td>T + 49</td>
<td>25</td>
<td>0.3</td>
<td>83</td>
<td>0.4</td>
<td>62</td>
</tr>
<tr>
<td>T + 51</td>
<td>28</td>
<td>0.6</td>
<td>46</td>
<td>0.5</td>
<td>56</td>
</tr>
<tr>
<td>T + 72</td>
<td>30</td>
<td>1</td>
<td>30</td>
<td>1</td>
<td>30</td>
</tr>
</tbody>
</table>

* Time (hours) since plate set up
No mod  No modulator added
CSA     Cyclosporin added
VER     Verapamil added

In those cells not exposed to further additions of modulator the range of IC50 for non pre-treated and CSA pre-treated cells is similar (IC50: 3 - 10ng/ml). However, for cells pre-treated with verapamil the range of values is higher (IC50: 10 - 30ng/ml). This indicates that these cells are more resistant to vincristine than their non verapamil pre-treated counterparts.

With the addition of modulator at various time points (with respect to time of vincristine addition), there appears to be less effect upon both IC50 and SR in pre-treated cells. In
addition those cells which have been pre-treated frequently require higher concentrations of vincristine to achieve the IC50, (compared with non pre-treated cells). So not only are verapamil pre-treated cells more vincristine resistant, but to a lesser extent CSA pre-treated cells also display this phenomenon. The mechanism for this phenomenon is unclear, it may be associated with an increased degree of P gp expression, induction of an alternative resistance mechanism or decreased expression of factors which promote cytotoxicity.
Figures 2.1la-b Sensitisation ratio for modulator pre treated cells receiving additional modulator at various times

Verapamil pretreated cells

CSA pretreated cells
Whilst the changes in SR seen with time of modulator addition appear to follow a similar pattern in modulator pre-treated cells (figure 2.11a-b) to that previously seen in non pre-treated cells (figure 2.10a), the scale of these changes is significantly diminished, such that there is no apparent benefit from varying the time of modulator addition in CSA pre-treated cells, and only a minimal benefit from adding modulator around the time of vincristine addition to cells pre-treated with verapamil.

It needs to be borne in mind however that the CSA pre-treated cells are unable to proliferate well, (for whatever reason), and this may mask or interfere with modulation.
2.11.4 Discussion and Conclusions

Whilst there are obviously several limitations to using this assay system to draw conclusions about reversal of MDR, it does appear that prolonged pre-incubation with a modulating agent does not improve chemosensitivity. If anything it renders these cells more resistant and less modulatable. It is recognised that the cell line used in this assay may not reflect cells within a tumour. However, as this line was not developed to be highly resistant, it is unlikely to overexpress the MDR gene. The cell lines used by Beidler (1993) over expressed the MDR gene, and it was this overexpression which was down regulated following prolonged exposure to modulator.
2.12 EFFECT OF CONTINUOUS VERSUS DISCONTINUOUS EXPOSURE TO MODULATING AGENTS

2.12.1 Introduction and aims

It has been reported that in order to achieve maximal modulation in vitro prolonged continuous administration of modulator is desirable.

Boiocchi et al (1992) demonstrated (in multidrug resistant colon carcinoma cells) that if verapamil was discontinued for even a short period of time (during washing cells) a significant effect upon drug extrusion occurred. Cass et al (1989) had previously demonstrated that multidrug resistant leukaemia cells exposed to vincristine and a modulator for a short period displayed greater cytotoxicity if they were then incubated with modulator alone.

These papers suggest that the effect on survival in cells treated with cytotoxics and modulators for a short period can be influenced by continuing to expose those cells to modulator even after the cytotoxic has been removed. Whilst this effect may be related to the cytotoxic effect of modulator alone, if it is due to increased modulation of MDR it may have important consequences for clinical modulation schedules.

In an attempt to investigate this phenomenon an experiment was designed to allow short cytotoxic exposure (with or without modulator) followed by various modulator conditions. The previously used experimental design was inappropriate to this type of study as it was too difficult to accurately remove medium from wells, wash the cells and then incubate in another solution.

In order to optimise the behaviour of cells using this schedule both the dose range of vincristine and concentrations of modulator had to be altered.
2.12.2 Experimental design

All cells had a three hour exposure to vincristine prior to plating out.

All cells had continuous exposure to modulator for the four day period after the study period.

The study period was designed to allow six different modulator exposures:

1. Continuously present throughout, including the wash periods.
2. No modulator pre treatment, added immediately after plating out.
3. No modulator pre treatment, added two hours after plating out.
4. Modulator pre treatment (three hours pre incubation), no modulator in wash period (30 minutes), modulator added immediately after plating out.
5. Modulator pre treatment (three hours pre incubation), no modulator in wash period (30 minutes), modulator added two hours after plating out.
6. Modulator pre treatment, modulator in wash period, then two hour period without modulator after plating out.

The cell line SK N BE was used for these experiments, cells were harvested for use in the exponential phase of growth. All medium, PBS and drugs used were warmed to 37°C prior to use.

A short exposure to vincristine was chosen, in conjunction with various schedules of exposure to modulator. In all there were nine different modulator treatment possibilities, (all cells were also exposed to a dose range of vincristine), these are illustrated in figures 2.12a-b.

Cells were harvested from tissue culture flasks and resuspended in medium at a cell density of $10^5$ to $10^6$ cells/ml. This cell suspension was then evenly divided between 30 prepared tubes. Each of these prepared tubes contained medium, vincristine (in a dose
range) and modulator. The final tube volume was made up to 10mls in each case with medium.

Vincristine was used in the following final tube concentrations; zero, 1, 10, 100, 1000 and 10 000 ng/ml. This concentration range had to be changed from that used in other experiments in order to produce a dose response curve encompassing the IC50 and approaching the IC80.

Modulator concentrations also had to be reduced in order not to be too toxic to the cells, i.e. to allow the cells to proliferate well in the subsequent MTT assay. Therefore tubes either contained no modulator (one set of 6 tubes), CSA at 1μg/ml (two sets of 6 tubes) or verapamil at 1μg/ml (two sets of six tubes).

All tubes were then incubated in a humidified 5% CO₂ incubator for three hours (longer than this resulted in cells sticking to the tubes).

**Figure 2.12a Schema of drug additions**

<table>
<thead>
<tr>
<th>Cells in tubes</th>
<th>Cells plated out</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>3 hrs</td>
<td>0.5 hrs</td>
</tr>
<tr>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>2 hrs</td>
<td>4 days</td>
</tr>
</tbody>
</table>

Incubation with VCR +/- modulator

Continuous exposure to modulator

Wash in PBS +/- modulator

Variable exposure to modulator
Figure 2.12b  Diagrammatic representation of the modulator treatment to which different groups of cells had been subjected

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Time

- CSA present continuously. No break
- 2 hour break in CSA exposure
- ~ 30 min break in CSA exposure
- ~ 2½ hour break in CSA exposure
- Verapamil present continuously. No break
- 2 hour break in verapamil exposure
- ~ 30 min break in verapamil exposure
- ~ 2½ hour break in verapamil exposure
- No pretreatment, modulator added early
- No pretreatment, modulator added 2 hours after plates set up
At the end of this incubation, the contents of each tube were spun (1000 rpm for 5 minutes) and washed twice in pre-warmed PBS. Those tubes which had not had modulator present during the incubation, and a set of tubes which had verapamil or CSA present during the incubation were washed in PBS alone. One set of tubes which had been incubated with CSA were washed in PBS with CSA in the same concentration as had been present during the incubation. One set of tubes incubated with verapamil were washed in PBS with verapamil in the same concentration as had been present during incubation.

All cells were then resuspended in medium (to allow sufficient cell suspension to add 100μl to 32 wells) and quickly plated out into prepared pre warmed 96 well plates.

Ten plates were set up, five with medium and modulator present at the time of cell addition, and five without. The latter set of plates had medium and modulator added after a further two hour period of incubation. Modulator was added to the plates to allow four different combinations; no modulator, verapamil 1μg/ml, CSA 1μg/ml, or verapamil and CSA at 1μg/ml, (concentrations refer to final well concentration).

Figure 2.12c illustrates how cells and modulator were distributed in a plate. Each plate was set up to include wells without further modulator addition (once cells plated out), these wells provided the control readings used in calculating the SR for a treatment. After all necessary additions had been made the plates were incubated undisturbed for 4 days, at this point they were read in the usual way.
Vincristine concentration is that to which cells were exposed during the initial 3 hour incubation.

<table>
<thead>
<tr>
<th>No Vincristine</th>
<th>VINC 1ng/ml</th>
<th>VINC 10ng/ml</th>
<th>VINC 100ng/ml</th>
<th>VINC 1000ng/ml</th>
<th>VINC 10000ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>No modulator</td>
<td>CSA 1μg/ml</td>
<td>Verapamil 1μg/ml</td>
<td>CSA 1μg/ml and Verapamil 1μg/ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.12.3 Results

The optical density readings of all plates were above 0.75 for the 'no vincristine' cells, this demonstrated that pre-treatment received by these cells was not detrimental to their ability to proliferate.

The IC50 in those cells which did not receive modulator after being placed in 96 well plates, (i.e. exposure to modulator only occurred prior to this), were similar within treatment groups, as shown in table 2.12a.

These figures provide some confirmation for the consistency of results obtained using this assay. It was expected that there would be no difference between those plates which had medium added before cells and plates which had medium added 2 hours after the cells. However what the above results do illustrate is that the IC50 values are significantly affected by the modulator pre-treatment. No modulator or 3 hours of verapamil have a similar IC50, suggesting little effect from short verapamil incubation.

3 hours of CSA has an intermediate effect, verapamil for 3 hours plus washes a greater effect, with the greatest reduction in IC50 seen in those cells pre-treated with 3 hours CSA and CSA in the PBS washes.
Table 2.12a  IC50 for cells which were not exposed to modulator after plating out

<table>
<thead>
<tr>
<th>Treatment (see figure 2.12b)</th>
<th>IC 50 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSA continuous.</td>
<td></td>
</tr>
<tr>
<td>(Pretreatment &amp; wash &amp; immediate addition of medium)</td>
<td>40</td>
</tr>
<tr>
<td>CSA with 2 hour break.</td>
<td></td>
</tr>
<tr>
<td>(Pretreatment &amp; wash &amp; delayed addition of medium)</td>
<td>40</td>
</tr>
<tr>
<td>CSA with 30 minute break.</td>
<td></td>
</tr>
<tr>
<td>(Pretreatment, plain PBS wash, immediate addition of medium)</td>
<td>3000</td>
</tr>
<tr>
<td>CSA with 2½ hour break.</td>
<td></td>
</tr>
<tr>
<td>(Pretreatment, plain PBS wash, delayed addition of medium)</td>
<td>2000</td>
</tr>
<tr>
<td>VER continuous.</td>
<td></td>
</tr>
<tr>
<td>(Pretreatment &amp; wash &amp; immediate addition of medium)</td>
<td>100</td>
</tr>
<tr>
<td>VER with 2 hour break.</td>
<td></td>
</tr>
<tr>
<td>(Pretreatment &amp; wash &amp; delayed addition of medium)</td>
<td>100</td>
</tr>
<tr>
<td>VER with 30 minute break.</td>
<td></td>
</tr>
<tr>
<td>(Pretreatment, plain PBS wash, immediate addition of medium)</td>
<td>600</td>
</tr>
<tr>
<td>VER with 2½ hour break.</td>
<td></td>
</tr>
<tr>
<td>(Pretreatment, plain PBS wash, delayed addition of medium)</td>
<td>700</td>
</tr>
<tr>
<td>No pretreatment, medium added immediately.</td>
<td>5000</td>
</tr>
<tr>
<td>No pretreatment, medium added after 2 hours.</td>
<td>4000</td>
</tr>
</tbody>
</table>

Immediate medium added to plates before cells.
Delayed medium added to plates 2 hours after cells.

N.B. Each 96 well plate contained wells which were only exposed to modulator prior to plating out (i.e. subsequent additions were of medium alone). It is these which are presented in table 2.12a, to illustrate the effect of such pretreatment. Other wells on the same plate will have had modulator added at the appropriate time (in the place of medium alone).

Sensitisation ratios were calculated for the benefit of pre-treatment, i.e. comparing no pretreatment with some pre-treatment (table 2.12b).

Table 2.12b  SR for the effect of various pre-treatment schedules

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSA: 3 hours plus wash</td>
<td>112</td>
</tr>
<tr>
<td>CSA: 3 hours only</td>
<td>7</td>
</tr>
<tr>
<td>VER: 3 hours plus wash</td>
<td>45</td>
</tr>
<tr>
<td>VER: 3 hours only</td>
<td>1.8</td>
</tr>
</tbody>
</table>
For the purposes of all further calculations the IC50 values used were the mean of the IC50 for immediate and delayed addition of medium, these were identical in two cases (see table 2.12a).

Sensitisation ratios were then calculated for each modulator treatment (i.e. modulator added to cells once they were in plates). Figure 2.12d displays these results which have been calculated using the IC50 from 'no modulator pre-treatment' wells. From this figure it is easy to appreciate that further modulation with verapamil after any pre-treatment is least effective, and that there is little variation in the degree of this modulation with various pre-treatment schedules.

Further modulation with CSA is however appreciable and similar with either verapamil or CSA pre-treatment. In this case there does appear to be a reduction in additional modulation with longer periods without modulator.

The greatest modulation was seen with CSA and verapamil used together following pretreatment with continuous CSA. The degree of additional modulation after CSA pre-treatment was related to duration of break in CSA presence, with the exception that the 30 minute gap whilst cells were washed in PBS proved to greatly decrease the amount of modulation.

This observation can also be seen in the other treatments. For instance, both CSA and verapamil alone display less modulation when administered after a 30 minute gap when compared to that seen following a 120 minute gap, and both CSA and double modulator (CSA and verapamil together) are less effective when given after this pre-treatment when compared to either continuous verapamil or verapamil with a 120 minute gap. (It should be remembered that in all cases the 30 minute gap occurred as the cells were being washed).
Figure 2.12d  Sensitisation Ratio to show variation with breaks in modulator pre-treatment
2.12.4 Discussion and Conclusions

The data presented here to some extent support the theory that continuous modulator provides the optimal schedule as the maximum SR for both CSA and verapamil was observed when cells had been pre-exposed without a break to either agent.

The effect of pre-treatment is of note (table 2.12b), with a dramatic increase in SR for those cells washed in modulator (1.8 increasing to 45 for verapamil, 7 increasing to 112 for CSA). As all cells were washed in an identical fashion, the only difference being addition of CSA, verapamil (or nothing) to PBS, it is unlikely that the wash itself resulted in a differential effect upon modulation. Washing cells without modulator may have resulted in intracellular vincristine effluxing from cells and as this vincristine would have then been discarded it would not be present for the following 5 day incubation. Conversely, those cells which were washed in modulator may have retained vincristine, due to the effect of modulator on P gp.

The superior potency of CSA may explain the failure of additional verapamil to produce any further modulation.

Verapamil pre-treated cells demonstrated most modulation when continuous verapamil was followed by CSA (similar in magnitude to CSA pre-treated cells). In all cases of verapamil pre-treatment, CSA or combination modulator (for the following 4 day incubation) was more effective than verapamil. This may be a reflection of the mechanism of action of verapamil as an MDR reversing agent. If verapamil only influences the P gp molecule, whereas CSA is able to also to interact with the plasma membrane, then the use of a schedule where verapamil follows CSA will not produce any additional benefit. However if the converse schedule is given (i.e. verapamil
followed by CSA) the ability of CSA to interfere with the function of P gp through a membrane active mechanism will be observed as increased modulation.

For those cells pre-treated with one of the CSA schedules, verapamil added a small benefit, however the effect of either CSA or both modulators together was more striking (particularly those cells which received continuous CSA followed by both modulators). This implies that although the potency of CSA at reversing MDR is greater than verapamil, it can be further increased by relatively simple means.
2.13 CONCLUSIONS

Given the previously discussed limitations of using paediatric cell lines in an *in vitro* assay (such as the MTT assay) a few general conclusions can be drawn.

The tissue of origin and previous chemotherapy are important in both the chemosensitivity and reversibility (of multidrug resistance) in these lines.

Other resistance mechanisms are likely to play an important part in some of the cell lines used (particularly the rhabdomyosarcoma lines).

Reversal of resistance using modulators is demonstrable. The amount of modulator required to demonstrate reversal approaches that used clinically in some cell lines.

CSA is demonstrably more potent than verapamil at reversing the MDR phenotype at concentrations which are relatively non toxic to these cells in culture.

Using verapamil and CSA together was only beneficial in specific circumstances, and was generally associated with increased toxicity.

When modulator and cytotoxic are both administered simultaneously for prolonged periods, there is a similar effect upon reversal of resistance when the modulator is started a few hours before or after the time at which the cytotoxic is started.

Prolonged pre-treatment of cells with modulator alone does not improve modulation, in fact it appears to make these cells more resistant and less modulatable.

If cells are exposed to cytotoxics for a short period, prolonged exposure to modulators increases cytotoxicity. This is particularly true for CSA, or CSA and verapamil together.

In this schedule there is an advantage (in terms of cytotoxicity) in giving combinations of modulators.
CHAPTER 3.
EXPRESSION OF THE MDR 1 GENE IN PAEDIATRIC TUMOUR CELL LINES
Results presented in Chapter 2 indicate that some of the cell lines investigated display a resistance and reversal pattern consistent with P gp overexpression. In order to be reasonably certain that MDR 1 gene overexpression is the cause of observed drug resistance, not only should the pattern of resistance and reversal be typical, but the cells under investigation should also over express the product of the MDR 1 gene.

In this chapter, the same panel of cell lines is investigated (using molecular methods), to determine which lines display other features of the MDR 1 phenotype.

All cell lines were subjected to each method of investigation. The presence of P gp expression was assessed using an immunohistochemical (IHC) technique with three monoclonal antibodies (C219, MRK 16 and JSB 1). MDR 1 mRNA expression was detected using northern blotting, reverse transcriptase polymerase chain reaction (RT PCR) and In Situ Hybridisation (ISH).

It is now well recognised that reliance on one method alone when investigating MDR 1 expression in tumour specimens is misleading. In several reported series there is marked discrepancy between results obtained with different techniques. This is observed both in clinical material (Toth et al 1992, Vergier et al 1993) and, to a lesser extent, in cell lines (Brophy et al 1994).

Many different ways of performing immunohistochemistry have been developed and are in use in both routine and research laboratories. Unfortunately none of these methods are sufficiently consistent when used to examine specimens for P glycoprotein expression, therefore none are currently in routine use for this purpose or standardised (methodology, antibody source or quantitation of result). Many groups have experienced
considerable difficulty in developing satisfactory procedures for use on fixed tissue, though some are confident that their results are reproducible (Chan et al 1990, 1991). Frozen or fresh material is less difficult to use, and several groups have reported encouraging results from these investigations. There is a general consensus that IHC is not as sensitive as western blotting in detecting P gp expression (Toth et al 1992) though Chan (1988) feels that her method is more sensitive. Toth demonstrated that for a cell line (formalin fixed, immunoperoxidase 'sandwich' method using C 219 and C 494) to express enough P gp to exhibit a positive result, that cell line needed to be at least five fold doxorubicin resistant (compared to parental line). However a positive result could be demonstrated by western blotting in a cell line which was only two fold doxorubicin resistant.

Many different antibodies raised against the P gp molecule have been used in published work. However three are commonly in use and their characteristics are becoming clear. Two of these are commercially available (C 219 and JSB 1), the other is widely used (MRK 16) and originates from Tsuruo's laboratory. The importance of using more than one antibody has been demonstrated many times, and certain cross reactivity patterns are now recognised. For example, C 219 is known to bind to the product of the MDR 3 gene, and to a muscle protein.

Immunohistochemistry is an attractive tool as it allows visualisation of the product of the gene of interest, however results obtained by this method need to be viewed in context, with a thought to the normal expression pattern of P gp and information about expression of the MDR 1 specific mRNA.

Northern blotting is well established as a way of assessing both the presence and size of a particular transcript in total cellular RNA. Briefly, total RNA is isolated from cells or
the specimen of interest. This is separated (by size) by electrophoresis through an agarose gel and transferred to a nylon membrane using capillary transfer (northern blotting). The RNA is covalently cross-linked to the membrane by ultraviolet irradiation. The membrane is then hybridised with a labelled oligonucleotide (probe) specific for the species of RNA to be investigated. If the probe is labelled radioactively, then the result can be visualised on photographic film.

Polymerase chain reaction (PCR) is now a commonplace method employed to investigate the DNA content of cells or samples. RNA can also be examined using this technique if a reverse transcription step is incorporated to transcribe RNA (extracted from the specimen of interest) into complementary DNA. This differs from genomic DNA as mRNA only consists of coding sequences. Specific regions of cDNA can then be amplified once the sequence of the region of interest is known. Short nucleotide sequences complementary to the cDNA need to be synthesised to act as primers for the reaction (gene specific 3' antisense oligonucleotide primers). A pair of primers is chosen to span an intron, this makes amplification of contaminating DNA unlikely due to its very large size. If it is amplified it is easy to distinguish (from the PCR product of interest) owing to the difference in expected size. If the sequence is not known or if the total mRNA complement is to be examined then random hexamers or oligo dTs can be used to prime the reaction. A reaction mixture is assembled which includes the cDNA of interest, a primer pair specific for the region to be amplified, a thermostable DNA polymerase enzyme and nucleotides for incorporation into the product. The mixture is heated up to 94°C to dissociate DNA into two single strands. When allowed to cool (to 57°C) the single stranded DNA comes into contact with, and binds to the oligonucleotide primers. As the temperature is brought up to 72°C the enzyme DNA
polymerase extends the primer to form a new double stranded DNA molecule. These reactions are repeated several times, typically up to forty. PCR exponentially amplifies the sequence of interest, the product can then be used to demonstrate that the region of interest is present in the sample examined by hybridisation of a labelled oligonucleotide probe.

The previous methods have examined RNA samples extracted from cells to determine if a particular sequence is present. In situ hybridisation techniques are able to demonstrate the presence of specific RNA sequences within the tissue or cells of interest without disturbing tissue distribution of RNA. The value of this method lies in being able to determine which cells within a sample are expressing a particular species of mRNA, this has important implications concerning the characteristics of those cells.

In situ hybridisation requires a short nucleotide sequence complementary to a region of mRNA of interest, this is usually called the 'antisense probe'. In addition as a negative control a second nucleotide sequence is used, this is commonly complementary to the antisense sequence and called the 'sense probe'. Both of these probes can be modified in such a way that they can be detected, for example incorporation of a radionucleotide or a fluorescent nucleotide will enable them to be visualised by photographic or fluorescent means. The tissues to be examined with this method need to be prepared in such a way that the mRNA is accessible to the probe which can hybridise to sequence specific regions.
3.2 METHODS

3.2.1 Immunohistochemistry

Cytospin preparation of cell lines

Cells were cytospun as described in the previous section. Three fixation methods were performed initially to compare morphological preservation. The triple fixation method described earlier was chosen as it provided the best intracellular detail. The other two fixation procedures tried were either 5 or 10 minutes in acetone (4°C). If possible slides were used immediately, alternatively unfixed cytospins were stored at ~70°C until required.

Monoclonal antibody characteristics

Three monoclonals were used as a panel, all three are said to be specific for the MDR 1 protein P gp, these were C 219, JSB 1 and MRK 16.

C 219 (Centocor) is a mouse monoclonal (IgG2a), the epitope it recognises is located on the internal surface of the plasma membrane. It is known that this monoclonal shows some cross reactivity with the protein product of the human MDR 3 gene and also with striated muscle (cross reacts with a 200kD glycoprotein which may be a type of myosin). C 219 (Kartner et al 1985) was reconstituted in water to give a 0.1μg/μl solution (working dilution), this was stored in aliquots at -20°C until use.

JSB 1 (Serotec) is also a mouse monoclonal (IgG1) which recognises an epitope on the internal surface of the plasma membrane and was raised by van der Valk in 1990. This is supplied as a ready to use ascites solution which is stored at 4°C.

MRK 16 (Hamada and Tsuruo 1986) was obtained from Dr Twentyman as a 500μg/ml solution this was further diluted to 20μg/ml (working dilution) and stored at -20°C until
use. This monoclonal (mouse IgG2a) has been shown to stain smooth muscle in stomach and intestine. It is specific for MDR 1 (does not cross-react with MDR 3) and recognises an external epitope of P glycoprotein.

**Immunohistochemical method**

An avidin/biotin affinity immunoperoxidase method which is commercially available (Immunotech) was used. In general manufacturers instructions were followed, though it was found that a step to block endogenous peroxidase activity was unnecessary (this enzyme is predominantly found in macrophages and other blood components, but also in renal tubules), and was in fact deleterious to the end result. The kit used was designed to be universal and to produce good results irrespective of the source of the primary antibody. The procedure is as follows: Slides were washed in PBS for 5 minutes (and not allowed to dry out from there on), protein blocking agent (supplied with the kit) was applied for five minutes followed by the primary antibody. This was applied at the working concentrations specified above, 20-40μl per cytospin were required. Incubation with the primary antibody was for at least 18 hours at 4°C. The positive control supplied with the kit proved to be inappropriate, (kappa and lambda light chains) but one cell line always came up strongly positive (H69 LX4) and could be used as a control. A negative control was also always used where the primary antibody was replaced by PBS, with all other steps performed in the same way. After overnight incubation with the primary antibody, the slides were washed in PBS (three washes of 2 minutes), and then incubated with the second antibody (biotinylated and polyvalent) for one hour at room temperature (all ensuing procedures took place at room temperature). Slides were washed in PBS (as above) and then incubated with the universal peroxidase reagent for one hour. Once this had been washed off (PBS for three x 2 minutes), chromogen
solution was placed onto each cytospin for 30 minutes (this reaction could be observed microscopically to ensure adequate time had elapsed). Chromogen was washed off in water and the cytospins counterstained with haematoxylin then mounted beneath coverslips in Gel Tol (aqueous mounting medium).

**Interpretation**

Antibody which has localised to its specific antigen (epitope on P gp) should appear as a red granular reaction product. The haematoxylin counterstain appears blue/purple.

Slides were examined with a standard light microscope and scored as negative (no red granular product), weak positive (+) if there was slight overall or stronger patchy staining. Medium positive (++) was allocated if there was either very strong granular staining but only on a proportion of cells or intermediate staining of most of the calls. Strong positive (+++) was only allocated if there was strong staining of most of the cells.
3.2.2 Northern Blotting

RNA extraction

Cells were cultured and harvested as described in the previous chapter.

Total cellular RNA was extracted from fresh or DMSO frozen cell pellets using the method published by Wilkinson (1988) and described below.

Cells ($10^5$ to $10^7$ cells) were pelleted and resuspended in ice cold tris-saline (400µl). After centrifugation at 3000 rpm ($4^\circ$C) for 30 seconds the supernatant was discarded. The remaining pellet was resuspended in 400µl ice cold tris-saline and 100µl ice cold NDD buffer, each tube was gently inverted ten times and then centrifuged at 3000 rpm ($4^\circ$C) for 30 seconds. The supernatant was transferred to a tube containing 500µl 1:1 phenol:chloroform, with 25µl 20% SDS and 15µl 5M NaCl. This was vortexed briefly and centrifuged at 13 000 rpm (room temperature) for two minutes. The upper aqueous layer was subjected to further extractions with an equal volume of phenol:chloroform (without SDS or NaCl) until the interface between the phases was clear. A final chloroform extraction removed traces of remaining phenol. RNA was precipitated from the aqueous phase by the addition of 1/10th volume 3M Sodium acetate (pH 5.5) then 2.5 volumes of ice cold ethanol. This was left at $-20^\circ$C for a minimum of 2 hours (or up to overnight). Samples were spun at 13 000 rpm for 15 minutes, the pellet washed with 80% ethanol in DEPC water and then air dried until all traces of ethanol had just evaporated. (Overdrying makes the RNA hard to redissolve fully). RNA was then quantified spectroscopically at OD 260nm, where an OD of 1 is obtained from a RNA concentration of 40µg/ml. This was done at 1/100 dilution (i.e. 10µl RNA in 1000µl water).
**Tris-saline** 25mM Tris. HCl pH 7.4, 130mM NaCl, 5 mM KCl.

**NDD buffer** 1% Nonidet P-40, 0.5% Na deoxycholate, 0.01% dextran sulphate (made up in Tris-saline)

**DEPC water** 0.1% Diethyl pyrocarbonate in distilled water, left to stand overnight then autoclaved.

**Gel electrophoresis and RNA transfer**

RNA was size-separated by electrophoresis through a denaturing agarose gel containing formaldehyde. 10μG total RNA per well was loaded and the gel was run at 30mV overnight (18 hours). A photograph of the gel (with ruler) taken under UV illumination showed positions of the 18S and 28S ribosomal RNA and the wells. The gel was then soaked in two changes of 10 x SSC for 20 minutes. The RNA was transferred from the gel to a nylon membrane (Zeta-probe, Promega) by capillary transfer (conventional northern blotting) using 10 x SSC at room temperature overnight. The gel was viewed under UV illumination to confirm complete (or near complete) transfer of RNA. The RNA was covalently bound to the nylon membrane by UV crosslinking and stored at 4°C.

**1% Gel:** 1g agarose, 10mls 10 x MOPS buffer, 87mls DEPC water, melted and allowed to cool to 60°C, 5.1mls 37% formaldehyde added in fume hood, gel poured and allowed to set in fume hood.

**Electrophoresis tank buffer:** 1 x MOPS buffer
10 x MOPS 0.2M MOPS (3-(N-morpholino)-proanesulphonic acid), 50mM sodium acetate, 10mM EDTA adjusted to pH 7.0 and autoclaved.

**Sample buffer:** 10 μl formamide, 2.5 μl formaldehyde, 2.4 μl 5 x MOPS buffer

*10 μg RNA* in 10 μl DEPC water, plus 15 μl sample buffer. Denatured at 65°C for 15 minutes and then loaded with 2 μl running buffer and 1 μl ethidium bromide (1 mg/ml).

**Running buffer:** 50% glycerol, 1 mM EDTA (pH 8.0), 0.25% bromophenol blue, 0.25% xylene cyanol FF.

**Probe preparation and labelling**

**MDR 1 probe**

Supplied by Prof. Borst (The Netherlands Cancer Institute), as the 5' 1.33 Kb of human MDR 1 cDNA clone 1.7 described in van der Bliek et al (1988) from position '98 to the EcoR1 site at position 1237 with respect to the ATG at position 425 of Chen et al (1986). This had been cloned into the EcoR1 site of the Pro mega vector pGEM3Zf (in the sense orientation with respect to the T7 promoter). The insert is specific for the human MDR 1 gene at high stringency (65°C, 0.1 x SSC)

**Preparation of plasmid DNA**

MDR 1 plasmid containing E Coli were grown on L-agar plates containing 0.01 mg/ml ampicillin. A single colony was inoculated into Terrific broth with ampicillin and grown in a shaking incubator overnight at 37°C in a 50 ml sterile tube. The cells were pelleted
by centrifugation at 3000rpm for 5 minutes, resuspended in 100μl lysis buffer and incubated on ice for 10 minutes. Then 200μl of freshly made detergent solution (0.2M NaOH, 1% SDS) was added, followed by a further 10 minute incubation on ice. After this 150μL 3M sodium acetate (pH 4.8-5.5) was added, the tube centrifuged (3000rpm, 10 minutes), and the supernatant collected. DNA was extracted by phenol/chloroform extraction and collected by ethanol precipitation as previously described.

**Restriction enzyme digestion**

Restriction enzyme digestion was carried out according to the manufacturers' instructions using the supplied buffers. EcoRI digestion released the 1.33Kb insert encoding part of the MDR 1 cDNA. BamHI digestion linearised the vector such that transcription from the T7 promoter gave a 1295bp riboprobe in the sense direction (i.e. sequence identical to the MDR 1 mRNA). BglII digestion linearised the vector at the opposite end of the insert such that transcription from the SP6 site gave a 984bp riboprobe in the antisense direction (i.e. sequence complementary to the MDR 1 mRNA).

For preparation of the template for riboprobe synthesis, 20μg of plasmid DNA was digested with the appropriate restriction enzyme then the DNA was purified using the Magic Prep system (Promega). The latter works on the principle of selective binding of the DNA to a particulate resin followed by multiple washes and then elution of the DNA at low salt concentrations into a small volume of water. Linearised template was stored at 4°C in aliquots of 1μg.
**Lysis buffer** 50mM glycine, 25mM Tris.HCl pH 8, 10mM EDTA, 2mg lysozyme.

**L-agar** Bacto tryptone 10g, sodium chloride 5g, yeast extract 5g, agar 15g to make 1 litre, sterilised by autoclaving.

**Terrific broth** Bacto tryptone 12g, yeast extract 24g, glycerol 4ml, KH$_2$PO$_4$ 2.31g, K$_2$HPO$_4$ 12.54g to make 1 litre, sterilised by autoclaving.

**Ampicillin** Was dissolved in sterile water, filter sterilised and added to agar at 10μg/ml.

**Actin probe**

This is a 700 bp HindIII / EcoR1 fragment of the β actin cDNA cloned into pBluescript. The insert was released by double digestion with EcoR1 / HindIII, gel purified and 50 - 100ng aliquots were used as a template for probe synthesis.

**Probe labelling**

The probes were labelled by random priming using the Stratagene PRIME-IT II kit. This enables synthesis of oligonucleotide sequences incorporating radiolabelled nucleotide by the Klenow fragment of DNA polymerase I. Labelling was carried out according to manufacturers' instructions. $^{32}$P CTP with dCTP buffer (containing no 'cold' CTP) was used. The percentage incorporation of radionucleotide into DNA was estimated from the proportion of counts precipitated onto Whatman GF/A filter by 10% trichloracetic acid, which quantitatively precipitates oligonucleotides of greater than 20
bases. Incorporation of greater than 30% was accepted as giving workable probe. Unincorporated nucleotides and protein were removed by passing the reaction mixture through a Sephadex column (‘Nick column’, Pharmacia) with TNE (10mM Tris pH8, 1mM EDTA, 100mM NaCl).

Hybridisation

The nylon membrane was prehybridised at 65°C for 10 minutes in Church and Gilbert hybridisation mixture with denatured sonicated salmon sperm at 200µg/ml. The probe was denatured (5 minutes at 95°C) and added to the hybridisation solution. Hybridisation took place overnight (18 hours) at 65°C in rotating glass bottles. After hybridisation the filters were washed to remove non-specifically bound probe. The initial wash was in 2 x SSC, 0.1% SDS at 65°C for 10 minutes, followed by 2 washes in 0.5 x SSC, 0.1% SDS at the same temperature. This was followed if necessary by a higher stringency wash in 0.1 x SSC, 0.1% SDS.

Excess wash solution was blotted off the filters which were then wrapped in Saran wrap for autoradiography.

**Church and Gilbert buffer** 0.5M Na₂HPO₄ (pH 7.2, with orthophosphoric acid), 7% SDS, 0.1mM EDTA

Autoradiography

Autoradiography of 32P-labelled probe bound to membranes was carried out using Fuji RX medical X ray film in cassettes which had tungsten intensifying screens. Plastic wrapped filters were placed in direct contact with the film and exposed at 70°C for various times. Films were developed using an automatic film processor (Fuji).
3.2.3 Reverse Transcriptase Polymerase Chain Reaction

Reverse Transcription

First strand cDNA was synthesised from total RNA using Superscript (Gibco). This enzyme is purified from E. Coli expressing the cloned pol gene of M-MLV RT from which the RNase H sequence has been deleted. It uses single stranded mRNA or DNA as a template in the presence of a primer to synthesise a complementary DNA strand.

RNA (1µg), oligonucleotides (0.5µg) and water (to make tube volume 11µl at this stage) were first incubated at 70°C for 10 minutes. After this 0.1M DTT (2µl), dNTPs (1µg of 10mM), Superscript enzyme (1µl) and enzyme specific buffer (4µl of 5 x buffer) were added and mixed. A one hour incubation at 37°C allowed cDNA to be transcribed, the reaction was then stopped by heating to 70°C for 10 minutes then placing the tubes on ice. cDNA was stored until use at -20°C.

Polymerase Chain Reaction (PCR)

This reaction was carried out in an automatic programmed heating block (Hybaid Omnigene). Appropriate controls were always included, i.e. negative controls were no RNA and no cDNA tubes, positive control was a known MDR 1 positive cDNA which had been synthesised in the same reverse transcription reaction as the test RNA.

PCR primers were synthesised commercially, and were chosen to be specific for the human MDR 1 gene, and the human B₂ microglobulin (B₂M) gene.
Orientation refers to the sequence of the corresponding mRNA. Sequences of the primers are read from left to right as 5' to 3'.

The MDR 1 cDNA sequence used is that reproduced in figure 1.4a from Chen et al 1986. The primers and internal oligonucleotide used as a probe are marked. The MDR 1 primer pair spans exons 21 and 22, and amplify a 157 bp fragment which is specific for RNA (amplification of any contaminating DNA in the RNA preparation would give a product of 2257kb).

The B$_2$ microglobulin gene sequence used is found in Gussow et al 1987. The primers and internal oligonucleotide used are marked. These primers span two introns, and generate a 120 bp product (the DNA product would be 2175bp).

Internal oligonucleotides were used to confirm specific sequence amplification as described later. The MDR 1 internal oligonucleotide is a 30 bp sequence, containing 50% GC bases, with a Tm of 60.9°C. The B$_2$ microglobulin internal oligonucleotide is a 20 bp sequence, with 55% GC bases and a Tm of 51°C.
PCR was performed using cDNA (1μl), 200nM dNTPs, 0.5μM primer pairs, Taq enzyme (0.1μl) with 2.5μl 10 x buffer (100mM Tris.HCl pH 8.3, 500mM KCl, 0.1% gelatin) and water (to make tube volume up to 25μl). Two drops of mineral oil were added to each reaction tube to minimise evaporation during the reaction. Appropriate positive and negative controls were included in each experiment.

The PCR program used gave a 3 minute initial denaturation at 94°C, followed by 1 minute at 57°C (to allow DNA to anneal with oligonucleotides), 2 minutes at 72°C (to extend the new double stranded molecule). This first cycle was followed by a total of 39 cycles identical to the first except for a shorter initial denaturation (30 seconds at 94°C).

**Gel electrophoresis of PCR products**

For PCR products of this size a horizontal 3% Nu Seive agarose gel run at 70mV for 3 hours provided adequate resolution of the two products when run together. 1 x TAE was used as the tank buffer. A 5μl aliquot of the PCR product was sufficient to determine the success of a PCR reaction, however for the purposes of subsequent capillary transfer and hybridisation a 20μl aliquot was used. A 1 Kb DNA size ladder (Gibco) loaded onto each gel allowed accurate size estimation of the bands. A photograph of the gel taken with UV illumination showed positions of the PCR fragments.

**Gel:**

3% Nu Sieve agarose in 1 x TAE with 2.5μl Ethidium Bromide

(10mg/ml) made to 50mls for mini and 100mls for midi gel.

**50 x TAE**

242g Tris base, 57.1 ml glacial acetic acid and 100ml 0.5M EDTA (pH 8.0) made up to 1 litre.
Gels to be hybridised with the internal oligonucleotide probes were run as midi (100ml) gels in the same way as above.

**Southern blotting**

The PCR products were transferred from the gel to a Zeta-probe (Promega) nylon membrane by capillary transfer in 0.4M NaOH. The gel was viewed under UV illumination to confirm complete transfer of DNA. This did not need to be UV cross linked. The membrane was stored at 4°C until required for hybridisation.

**End labelling of oligonucleotide probes**

The internal oligonucleotides described above were used to confirm the presence of the correct, specific PCR products. These oligos were end labelled with $^{32}$P ATP in a phosphate transfer reaction catalysed by polynucleotide kinase. This was achieved using 10pmol oligo, 2μl 10 x buffer (One Phor all PLUS) 15μl water, 1μl $^{32}$P ATP and 1μl PNK enzyme, these were mixed and incubated for one hour at 37°C. Unincorporated nucleotides and protein were removed by passing the reaction mixture through a 'Nick column' with TNE as described previously.

**Hybridisation**

The nylon membrane was prehybridised at 55°C for 10 minutes in Oligo-Hyb buffer. The $^{32}$P-labelled oligonucleotide probe was then added to the hybridisation buffer. Hybridisation took place overnight (18 hours) at 55°C for the MDR 1 internal and 45°C for the B$_2$ microglobulin internal probe.

After hybridisation the filters were washed to remove non-specifically bound oligonucleotide. The initial wash was in 2 x SSC, 0.1% SDS at 55°C (or 50°C
depending upon which probe was used), 10 minutes, followed by 2 washes in 0.5 x SSC, 0.1% SDS at the same temperature. This was followed if necessary by a high stringency wash in 0.1 x SSC, 0.1% SDS. The filters were blotted and wrapped in Saran wrap for autoradiography.

**Oligo-Hyb buffer** 0.1% SDS, 0.1% Sodium pyrophosphate, 0.05% BSA, 0.05% PVP, 0.05% Ficoll, 5 x SSC.

**Autoradiography**

This was carried out in the same way as previously described.
3.2.4 In Situ Hybridisation

**Cytospin preparation of cell lines**

Each cell line was cultured and harvested as previously described (chapter 2). Cell suspensions (10^6 cells/ml) in PBS were made immediately prior to use. 100μL of cell suspension was added to each cytospin funnel. These cells were spun out onto clean (TESPA coated) slides using a Shandon cytocentrifuge at 500rpm for 5 minutes. Each slide was air dried (5 minutes) and then fixed using the 'triple fixation method' described by Chan et al (1988). This involved 5 minutes in 0.46% formaldehyde (in PBS) at 0°C, followed by two washes in PBS (0°C), then 5 minutes in 95% ethanol (20°C) and 10 minutes in acetone (20°C). Slides were again air dried and used immediately. If slides were to be stored they were kept unfixed (after air drying) at 70°C. These cell preparations were then prepared for hybridisation in the same way as histological sections described below starting at the indicated point.

**Preparation of TESPA coated slides**

Clean slides were treated with 10% HCl in 70% alcohol (10 seconds), followed by 10 seconds in distilled water, and 10 seconds in 95% alcohol. These were dried in an oven at 150°C and allowed to cool. Once cool they were dipped in 2% TESPA (3 aminopropyltriethoxysilane) in acetone (10 seconds) followed by 2 washes in acetone then one in distilled water (10 seconds each) and dried (42°C). If possible slides were used fresh, or stored in an air tight box.
Treatment of glassware to destroy RNase activity.

Bake at 200°C for 1-2 hours. If delicate rinse in 95% methanol, then soak in 3% aqueous hydrogen peroxide (10 minutes), rinse in DEPC treated water, dry and protect from dust.

Pre hybridisation treatment of cells

All glassware and solutions were RNase free. Slides were handled in batches of 20.

Cell line cytospins started pretreatment with rehydration through an ethanol series (two 2 minute dips in each of 100%, 90%, 70%, 50% and 30% ethanol/sterile water).

All slides were acetylated with acetic anhydride to reduce non specific binding (two 10 minute washes in 0.1M triethanolamine pH 8 with 625μl acetic anhydride per 200mls).

Slides were washed in PBS (5 minutes), dehydrated through ethanol series (1 minute each), left in 100% ethanol for three 5 minute periods and then allowed to air dry.

These slides were stored desiccated and used within one day.

PFA

400 mls PBS heated to 65°C then 16g paraformaldehyde added with some 5M NaOH to dissolve the PFA. Allowed to cool to room temperature and then pH adjusted with concentrated HCl. This is used immediately or stored overnight at 4°C.

Preparation of $^{35}$S labelled riboprobes

The MDR 1 specific sense and antisense linearised plasmid templates described in 3.2.2 were used, together with an actin template supplied by Ambion. Riboprobes were made using the Ambion MAXIscript kit and reagents supplied (except SP6 from Pharmacia).
For each labelling reaction 1μg template DNA was used. Reagents were added in the
following order at room temperature (to prevent precipitation of DNA): 2μl 10 x
transcription buffer, 1μl each of 10mM rCTP, rATP and rGTP, 1μl 200mM DTT,
template DNA in 7μl water, 5μl 35S rUTP (>1mCi/100μl), 1μl RNase inhibitor, and 1μl
enzyme (T7 polymerase for actin and MDR 1 sense, SP6 polymerase for MDR 1
antisense). After mixing, the reaction was incubated for one hour at 37°C, with addition
of 1μl further enzyme at 30 minutes. After this incubation template DNA was removed
by the addition of 1μl DNase (37°C for 15 minutes) and this reaction stopped by adding
2μl 0.2M EDTA. To estimate incorporation of radiolabel into RNA, a 1μl aliquot of the
reaction mixture was spotted onto each of two Whatman GF/A filters. One filter was air
dried, whilst the other was washed twice with ice cold TCA to remove unincorporated
nucleotides and once with cold ethanol. After drying the filters were placed in 10 mls of
Aquasol scintillation fluid and specific activity and percentage incorporation were
estimated in a scintillation counter standardised for 35S emissions.

The probe was purified by phenol/chloroform extraction followed by ethanol
precipitation to remove unincorporated label, by adding 1/10 volume 5M ammonium
acetate and 3 volumes ethanol with 5μg yeast RNA to aid precipitation. This was left at
'20°C for 20 minutes and then washed in 80% ethanol in DEPC water.

As the MDR 1 probes were both long (MDR 1 antisense 984bp, MDR 1 sense 1295bp),
a limited alkaline hydrolysis was then performed to produce shorter fragments which
could more readily enter the target cells (approximately 150bp is ideal). This was
carried out with 80mM NaHCO3, 120mM Na2CO3 at 60°C for a time (maximum 70
minutes) estimated using the equation overleaf.
Time (minutes) = (Lo - Lf) / (0.11 x Lf x Lo)

Lo = Transcript size (Kb)
Lf = Desired fragment length (i.e. 0.15Kb)
0.11 = Number of scissions per minute

Time for antisense = 52 minutes
for sense = 54 minutes

Following digestion, the solution was neutralised with ammonium acetate and the labelled riboprobe recovered by ethanol precipitation. After reconstitution in 20μl TE/DTT, the specific activity of the probe was estimated as described above. A DTT concentration of 50mM was maintained at all stages to prevent oxidation of $^{35}$S. The probe was stored overnight (if required) in DEPC water at 70°C. Each probe was further diluted to a concentration of $1 \times 10^5$ disintegrations per minute per microlitre in hybridisation mixture.

**TE buffer** 10mM Tris-HCl pH8, 1mM EDTA

**Hybridisation mixture:**
50% deionised formamide, 10% dextran sulphate, 1 x Denhardt's solution 20mM Tris pH8.0, 0.3M NaCl, 5mM EDTA pH8.0, 0.5 mg/ml yeast RNA, water and DTT added to 50mM immediately prior to use.

20 x Denhardt's is 4g bovine serum albumin, 4g Ficoll and 4g polyvinyl pyrollidine per litre.
Hybridisation and post hybridisation washing

Riboprobes (as prepared above) were denatured (80°C for 2 minutes) and applied directly to the dried tissue section which was overlaid with a siliconised coverslip (approximately 20µl per 20mm² coverslip). The slides were hybridised overnight (18 hours) in a sealed box (containing tissue soaked in 50% formamide, 5 x SSC), at 55°C (either in a water bath or hybridisation oven). After this period of hybridisation the slides were washed for 30 minutes at 50°C in 5 x SSC, 10mM DTT to remove the coverslips and excess probe. Then followed a series of washes starting with a high stringency wash in 50% formamide, 2x SSC, 0.1M DTT (20 minutes at 60°C), with three 10 minute washes in NTE to remove traces of DTT. All sections were then subjected to RNase A treatment to digest probe which was not bound in a stable duplex, (40µg/ml RNase A, pH 7.5 in NTE for 30 minutes at 37°C). A second high stringency wash followed, as above. The sections were then washed in 2 x SSC for three periods of 10 minutes, then 0.1 x SSC for three periods of 10 minutes. They were then dehydrated through an ethanol series (each containing 0.3M ammonium acetate pH7) and air dried.

NTE 0.5M NaCl, 10mM Tris, 5mM EDTA pH7.5.

Siliconisation of coverslips:

Coverslips were soaked overnight in 100% alcohol, rinsed in fresh 100% alcohol, dipped in silane solution for 20 seconds, dried, then finally dipped in 100% alcohol before being dried ready for use. If possible coverslips were prepared fresh each time.
 Autoradiography of slides

Slides were coated in a 50% solution of Ilford K5 emulsion (melted, diluted with warm water, glycerol added to 1%), and air dried on racks in a light proof box (overnight). They were then packed into sealed light proof slide boxes with silica gel and exposed at 4°C for 21 days.

Slides were developed in Kodak D19 for 2 minutes at room temperature, development stopped with 1% acetic acid, 1% glycerol for 1 minute. Slides were fixed for 4 minutes in 30% Sodium thiosulphate, and washed twice in water (10 minutes each). Sections were then stained in Giemsa (0.1% made up in BDH buffer and water) or Haematoxylin, washed, air dried and mounted beneath coverslips using DPX mounting fluid.

Slides were examined and photographed using bright field illumination to display tissue detail and dark field illumination to enhance visibility of the areas of probe binding indicated by overlying silver grains.
3.3 RESULTS

3.3.1 Immunohistochemistry

From the comparison of fixation methods for these cytospun cell lines (figure 3.3a) it became apparent that the triple fixation method provided superior morphological detail. This fixation method was adopted for further experiments on the assumption that cytoplasmic structures and antigenic epitopes would be more intact in those cells which displayed more ultrastructural detail.

Figure 3.3a Morphology of cell line (RMS) following different fixation methods (x40)

i) Triple fixation method

ii) 5 minutes in acetone

iii) 10 minutes in acetone
Plain morphology of each line is shown (following Giemsa staining) in figure 3.3b. As can be seen from this the morphology and preservation of detail in the cell lines investigated is variable, although in some cases there was good cytoplasmic detail.

Figure 3.3b  Morphology of all cell lines (except RMS) following triple fixation and Giemsa staining  (x 40)

i)  H69 LX4

ii)  H69 P

iii)  CCRF CEM

iv)  HX 142

v)  SK N BE

vi)  SK N SH

vii)  RD

viii)  HS

ix)  HX 170
Table 3.3a summarises the IHC results for the panel of cell lines, figure 3.3c shows examples of some of these positive lines. Antibody staining was cytoplasmic in all cases of positivity with stronger membrane staining (than cytoplasm) in a few cases.

Figure 3.5c  Immunohistochemistry of four cell lines with each monoclonal antibody (x 40)

Cell line HX 170

C 219  JSB 1

MRK 16
Cell line H69 LX4

C 219

JSB 1

MRK 16
Table 3.3a  Summary of immunohistochemistry results

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Neg. control</th>
<th>C219</th>
<th>JSB 1</th>
<th>MRK 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>H69 P</td>
<td>Neg</td>
<td>++</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>H69 LX4</td>
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<td>+++</td>
<td>+++</td>
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<tr>
<td>CCRF CEM</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>+</td>
</tr>
<tr>
<td>RD</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>+</td>
</tr>
<tr>
<td>RMS</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>HS</td>
<td>+ / Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>+</td>
</tr>
<tr>
<td>HX 170</td>
<td>+ / Neg</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>SK N BE</td>
<td>Neg</td>
<td>+</td>
<td>++</td>
<td>+</td>
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<td>SK N SH</td>
<td>Neg</td>
<td>+</td>
<td>Neg</td>
<td>+++</td>
</tr>
<tr>
<td>HX 142</td>
<td>Neg</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Neg. control buffer only in primary antibody step

Neg = negative result

+, ++, +++ = positive result, as described in methods section

From the above table it is easy to see that interpretation based on a single antibody result is misleading. The two cell lines which came up positive when subjected to 'negative control' conditions displayed a (very) few strongly staining cells which were small and looked quite different from others in the cytospin preparation. It is most likely that these represent non viable cells which may take up the second antibody and therefore undergo the immunoperoxidase reaction. Of those cell lines which appeared to be weakly positive (i.e. +), three displayed strong positivity on a few cells only (RD, HS and HX 142), the others had weak uniform staining. Explanations for this type of positivity include;

a) Contamination with cells from another cell line, this is unlikely to have occurred at the time of slide preparation as great care was taken to ensure each cell line was centrifuged separately.

b) The cell line culture may have become contaminated, again this is unlikely because given the similar culture conditions of all cell lines, any contaminating line should
proliferate well and so give rise to a larger proportion of cells on the cytospin preparation.

c) Damage to antigenic epitopes or cytoplasmic structures during fixation or processing.

Defining 'overall positive IHC' to be any positive result from two or more antibodies the following cell lines are P glycoprotein positive: H69 LX4 (small cell lung), HX 170 (rhabdomyosarcoma), SK N BE, SK N SH and HX 142 (all neuroblastoma).

It is of interest that the parental (MDR negative) cell line (H69 P) reacts with the C 219 monoclonal, and that even though C 219 is known to recognise a myosin protein, only one of the rhabdomyosarcoma lines (HX 170) was positive with this antibody. H69 LX4 was not as strongly positive with C 219 as might be predicted both from the level of mRNA specific MDR 1 expression on RT PCR and in comparison with the degree of positivity seen with the other two monoclonals. This observation has not previously been reported in published work.
3.3.2 Northern Blotting

The MDR 1 mRNA is 4.5Kb and will therefore run to approximately the same position on the agarose gel used as 28S ribosomal RNA. The actin mRNA is 1.1Kb and runs to approximately the same position as the 18S ribosomal RNA.

Figure 3.3d shows the RNA stained with ethidium bromide and illuminated with UV light. The quality of RNA was such that some samples needed to be re run. However RNA of acceptable quality was run on the two gels shown and was transferred by capillary action onto the nylon membrane a previously described. Figure 3.3e shows autoradiography of the MDR 1 probe hybridised to RNA, and figure 3.3f shows the actin probe. These figures are a composite of different exposures on two membranes.

Figure 3.3d Photograph of RNA separated by electrophoresis, stained with ethidium bromide and illuminated by UV light
Figure 3.3e  Autoradiograph of MDR 1 probe hybridised to cell line RNA

<table>
<thead>
<tr>
<th></th>
<th>SH</th>
<th>HS</th>
<th>170 LX4 BE CEM</th>
<th>142</th>
<th>RD</th>
<th>RMS</th>
<th>P</th>
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</thead>
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<td><img src="image1.png" alt="Image" /></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image1.png" alt="Image" /></td>
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</tr>
<tr>
<td>18S→</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image1.png" alt="Image" /></td>
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<td><img src="image1.png" alt="Image" /></td>
<td><img src="image1.png" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.3f  Autoradiograph of actin probe hybridised to cell line RNA

<table>
<thead>
<tr>
<th></th>
<th>SH</th>
<th>HS</th>
<th>170 LX4 BE CEM</th>
<th>142</th>
<th>RD</th>
<th>RMS</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>28S→</td>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>18S→</td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>
The differences in signal intensity is largely due to inaccuracies in RNA quantitation and loading as can be seen from the actin result. The cell lines in which detectable MDR 1 mRNA is found on northern blotting are H69 LX4, SK N BE, HX 142, SK N SH and HS. The signal from H69 LX4 is not as intense as expected, this is probably due to partially degraded RNA. However repeat experiments were unable to improve upon this.
### 3.3.3 Reverse Transcription Polymerase Chain Reaction

Each cell line was treated using the method described above, and the PCR products separated by electrophoresis on an agarose gel. Figure 3.3g shows such a gel stained with ethidium bromide under UV illumination.

**Figure 3.3g** PCR products separated by electrophoresis and stained with ethidium bromide

Interpretation of the stained gel in figure 3.3g indicates that the $\beta_2$ microglobulin PCR fragment is amplified in all cell lines, although the H69 LX4 line consistently shows reduced amplification compared with the other lines (even when the reaction is performed in a separate tube to the MDR 1 PCR reaction). Several cell lines apparently amplify the MDR 1 fragment, these include H69 LX4, SK N BE, HX 142, SK N SH and CCRF CEM.

In all these cell lines a 157 bp product is seen, consistent with MDR 1 expression. Using the method described above it did not prove possible to confirm the nature of the PCR product using MDR and $\beta_2$ microglobulin internal oligo-probes. The reasons for this remain unclear.
3.3.4 **In Situ Hybridisation**

When examining the results of these experiments it was necessary to compare the background number of silver grains in an area without cells with the number of silver grains overlying a cellular area on each slide. Additionally the silver grain count (per area) following treatment with the sense and antisense probes needs to be compared. A strongly positive result can only occur if the silver grain count with the antisense probe is much higher than with the sense probe, and the grain count over cellular areas is much higher than background counts. A further (actin antisense) probe was used, this is expected to provide a strongly positive result in all cells and was used as a positive control for each specimen.

When the cell line cytospins were examined they were scored on the basis of estimated grains per cell. This was translated into four categories: low (less than 5 grains/cell), medium (10-30 grains/cell), high (30-100 grains/cell) and very high (more than 100 grains/cell). These results for the cell lines are presented in table 3.4a. In most cases the background was approximately 5 grains/cell, in some cases it was higher than this. If the background staining was the same order of intensity as the staining over cells the overall result (of MDR mRNA expression) was considered to be 'low' as no further interpretation could be made. Cases where this arose are marked (*). Figure 3.3h shows representative areas of some MDR positive (by ISH) cytospin preparations taken under dark and bright field microscopy.
### Table 3.4a  
In situ hybridisation results for cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>MDR sense</th>
<th>MDR antisense</th>
<th>Actin antisense</th>
<th>Overall result*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H69 LX4</td>
<td>Low</td>
<td>Very High</td>
<td>Very High</td>
<td>Positive</td>
</tr>
<tr>
<td>H69 P</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Negative</td>
</tr>
<tr>
<td>CCRF CEM</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Negative</td>
</tr>
<tr>
<td>RMS</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Negative</td>
</tr>
<tr>
<td>RD</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Negative</td>
</tr>
<tr>
<td>HS</td>
<td>Low</td>
<td>Low*</td>
<td>High</td>
<td>Negative</td>
</tr>
<tr>
<td>HX 170</td>
<td>Medium</td>
<td>Medium</td>
<td>High</td>
<td>Negative</td>
</tr>
<tr>
<td>HX 142</td>
<td>Medium</td>
<td>Very High</td>
<td>High</td>
<td>Positive</td>
</tr>
<tr>
<td>SK N BE</td>
<td>Low</td>
<td>High</td>
<td>Very High</td>
<td>Positive</td>
</tr>
<tr>
<td>SK N SH</td>
<td>Low*</td>
<td>Medium</td>
<td>High</td>
<td>Positive</td>
</tr>
</tbody>
</table>

* overall result cell lines which appear to be over expressing the MDR 1 mRNA

---

**Figure 3.3h**  
Photographs of ISH on cell lines under dark and bright field microscopy
3.4 DISCUSSION

The IHC results presented in table 3.3a illustrate some of the difficulties in interpretation, with only four (out of ten) cell lines displaying the same result for all three monoclonal antibodies. Even with cell lines where it is assumed that the cell population is homogeneous, gross differences are still seen in the staining of cells in different parts of the same cytospin. Interpretation of histological specimens where the cell population is known to consist of a variety of different cell types is thus even more difficult.

The 'cross reactivity' of these antibodies with antigenic sites (other than P glycoprotein) is likely to occur more frequently in tissue specimens than in cell lines. In addition to those immunoreactivity patterns already mentioned, C219 and MRK 16 have been shown to immunoreact with stromal macrophages and endothelial cells within NHL and non lymphoid tumours (Schlaifer et al 1992). Although it is possible that this in fact represents P gp expression at these sites.

In the previous chapter it was suggested that out of the ten cell lines investigated, only four exhibited resistance patterns which were reversible using verapamil or cyclosporin. These cell lines were H69 LX4 and the three neuroblastoma lines (SK N BE, SK N SH and HX 142). All four of these lines appear to express enough P gp to be considered positive using this IHC method. The rhabdomyosarcoma line HX 170 also appears positive by this IHC method, however the only experimental circumstance in which it displayed reversal of resistance was when etoposide and verapamil were used in combination.
The technique of northern blotting is known to be relatively insensitive when compared with RT PCR, however in this case one cell line (HS) was apparently positive by northern but not by RT PCR, the reasons for this are unclear.

The results obtained for ISH on cytospin preparations of cell lines are reproducible and (despite a high level of background staining in some cases) are probably a valid reflection of the mRNA expression in those cells.
3.6 CONCLUSIONS

Table 3.6a summarises the MDR status of the cell lines investigated using four methods (northern blotting, RT PCR, ISH and IHC), and those with a resistance pattern reversible with verapamil or CSA (from chapter 2).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Northern</th>
<th>RT PCR</th>
<th>ISH</th>
<th>IHC</th>
<th>Reversible in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>H69 LX4</td>
<td>SCLC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H69 P</td>
<td>SCLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCRF CEM</td>
<td>ALL</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMS</td>
<td>Rhabdo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>Rhabdo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HS</td>
<td>Rhabdo</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HX 170</td>
<td>Rhabdo</td>
<td></td>
<td>+</td>
<td></td>
<td>(+)</td>
</tr>
<tr>
<td>HX 142</td>
<td>NBL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SK N BE</td>
<td>NBL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SK N SH</td>
<td>NBL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ positive result

The known positive SCLC cell line (H69 LX4) and all of the neuroblastoma cell lines are positive by all four methods, and on in vitro testing. Two of the rhabdomyosarcoma cell lines (RMS and RD), and the known negative SCLC line (H69 P) are negative by all methods. However there are three lines with mixed results, CCRF CEM is positive by RT PCR, and on IHC by one antibody (MRK 16). HS was positive on northern and also on IHC by MRK 16, and HX 170 was positive by IHC but not by any other method. None of these lines displayed reversible resistance patterns on in vitro testing.

It is of interest that CCRF CEM is used in many studies of MDR as a resistant cell line once resistance has been developed by sequential exposure to cytotoxic agents.

Few published papers compare IHC with ISH, RT PCR and northern blotting. However two have used slot or dot blotting in place of northern blotting. A report using these
techniques (IHC with MRK 16 only) on tumour cells from children with leukaemia or lymphoma (Brophy et al 1994) found that there was 39% complete agreement between methods (36 samples), with the highest concordance between ISH and RT PCR (80%). Sarcoma tissue was examined in 22 cases by Vergier (1993), with ISH, dot blot/northern and IHC (using three monoclonal antibodies). ISH perfectly correlated with dot blot hybridisation in the only case positive by all methods (this case was positive with all three monoclonal antibodies). C 219 was not positive in any other case, JSB 1 marked stromal cells in one other case, and MRK 16 marked stromal cells in seven other cases and tumour cells in a further seven (these samples were not positive by any other method). Positive and negative cell lines were used as controls, the positivity of these cell lines by IHC is not stated, though it is implied that the lines known to express P gp were positive by all methods.

The results obtained from this work again emphasise the importance of not relying on a single method of determining MDR status, unfortunately the most specific method (ISH) from this study is also the most complex and time consuming and moreover, did not give a clear result when used on tissue sections (not shown). The convenience and reliability of methods is of extreme importance when deciding which should be included in a panel to screen samples for MDR status.
CHAPTER 4
MODULATION OF CLINICAL DRUG RESISTANCE USING CYCLOSPORIN A
4.1 INTRODUCTION

4.1.1 Cyclosporin characteristics

Cyclosporin A, a natural metabolite of the fungal species *Tolypocladium inflatum* was first discovered in a soil sample from Norway in 1976. It is a neutral lipophilic cyclic endecapeptide and is distinctive for three reasons: One of the amino acids had never previously been isolated or known in free form, ten of the eleven amino acids are aliphatic and it has a rigid ring structure. Cyclosporin A and several structural cogners (with substitutions or deletions on the ring structure), have now been fully synthesised and are available, however of these, CSA remains the most active immunosuppressive agent (Kahan 1985).

Cyclosporin is commonly used in both oral and intravenous preparations to provide immunosuppression to patients who have received a bone marrow or organ transplant (Kahan 1989). In the context of modulation of drug resistance only the parenteral preparation has been used to date. As CSA is poorly soluble in aqueous solutions the intravenous preparation is stabilised in castor-oil (cremophor). It appears that cremophor itself is not without activity in multidrug resistant cells (Schuurhuis et al 1990) which may complicate evaluation of cyclosporin per se.

CSA circulates bound to low density lipoproteins and chylomicrons and also in a free form. The amount of free circulating CSA (determined following separation from plasma by dialysis and measured by HPLC) does not correlate with either the measured serum concentration or toxicity (Lindholm et al 1988). It is likely that bound CSA acts as a reservoir which is directly transferable from the blood into cell membrane structures.

CSA is metabolised by the liver and excreted in both faeces and urine (Maurer 1985). Hepatic metabolism occurs via isoenzymes of the cytochrome P-450 enzyme
superfamily. Drugs which induce or inhibit these enzymes will interfere with CSA metabolism. Virtually no unmetabolised CSA is found in either bile or urine after an intravenous dose. The majority of CSA metabolites are excreted via the biliary system, (this has obvious implications in cases of hepatobiliary dysfunction), and a degree of enterohepatic circulation occurs. The importance of CSA metabolites in either efficacy or toxicity is unclear, though there is a suggestion that one of the metabolites (AM1) has significant immunosuppressive activity (Kahan 1993). It is now well recognised that children have a higher clearance rate of CSA than adults (up to 40% higher), and therefore may need a different dose and schedule (Kahan and Grevel 1988). Patients with hyperbilirubinaemia or raised alanine aminotransferase have reduced clearance of CSA and its metabolites. Renal impairment does not significantly influence CSA metabolite elimination as so little is excreted by this route (6% compared with more than 90% in the bile).

Tissue distribution studies have been reported and indicate that (as expected from the drugs high lipophilicity) fatty tissues contain the highest concentrations. In addition, pancreas, adrenal and to a lesser extent liver have high levels, (the brain has very low levels). This widespread tissue distribution helps to explain the large apparent volume of distribution.

Following intravenous administration, an elimination half life (of both CSA and its metabolites) in the order of 6-8 hours occurs, with clearance between 2-32 mls/min/kg. The wide variation in clearance reflects rapid elimination in children and much slower elimination in the elderly, or those with hepatic dysfunction (Wood and Lemaire 1985). Several different methods to measure CSA concentration have now been developed, CSA is stable in serum at room temperature for several days, but whole blood samples need to be assayed quickly to prevent CSA release from cells (Smith et al 1983).
assays can measure the CSA concentration in whole blood, plasma or serum, however the concentration in whole blood is consistently higher than that in plasma or serum (due to CSA within red blood cells).

The two main assay techniques in use are high performance liquid chromatography (HPLC) and radioimmunoassay (RIA). The first to be developed was HPLC and it remains the more accurate, however it requires a large sample and a higher degree of expertise. Although RIA is now the most commonly used method earlier assays were unable to distinguish between CSA and its metabolites.

Monitoring of plasma concentrations of CSA is important both to achieve a therapeutic level to prevent graft versus host disease (GvHD) in bone marrow transplant patients, and graft rejection in solid organ transplant recipients, and to avoid the toxicity associated with high concentrations. Whilst the concept of a therapeutic level of CSA is clear, it is not certain what that level needs to be in different clinical situations. Additionally there is a great degree of variation in pharmacokinetics between patients making accurate prediction of the dose required difficult. There are many reported series which have examined the relationship between CSA dose or concentration and transplantation success. Data from recipients of heart, kidney or bone marrow transplants indicate that an oral dose of more than 3mg/kg is required for maximum efficacy, but that doses of more than 10mg/kg are associated with toxicity (Mason 1992). Some studies demonstrate increased GvHD in bone marrow transplant patients with low CSA concentrations (Yee et al 1988), whilst other studies demonstrate no correlation between CSA concentration and development of GvHD (Deeg et al 1985, Barrett et al 1982,). In the context of bone marrow transplantation a CSA concentration of between 150-300ng/ml is usually considered adequate. In solid organ transplant patients a concentration of between 250-500ng/ml is generally aimed for, though in the
case of renal transplants this is kept lower initially when the transplanted kidney is at its most vulnerable (Kahan 1993, Haverich 1992). Concentrations lower than 200ng/ml are associated with a higher incidence of graft rejection and loss.

There are many adverse effects associated with CSA, some of these are seen with chronic administration (hypertrichosis and gingival hyperplasia), others are more commonly associated with CSA concentration (renal impairment or hypertension). Renal damage occurs as a result of constriction of the afferent glomerular arteriole leading to a reduced glomerular filtration rate often in association with tubular damage. Renal impairment appears to correlate with the trough CSA concentration rather than the peak concentration achieved, a trough lower than 400ng/ml has been advocated in bone marrow transplant patients (Hows et al 1983). More recently it has been suggested that area under the concentration curve gives a better indication for dose adjustments (Grevel 1993). The incidence of renal damage is increased with concurrent use of other nephrotoxic agents (Hows et al 1981). Hypertension and convulsions are recognised in patients receiving CSA, and these may be more frequent in children than in adults (Joss et al 1982).

Arellano et al (1991) reviewed all reported cases of CSA overdose (twenty seven). Two of these were intravenous (5 intramuscular, twenty oral) and the dose received ranged from 30 to 400mg/kg. Toxicity was as expected from the known adverse effects of CSA and generally self limiting. Neonates fared particularly badly (one died) with hypotension, wheezing, metabolic acidosis and renal failure. The suggested management of an overdose of CSA is to reduce further absorption (if within six hours of oral administration), and to consider plasmapheresis (although this is of limited value because CSA has a large volume of distribution and as much as 60% of circulating CSA may be bound to blood cells). Of the two patients who received an intravenous dose,
one was a neonate (CSA 400mg/kg) and the other an adult (30mg/kg). They both survived, the neonate was seriously ill, but the adult only experienced abdominal pain and hyperbilirubinemia. No other patient was known to have developed a raised bilirubin, though two others had elevated liver enzymes.

Two of the more comprehensive reports of an overdose of CSA both involve an oral 5000mg dose, equivalent to 150mg/kg in an eleven year old bone marrow transplant patient (Kruger et al 1988), and 93mg/kg in a 31 year old kidney transplant patient (Schroeder et al 1986). Peak measured CSA concentrations (at 8-10 hours after ingestion) were 8200ng/ml (in the child) and 2600ng/ml (in the adult) by RIA. In both cases the level had fallen below 100ng/ml by 100 hours post ingestion. Both patients felt unwell with vomiting, drowsiness and headache, there were no hypertensive problems. Although the renal transplant patient lost the transplanted kidney from rejection 39 days after the CSA overdose the other patient did not suffer any renal toxicity.
4.1.2 CSA as an MDR modulating agent

As discussed in the first chapter, cyclosporin has been used as a modulating agent in both children and adults with resistant or relapsed malignant disease. Reported studies using CSA have employed various schedules and doses, in all cases so far published it has been administered intravenously. The majority of these studies employ a loading dose (up to 6mg/kg given over 1-2 hours) followed by a continuous infusion (up to 24mg/kg/day for 120 hours). From dose escalating studies a loading dose of 5-6mg/kg with a continuous infusion of 15-18mg/kg/day has been suggested on the basis of toxicity (Bartlett et al 1994, Yahanda et al 1992 and List et al 1993). The remainder of reported studies (including most of those on children) use a short intravenous infusion (one to three hours) given once or twice a day. The doses used in these cases is variable, for example 3mg/kg in one hour given seven hours and one hour prior to the chemotherapy (Verweij et al 1990), 4mg/kg in one hour on a daily basis for five days (Sonneveld and Nooter 1990), and dose escalating up to 33mg/kg in three hours given on two consecutive days, (Chan et al 1992 a & b, and 1994).

In most of these studies the toxicity of treatment (CSA in combination with a cytotoxic agent) was greater than expected, in some cases this was attributed to the modulating agent and in others to an increase in (expected) toxicity from the cytotoxic agent (for example increased vincristine neurotoxicity, Weber et al 1993). In both of these instances the dose of the suspected agent was modified, i.e. not increased further in dose escalation studies or dose reduced (Bartlett et al 1994, Thiessen et al 1992). From pharmacokinetic studies performed a significant effect on the clearance of both etoposide and doxorubicin has been demonstrated in adult patients given either of these agents together with CSA. The dose adjusted AUC of doxorubicin was increased by 55% when given in conjunction with CSA (Bartlett et al 1994), similar toxicity was
seen following a 60% doxorubicin dose reduction (when CSA and doxorubicin was compared with cytotoxic alone). When etoposide was given with CSA an 80% increase in dose adjusted AUC was seen when compared with etoposide alone (Lum et al 1992), this group suggested that a 60% etoposide dose reduction might be required when combination therapy is used in order to avoid unacceptable toxicity.

The toxicity seen with combination therapy was variable, reported problems include hypertension, myelosuppression, nausea, headache, hypomagnesaemia and some nephrotoxicity. Some patients appeared to experience pain or discomfort (not related to known sites of disease) in association with the CSA infusion.

Most studies have measured CSA concentrations and attempted to correlate these with the concentration needed to reverse MDR \textit{in vitro} (Slater 1986, Twentyman and Wright 1991a), which is in the order of 100-10 000ng/ml. Most clinical studies aim to attain at least 3000ng/ml (the lowest dose shown to be effective by Slater et al). The continuous infusion schedules (15mg/kg/day) are able to achieve 1000-4000ng/ml without unacceptable toxicity. The only short infusion schedule which has published results of CSA concentration is that from Verweij et al (1990) who used 3mg/kg in one hour given twice (at one and seven hours prior to chemotherapy), the peak CSA concentration achieved was at least 13 500ng/ml.

It is not clear how important the differences in CSA metabolism between children and adults are in the type of schedules discussed. It is likely that whilst the peak level of CSA will be similar in both the concentration during a continuous infusion or the AUC following a short infusion will be lower in children. In addition children may experience greater toxicity (than adults) from the CSA element of combination treatment.
4.1.3 Study objectives

The principle aims of this study were:

1. To investigate the feasibility of using either a continuous or a short infusion of CSA as a MDR modulating agent in paediatric patients.
2. To assess toxicity of both schedules.
3. To measure the CSA concentration achieved in each schedule.
4. To measure etoposide pharmacokinetics with and without CSA in both schedules.
4.2 TRIAL DESIGN

4.2.1 Patient characteristics

Eight patients with relapsed or resistant paediatric malignancy have so far been recruited into this study. Three of the patients had rhabdomyosarcoma (one embryonal, two alveolar), two neuroblastoma and one each; acute lymphoblastic leukaemia, Ewing's sarcoma and osteosarcoma. The patients age at the time of treatment was 5 - 17 years (median 14.5 years) and six of the patients were male.

Informed consent was obtained from the parents and patients (if old enough) and this study was approved by the local ethics committee.

Table 4.2a shows the previous treatment received by these children, all had previously had intensive treatment which included etoposide, details of this are shown in table 4.2b.
### Table 4.2a  Details of previous treatment

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease</th>
<th>Age</th>
<th>Interval</th>
<th>Primary and second line treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RMS</td>
<td>15 years 4 months</td>
<td>MMT 89, Melphalan ABMT</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Ewing's</td>
<td>16 years 14 months</td>
<td>High risk Ewing's protocol, surgery, Bu/Melph ABMT, RT</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ALL</td>
<td>17 years 0 months$^*$</td>
<td>UKALL XD, UKALL R1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Osteo</td>
<td>17 years 3 months</td>
<td>Cisplatin/Dox, Cyclo/Etop, HD MTX</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>RMS</td>
<td>6 years 15 months</td>
<td>VACA, Etop/Ifos, Etop/Vinc/Actino &amp; verapamil, surgery, RT</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>NBL</td>
<td>6 years 0 months$^*$</td>
<td>OPEC/OJEC</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>NBL</td>
<td>5 years 12 months</td>
<td>OPEC/OJEC, Melphalan ABMT, oral etoposide</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>RMS</td>
<td>14 years 4 months</td>
<td>MMT 89, Melphalan ABMT</td>
<td></td>
</tr>
</tbody>
</table>

* at relapse or progression  
$^*$ between start of last remission and relapse  
$^*$ progressed through second line treatment  
$^*$ failed to respond to primary treatment

Bu/Melph  Busulphan and Melphalan  
HD MTX  High dose methotrexate  
High risk Ewing's protocol  Ifosfamide, Vincristine, Doxorubicin, Etoposide, Actinomycin D  
MMT 89  Carboplatin, Epirubicin, Vincristine, Ifosfamide, Actinomycin D, Etoposide.  
OJEC  Vincristine, Carboplatin, Etoposide, Cyclophosphamide  
OPEC  Vincristine, Cisplatin, Etoposide, Cyclophosphamide.  
UKALL R1  Vincristine, Asparaginase, Dexamethasone, Epirubicin, 6 Thioguanine, Methotrexate, Etoposide, Cytosine Arabinoside, 6 Mercaptopurine,  
UKALL XD  Vincristine, Asparaginase, Prednisolone, Adriamycin, 6 Thioguanine, Methotrexate, Etoposide, Cytosine Arabinoside, 6 Mercaptopurine.  
VACA  Vincristine, Adriamycin, Cyclophosphamide, Actinomycin D.
Table 4.2b  Prior exposure to etoposide

<table>
<thead>
<tr>
<th>Patient</th>
<th>Prior etoposide exposure</th>
<th>Total etoposide dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200 mg/m^2/day x 3 days, 3 courses</td>
<td>1800 mg/m^2</td>
</tr>
<tr>
<td>2</td>
<td>100 mg/m^2/day x 5 days, 3 courses</td>
<td>1500 mg/m^2</td>
</tr>
<tr>
<td>3</td>
<td>200 mg/m^2/day x 3 days, 2 courses</td>
<td>1200 mg/m^2</td>
</tr>
<tr>
<td>4</td>
<td>100 mg/m^2/day x 5 days, 2 courses, 150 mg/m^2/dose x 4, 200 mg/m^2/day x 4 days, 2 courses</td>
<td>3200 mg/m^2</td>
</tr>
<tr>
<td>5</td>
<td>100 mg/m^2/day x 5 days, 3 courses, then 150mg/m^2/day x 3 days, 6 courses</td>
<td>4200 mg/m^2</td>
</tr>
<tr>
<td>6</td>
<td>200 mg/m^2/course, 7 courses</td>
<td>1400 mg/m^2</td>
</tr>
<tr>
<td>7</td>
<td>200 mg/m^2/course, 6 courses, oral etoposide 50 mg/m^2/day x 21 days, 2 courses</td>
<td>3300 mg/m^2</td>
</tr>
<tr>
<td>8</td>
<td>200 mg/m^2/day x 3 days, 3 courses</td>
<td>1800 mg/m^2</td>
</tr>
</tbody>
</table>

Table 4.2c  Baseline assessment

<table>
<thead>
<tr>
<th>Patient</th>
<th>WCC</th>
<th>Hb</th>
<th>Plt</th>
<th>Na</th>
<th>K</th>
<th>Urea</th>
<th>Bili</th>
<th>Creat</th>
<th>Mg</th>
<th>EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.8</td>
<td>12</td>
<td>139</td>
<td>138</td>
<td>3.7</td>
<td>3.0</td>
<td>8</td>
<td>51</td>
<td>0.79</td>
<td>105</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>14.7</td>
<td>201</td>
<td>142</td>
<td>3.9</td>
<td>4.1</td>
<td>13</td>
<td>69</td>
<td>0.8</td>
<td>104</td>
</tr>
<tr>
<td>3</td>
<td>3.8</td>
<td>9</td>
<td>156</td>
<td>121</td>
<td>3.8</td>
<td>1.9</td>
<td>10</td>
<td>45</td>
<td>0.68</td>
<td>86</td>
</tr>
<tr>
<td>4</td>
<td>12.4</td>
<td>5.9</td>
<td>150</td>
<td>121</td>
<td>4.6</td>
<td>5.9</td>
<td>15</td>
<td>106</td>
<td>0.63</td>
<td>62</td>
</tr>
<tr>
<td>5</td>
<td>13.5</td>
<td>7.1</td>
<td>220</td>
<td>137</td>
<td>4.2</td>
<td>2.6</td>
<td>10</td>
<td>46</td>
<td>0.97</td>
<td>109</td>
</tr>
<tr>
<td>6</td>
<td>11.5</td>
<td>8.0</td>
<td>281</td>
<td>138</td>
<td>4.0</td>
<td>6.4</td>
<td>15</td>
<td>50</td>
<td>0.73</td>
<td>108</td>
</tr>
<tr>
<td>7</td>
<td>5.1</td>
<td>8.5</td>
<td>329</td>
<td>136</td>
<td>3.7</td>
<td>3.6</td>
<td>4</td>
<td>34</td>
<td>0.78</td>
<td>145</td>
</tr>
<tr>
<td>8</td>
<td>2.7</td>
<td>8.9</td>
<td>40</td>
<td>137</td>
<td>3.7</td>
<td>3.0</td>
<td>11</td>
<td>66</td>
<td>0.68</td>
<td>79</td>
</tr>
</tbody>
</table>

Bili  Bilirubin (μmol/l)  Creat  Creatinine (μmol/l)
Hb    Haemoglobin (g/dl)  K     Potassium (mmol/l)
Mg    Magnesium (mmol/l) Na     Sodium (mmol/l)
Plt   Platelets (x 10^9/l) Urea  Urea (mmol/l)
WCC   White blood cell count (x 10^9/l)
EDTA  Glomerular filtration rate assessed by EDTA clearance (mls/minute/1.73m^2)

All patients were assessed prior to entry to provide information on baseline haematological, biochemical, hepatic and renal function (table 4.2c). Patients were excluded from this study if they had evidence of liver dysfunction or hyperbilirubinaemia, or had a glomerular filtration rate (GFR) less than 60 mls/minute/corrected surface area.
Whilst this was primarily a phase I/II study, all patients had disease in accessible or monitorable sites, these are shown in table 4.2d together with the mode of assessment.

### Table 4.2d  Disease sites and assessment at entry to study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sites of disease</th>
<th>Mode of assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Soft tissue disease in arm</td>
<td>Clinical examination and MRI</td>
</tr>
<tr>
<td>2</td>
<td>Pulmonary metastases</td>
<td>CXR</td>
</tr>
<tr>
<td>3</td>
<td>Bone marrow and peripheral blood</td>
<td>Blood film</td>
</tr>
<tr>
<td>4</td>
<td>Pulmonary metastases</td>
<td>CXR</td>
</tr>
<tr>
<td>5</td>
<td>Nodal and extranodal disease in arm</td>
<td>Clinical examination and MRI</td>
</tr>
<tr>
<td>6</td>
<td>Bone and bone marrow, abdominal primary</td>
<td>Bone marrow aspirates and trephines, bone scan, mIBG scan, urinary catecholamines, abdominal USS</td>
</tr>
<tr>
<td>7</td>
<td>Supraclavicular mass and bone marrow</td>
<td>Clinical examination, mIBG scan, urinary catecholamines</td>
</tr>
<tr>
<td>8</td>
<td>Pelvic disease extending into thigh</td>
<td>Clinical examination and CT scan</td>
</tr>
</tbody>
</table>

MRI  Magnetic Resonance Imaging  
CXR   Chest X ray  
mIBG scan  Metaiodobenzyl Guanidine uptake scan  
USS   Ultrasound scan  
CT scan  Computerised axial tomography
4.2.2 **Treatment protocols**

During the period of study two different treatment schedules were evaluated, one using a continuous infusion of CSA preceded by a loading dose and the other employing daily three hour CSA infusions. For convenience these will be termed schedules A and B respectively.

All patients received one dose of etoposide alone (i.e. no CSA) in order to evaluate etoposide pharmacokinetics. Ideally this was given before the first combination treatment, however in 4/8 cases this single agent chemotherapy was delayed until the patient had received one or more courses of combination treatment. The dose of etoposide used was 150 mg/m\(^2\) in all cases except one. This patient (number 8) had a 50% dose reduction for the first combination treatment because of thrombocytopenia post autologous bone marrow transplantation.

In all cases (when given as either a single agent or in combination) etoposide was diluted in 2mls/mg Normal Saline and given as an intravenous infusion over one hour.
Schedule A

A CSA loading dose (5 mg/kg) was diluted in 100mls Normal Saline and given over two hours. This was immediately followed by a continuous infusion of CSA (0.0625 mg/kg/hr) for the next 60 hours, given in Normal Saline.

Etoposide (150 mg/m²/dose) daily for three days was given in all cases (except patient 8). The first dose started at the end of the CSA loading dose.

If a response or stable disease was documented after the first two courses of CSA with etoposide further cytotoxic agents were added.

Figure 4.2a outlines the treatment regimen in schedule A.
Schedule B

In this regimen a daily three hour CSA infusion (30 mg/kg/day) was given, each dose diluted in 250mls Normal Saline. Etoposide was given in the same manner as in schedule A, each dose starting one hour into the CSA infusion. No patient to date has received schedule B without first receiving (and tolerating) schedule A. Figure 4.2b outlines the treatment in schedule B.

**Figure 4.2b** Outline of chemotherapy administration in schedule B

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CSA infusion</strong></td>
<td>T0 - T3</td>
<td>T0 - T3</td>
<td>T0 - T3</td>
</tr>
<tr>
<td>(30 mg/kg/dose)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ETOPOSIDE</strong></td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>(150 mg/m²)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

T0 = Time zero hours, i.e. start of CSA infusion

T3 = Time plus 3 hours, i.e. end of CSA infusion
4.2.3 Monitoring and other considerations

All patients were closely observed during each course of treatment particularly at the
start of every CSA infusion. Experience showed that this was when anaphylactic or
allergic reactions were most likely to occur. Blood pressure and pulse were measured
four hourly, and each patient was weighed daily. Serum biochemistry (which included
urea, creatinine, bilirubin and magnesium) was measured daily.

Nausea and vomiting were treated with ondansetron. Pain or discomfort which occurred
in association with these schedules was treated as necessary, this included opiates in
some patients. Some patients required antihypertensive treatment, this was administered
if the diastolic blood pressure rose above the 90th centile for height. The agent of choice
in this institution (nifedipine) was not used for patients in this study as it has potential
modulating activity. In its place clonidine was used as suggested by Yahanda et al
4.2.4 CSA pharmacokinetics

Samples were taken from each patient to estimate the CSA concentrations achieved. Blood samples were taken from a double lumen central line by a single operator if possible. These samples were not taken from the same lumen as had been used to administer CSA. If a CSA infusion was in progress at the time of sampling, it was discontinued and 10mls Normal Saline flushed through the sampling lumen prior to withdrawing a specimen. Patients receiving schedule A had 2 or 3 CSA concentration measurements each taken 24 hours apart. This allowed an estimation of the steady state concentration achieved with a continuous infusion.

Patients receiving schedule B had blood sampling at several time points to determine the peak concentration achieved, the speed of elimination and completeness of CSA elimination by 24 hours (i.e. the time that the next dose was given). Insufficient time points were available for complete pharmacokinetic evaluation, but it was necessary to assess CSA concentrations achieved in order to allow comparison to be made with schedule A and other reported studies.

CSA was measured in whole blood using a ELISA type EMIT assay by a routine biochemistry laboratory. The assay used is specific for CSA (i.e. it does not measure metabolites of CSA) and reproducible across the expected therapeutic range. However concentrations in excess of 1500ng/ml are less accurately measured, in part this is due to the need to further dilute these samples with matched whole blood.
4.2.5 Etoposide pharmacokinetics

All patients had samples taken following etoposide administration which allowed an assessment of etoposide pharmacokinetics and variations in this when etoposide was given with CSA. Samples were taken by a single operator if possible, from the opposite lumen of a double lumen hickman line to that used for etoposide infusions. Intrainfusion or end of infusion samples were taken once the infusion had been stopped (for a minute) and a saline flush (10mls) given through the sampling lumen. At least seven time points were taken if possible. Samples of serum were stored at 20°C until the assay was performed. All etoposide assays were performed by The Cancer Research Unit, Newcastle using a standard HPLC method (Lowis et al 1993).
4.2.6 Toxicity assessment

In addition to those assessments previously mentioned (blood pressure, pulse, weight, biochemistry) the following were also monitored; full blood count, renal function (EDTA clearance), the development of mucositis, anaphylaxis or hypersensitivity, skin rash, pain, constipation and headache. Patients were not routinely seen between courses unless an adverse event was anticipated. It is likely that some asymptomatic effects of this treatment (for example hypomagnesemia) were not measured or recorded.

4.2.7 Disease assessment

The patients were seen and assessed clinically before each course, however formal disease status assessment was only undertaken after the second course. This was with at least one of the methods listed in table 4.2d. Full assessment of all disease sites was undertaken if required to facilitate decisions concerning alterations in the overall management plan.
4.3 RESULTS

4.3.1 Treatment given

All patients initially received full dose etoposide with a continuous infusion of CSA, except patient 8 who was electively given a 50% etoposide dose reduction for pre-existing thrombocytopenia. One patient only received one course of combination treatment as disease progression then became evident (patient 3). One patient (number 2) was given a total of four courses of etoposide and CSA as he failed to tolerate the addition of further cytotoxic agents. In all 17 courses of continuous infusion CSA with etoposide (and no other agents) were given.

4.3.2 Toxicity

Observed events in patients receiving schedule A and etoposide alone

Eight patients received a total of 17 courses of this regimen, adverse events are summarised in table 4.3a.

The first two patients received antiemetics once nausea became evident, both experienced WHO grade 3 nausea and emesis. Subsequent patients all received elective antiemetics (ondansetron alone), 4/15 experienced nausea despite this.

As previously mentioned pain and discomfort has been reported as a problem with this type of regimen, 7/17 patients receiving this treatment experienced discomfort which was severe enough to warrant opiate analgesia. This discomfort was always short lived and did not persist once the treatment course had finished. Known sites of disease were not apparently involved, and the discomfort experienced bore no relation to other toxicity or to any response seen.

Hypertension requiring antihypertensive management was seen in three courses (two patients), in both cases it was short lived and responded promptly to clonidine.
Hyperbilirubinaemia was minimal and WHO grade 3 following one course, grade 3 following two courses, grade 0 or 1 following fourteen courses.

Whilst hypomagnesaemia was only confirmed following 4/17 courses (lower than 0.5mmol/l, i.e. normal range for laboratory), oral magnesium supplementation was given following seven courses.

One patient developed mucositis (number 3) at a time when he was pancytopenic with circulating leukaemic blasts.

Myelosuppression was observed in three patients (patient 3 with relapsed leukaemia, and patients 2 and 8 who had both had an autologous bone marrow transplant). Thrombocytopenia followed 6/17 courses, neutropenia was also present in 4 of these courses (three patients required admission for fever).

Schedule A with etoposide alone did not result in any renal, gastrointestinal or skin toxicity.

Other events noticed include headache (one patient), facial flushing (two patients), fleeting rash (one patient) and puffiness in the face (one patient), these were all attributed to the CSA infusion. One patient developed chicken pox between courses, and another (patient 3) developed an anal fissure whilst neutropenic.
<table>
<thead>
<tr>
<th>Adverse event</th>
<th>Schedule A</th>
<th>Schedule B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nausea or vomiting (despite antiemetics)</td>
<td>4/15 courses</td>
<td>0/3 courses</td>
</tr>
<tr>
<td>Pain requiring opiates</td>
<td>7/17</td>
<td>0/3</td>
</tr>
<tr>
<td>Hypertension requiring treatment</td>
<td>3/17</td>
<td>0/3</td>
</tr>
<tr>
<td>Hyperbilirubinaemia (WHO grade 2 / 3)</td>
<td>3/17</td>
<td>0/3</td>
</tr>
<tr>
<td>Hypomagnesaemia</td>
<td>4/17</td>
<td>0/3</td>
</tr>
<tr>
<td>Magnesium supplements used</td>
<td>7/17</td>
<td>0/3</td>
</tr>
<tr>
<td>Mucositis</td>
<td>1/17</td>
<td>0/3</td>
</tr>
<tr>
<td>Ileus</td>
<td>0/17</td>
<td>0/3</td>
</tr>
<tr>
<td>Constipation</td>
<td>0/17</td>
<td>0/3</td>
</tr>
<tr>
<td>Renal impairment</td>
<td>0/17</td>
<td>1/3</td>
</tr>
<tr>
<td>Skin reaction</td>
<td>0/17</td>
<td>0/3</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>4/17</td>
<td>3/3</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>6/17</td>
<td>3/3</td>
</tr>
<tr>
<td>Febrile neutropenia</td>
<td>3/17</td>
<td>3/3</td>
</tr>
<tr>
<td>Decline in EDTA clearance</td>
<td>0/17</td>
<td>1/3</td>
</tr>
</tbody>
</table>
Observed adverse events seen with schedule B

Two patients have so far received a total of three courses of schedule B, one patient received one course with etoposide and vincristine followed by etoposide, vincristine and actinomycin D. The other patient (number 6) received etoposide, vincristine and actinomycin D having tolerated this with schedule A.

The toxicity experienced is included in table 4.3a.

Providing additional dexamethasone is given with full dose ondansetron there was minimal nausea and vomiting. No hypertension, pain or discomfort, hyperbilirubinaemia, hypomagnesaemia or skin problems were encountered. However myelosuppression (neutropaenia and thrombocytopenia) were evident after all three courses with admission for parenteral antibiotics in each case for fever. One patient developed chicken pox during a neutropenic period.

One patient (number 7) who had developed a chronic watery diarrhoea during relapse of neuroblastoma required frequent admissions for fluid and electrolyte support between courses. Reversible renal impairment was observed following a period of dehydration at home (EDTA 9mls/min/1.73m², potassium 1.4mmol/l, urea 23mmol/l, creatinine 198µmol/l). His diarrhoea was attributed to a grossly elevated VIP (vasoactive intestinal peptide) with a moderately elevated somatostatin, both presumably secreted from sites of neuroblastoma.

One of the patients experienced a characteristic fleeting rash with perioral dysasthesia with the start of each CSA infusion.
4.3.3 Cyclosporin pharmacokinetics

Cyclosporin A concentration was measured in every course of treatment given.

In those courses using a continuous infusion of CSA (schedule A), two or three CSA concentration values are available for each course. At least one of these (usually two) is taken at a time in the infusion when the patient was assumed to be in a steady state, (i.e. day 2 or 3). Table 4.3b shows the steady state serum CSA concentrations achieved in these patients. It is of note that the lowest CSA concentrations occurred in the youngest patients (not shown), and that CSA concentration did not appear to correlate with toxicity (although this effect may have been masked by the more significant effect on toxicity of additional cytotoxic agents).

Table 4.3b Maximum CSA concentration measured

<table>
<thead>
<tr>
<th>CSA concentration</th>
<th>Number of courses</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 1000ng/ml</td>
<td>0</td>
</tr>
<tr>
<td>1000 - 1500ng/ml</td>
<td>12</td>
</tr>
<tr>
<td>&gt; 1500ng/ml</td>
<td>5</td>
</tr>
</tbody>
</table>

In 10 courses the CSA concentration was measured 4 - 7 hours after the end of the infusion, levels between 198 - 732ng/ml were present at this time. Most of these (8) were taken at 6 hours, of those taken earlier, the concentration at 4 hours was 732 and 378ng/ml. As might be expected the lower CSA concentrations occurred in those children who had a lower steady state CSA level (table 4.3c). The highest CSA concentration after the end of the continuous infusion occurred in a patient (number 4) who had a steady state CSA concentration of greater than 1500ng/ml and who experienced severe toxicity following that course (he also received vincristine and actinomycin D) with a transiently raised urea and creatinine.
Table 4.3c  CSA concentrations after the end of the infusion

<table>
<thead>
<tr>
<th>Maximum CSA concentration recorded</th>
<th>Time after end of infusion (hours)</th>
<th>CSA concentration at this time</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;1500ng/ml</td>
<td>6</td>
<td>198-642ng/ml (3 courses)</td>
</tr>
<tr>
<td>&gt;1500ng/ml</td>
<td>4</td>
<td>732ng/ml (1 course)</td>
</tr>
<tr>
<td>1000-1500ng/ml</td>
<td>6</td>
<td>254-438ng/ml (5 courses)</td>
</tr>
<tr>
<td>1000-1500ng/ml</td>
<td>4</td>
<td>378ng/ml (1 course)</td>
</tr>
</tbody>
</table>

Three courses of schedule B were monitored with a CSA concentration profile following one or two infusions. The peak CSA concentration during the infusion was measured in excess of 13 000ng/ml (patient 6, taken at the end of infusion) in one course and at least 10 000ng/ml in the other two. Figure 4.3a shows the CSA profiles following schedule B in two patients. It is clear that whilst this schedule achieves very high peak CSA levels they fall fairly promptly after each infusion. However, even 24 hours after each CSA infusion there is still appreciable circulating CSA (more than 200ng/ml). Despite the presence of CSA at the time of the second (or third) infusion, the peak concentration and apparent rate of clearance are similar between day one and three. As previously stated this schedule was well tolerated, although it produced more profound myelosuppression and nausea than expected. The myelosuppression was attributed to an increase in cytotoxic toxicity and etoposide pharmacokinetics were evaluated (see 4.3.4).

The overall exposure to CSA may in fact be very similar in schedule A and B, though the profile is clearly different.
Figure 4.3a  CSA profile
4.3.4 **Etoposide pharmacokinetics**

Etoposide concentrations were measured in a standardised way with reproducible results. Concentrations for each time point were analysed using a computer program (ADEPT II) which enables pharmacokinetic evaluation and analysis of this data. From the blood samples taken the α (distribution) and β (elimination) half lives (T½ α and T½ β respectively), clearance, apparent volume of distribution (Vdss) and area under the concentration curve (AUC) were obtained. These analyses were performed at The Cancer Research Unit in Newcastle upon Tyne.

Table 4.3d gives the summary of these results for each patient (if possible at least two etoposide profiles were performed on each patient, one with and one without CSA).

<table>
<thead>
<tr>
<th>Patient</th>
<th>VP 16 and CSA (schedule)</th>
<th>VP 16 dose (mg/m²)</th>
<th>T½ α (mins)</th>
<th>T½ β (mins)</th>
<th>Clearance (l/min/m²)</th>
<th>Vdss (l/m³)</th>
<th>AUC (ng/ml)</th>
<th>Max CSA (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Schedule A</td>
<td>164</td>
<td>5.9</td>
<td>398</td>
<td>12.6</td>
<td>6.98</td>
<td>13</td>
<td>1035</td>
</tr>
<tr>
<td>2</td>
<td>Alone</td>
<td>150</td>
<td>1.04</td>
<td>211</td>
<td>15.6</td>
<td>4.6</td>
<td>9.6</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Schedule A</td>
<td>150</td>
<td>74</td>
<td>619</td>
<td>8.1</td>
<td>6.7</td>
<td>18</td>
<td>&gt;1500</td>
</tr>
<tr>
<td>3</td>
<td>Alone</td>
<td>150</td>
<td>38</td>
<td>375</td>
<td>14</td>
<td>5.7</td>
<td>10</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Schedule A</td>
<td>142</td>
<td>9.2</td>
<td>236</td>
<td>15</td>
<td>4.8</td>
<td>9.4</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>142</td>
<td>118</td>
<td>1489</td>
<td>5.8</td>
<td>11</td>
<td>24.5</td>
<td>&gt;1500</td>
</tr>
<tr>
<td>4</td>
<td>Alone</td>
<td>149</td>
<td>17</td>
<td>135</td>
<td>35</td>
<td>5.6</td>
<td>4.2</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Schedule A</td>
<td>146</td>
<td>25</td>
<td>210</td>
<td>18</td>
<td>5.1</td>
<td>8</td>
<td>1041</td>
</tr>
<tr>
<td>5</td>
<td>Alone</td>
<td>146</td>
<td>22</td>
<td>174</td>
<td>22</td>
<td>5</td>
<td>6.8</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Schedule A</td>
<td>146</td>
<td>137</td>
<td>914</td>
<td>12</td>
<td>10.7</td>
<td>12.5</td>
<td>1455</td>
</tr>
<tr>
<td>6</td>
<td>Alone</td>
<td>150</td>
<td>2.7</td>
<td>111</td>
<td>27</td>
<td>4.1</td>
<td>5.5</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Schedule A</td>
<td>150</td>
<td>27</td>
<td>234</td>
<td>18</td>
<td>ND</td>
<td>8.3</td>
<td>1320</td>
</tr>
<tr>
<td></td>
<td>Schedule B</td>
<td>150</td>
<td>1.5</td>
<td>279</td>
<td>12</td>
<td>4.5</td>
<td>12.5</td>
<td>&gt;7500</td>
</tr>
<tr>
<td>7</td>
<td>Schedule A</td>
<td>75</td>
<td>41</td>
<td>400</td>
<td>10.2</td>
<td>5</td>
<td>7.3</td>
<td>1500</td>
</tr>
</tbody>
</table>

NA  Not applicable
ND  Not done
As can be seen from the above table the results obtained are incomplete. This is in part due to sampling difficulties (patients 1 and 2) yielding insufficient time points to allow interpretation. Patient 8 had not received the etoposide alone course at the time of most recent etoposide analysis (nor had patient 6 with schedule B).

There are therefore four paired (etoposide with and without CSA) sets of results for schedule A and one for schedule B.

Of the patients with paired sets of results, patients 2, 5 and 6 had etoposide alone before (ever) receiving either schedule A or B. Patients 4 and 7 had their first course of schedule A (i.e. CSA by continuous infusion) 21 days before etoposide alone, this may have influenced etoposide pharmacokinetics.

Results which were obtained from questionable sample series and the results from patient 8 were excluded, the remaining pharmacokinetic data are displayed in table 4.3e.

<table>
<thead>
<tr>
<th>Table 4.3e</th>
<th>Ranges obtained from reliable data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of courses</td>
</tr>
<tr>
<td>Etoposide alone</td>
<td>3</td>
</tr>
<tr>
<td>Schedule A</td>
<td>5</td>
</tr>
<tr>
<td>Schedule B</td>
<td>1</td>
</tr>
</tbody>
</table>

From the data presented in table 4.3e it is clear that etoposide pharmacokinetics when it is given alone differ from when it is given with CSA. The latter results in an increase in both the half life (α and β) and AUC, with a decrease in clearance.

Only one set of results are available for schedule B, however, whilst the α half life is much shorter (than for etoposide alone), the β half life and AUC are increased and clearance is decreased when compared with the etoposide alone course.
If the trend in individual patients is considered, similar findings are observed with an approximate halving of clearance and doubling of the AUC. This is seen in figures 4.3b-d which show the etoposide concentration/time curves with and without CSA. Figure 4.3d shows the effect of CSA given as schedule B with etoposide and with etoposide and vincristine together, there is no additional effect upon the concentration/time curve from vincristine. Whilst the exposure to etoposide is similar in this patient (number 7) when CSA in either schedule A or B is used, there is the suggestion from figure 4.3d that schedule B results in a higher etoposide AUC (supported by the calculated AUC in table 4.3d).
Figure 4.3b-d Etoposide profiles

Figure 4.3b
Patient 4, CSA given as schedule A

Figure 4.3c
Patient 6, CSA as schedule A

Figure 4.3d
Patient 7, all schedules
As the number of courses examined is so small it is not possible to comment on the effect of receiving CSA as part of schedule A before the pharmacokinetics of etoposide alone are assessed. Nor can any interpretation of the effect of CSA concentration achieved (during the CSA continuous infusion) on etoposide pharmacokinetics be made. Though if data from patients 2, 4, 6 and 7 are examined the clearance only decreases by more than 50% in patient 4, and the AUC more than doubles only in patients 4 and 6. Patients 2 and 4 both had serum CSA concentrations greater than 1500ng/ml, whilst patients 6 and 7 had CSA concentrations less than 1200ng/ml.
4.3.5 Responses

Of the eight patients given this treatment three, (patients 1, 3 and 8) failed to respond to the first two courses.

Patient 2 (Ewings' sarcoma) had a partial response (more than 50% reduction in lung metastasis on CXR) after two courses and went on to receive a further four courses of CSA with etoposide. One of these also contained vincristine and actinomycin D, and the other vincristine (with etoposide and CSA). However as the toxicity he experienced from these courses was severe his last two courses were with CSA and etoposide alone. After he had received a total of six courses (with minimal disease visible on CXR) he went on to receive radiotherapy to his lungs and remains well twelve months later.

Patient 4 (osteosarcoma) had stable disease after two courses. The third course of treatment contained additional vincristine and actinomycin D, however he experienced severe toxicity from this and further treatment was not given due to disease progression.

Patient 5 responded to the first two courses (partial response in nodal and extranodal rhabdomyosarcoma), further courses contained additional vincristine which was well tolerated (reduced vincristine doses - 30% of desired). The principal toxicity of additional vincristine was constipation. The remission obtained (more than 50% reduction in measurable disease) was consolidated with radiotherapy, and he remains well six months later.

Patient 6 (neuroblastoma) had additional drugs (both vincristine and actinomycin D) in reduced doses from the third course. These were well tolerated, as was a switch from CSA delivery via schedule A to schedule B. The principal toxicity experienced was myelosuppression which followed all courses with additional cytotoxic agents. The response in metastatic disease sites after seven courses (on mIBG scan) has allowed consolidation with mIBG therapy.
Patient 7 (neuroblastoma) had stable disease after the first two courses, so he went on to receive a further two courses with additional (dose reduced) vincristine and actinomycin D in conjunction with CSA in schedule B. However disease progression became evident after the fourth course and further treatment was discontinued.
4.4 **DISCUSSION**

Providing suitable patients are selected for this treatment (no renal or hepatic dysfunction) the toxicity is manageable although in some cases more severe than expected. The degree of acceptable toxicity in this group of patients needs to be considered and adjustments made as necessary. Whilst etoposide and schedule A cyclosporin produced minimal toxicity the effect of etoposide alone with CSA in schedule B has not yet been evaluated and may also be minimal.

The CSA schedules (A and B) were both well tolerated providing adequate antiemetics were given and the patients monitored during the infusion (in case of hypersensitivity or hypertension) with magnesium supplements provided as necessary.

Schedule A generally provided CSA concentrations of greater than 1000ng/ml, whilst it is likely that this is sufficient to modulate P glycoprotein, some *in vitro* data suggests that at least 3000ng/ml is necessary (Slater et al 1986). If this is the case then schedule B provides a more effective way of producing this serum level of CSA, with a concentration greater than 3000ng/ml for 8-10 hours after the start of each three hour infusion. When etoposide is given alone elimination is nearly complete in this time, when given with CSA an etoposide concentration of 5-10μg/ml is present ten hours after the start of the etoposide infusion. It appears that using CSA in this schedule provides a higher CSA concentration for the duration over which it is required.

There are no known surrogate markers for efficacy (of modulating MDR) when CSA is used as part of combination therapy. Hyperbilirubinaemia has been suggested (Lum et al 1992) but it is now thought that this is mediated via the product of the MDR 3 gene. It is of note that one of the reported patients who received an overdose of CSA (30mg/kg intravenously) developed transient hyperbilirubinaemia (Arellano et al 1991).
The effect of CSA on etoposide pharmacokinetics is similar to published studies with an increase in the AUC and a decrease in clearance (Lum et al 1992). The effect of using CSA as a modulating agent may therefore be obscured by the dose escalation of etoposide which occurs when CSA is present. In order to clarify this it is necessary to modify the etoposide dose (probably on an individual basis) to achieve the same AUC in each patient with and without CSA. This type of modification is being made in some ongoing adult trials.

If possible single agent etoposide was given before the patient had combination therapy with CSA. In those cases where this was not possible (i.e. CSA and etoposide were given 21 days before etoposide alone) it is unlikely that CSA had any appreciable effect on subsequent etoposide pharmacokinetics (21 days later) as even in a patient receiving an overdose (150mg/kg orally - Kruger et al 1988) the CSA level had fallen to 100ng/ml five days later. As it appears a higher concentration (of CSA) is required it is unlikely that sufficient CSA will be present in serum or tissues by 21 days to modulate etoposide pharmacokinetics or cytotoxicity.
CONCLUSIONS

This type of regimen is feasible with acceptable toxicity providing adequate precautions are taken. If schedule B is used the treatment courses can be given as an outpatient, however monitoring between courses is required when additional cytotoxic agents are given in case of neutropenia. If this type of study on modulation of resistance is to be taken further with assessment of responses it will be important to compare the response to etoposide alone with etoposide (given to the same AUC) and CSA. This is an acceptable approach in some adult practices, but is more difficult to perform in a paediatric group where it may be difficult to justify repeating the same dose of a drug which is known to have failed previously. Failure to respond to previous courses containing etoposide may have to be used as evidence of etoposide resistance.
CHAPTER 5.

CONCLUSIONS
MDR expression has been demonstrated in paediatric tumour cell lines (Neuroblastoma and Rhabdomyosarcoma). The need to use more than one technique is emphasized, as reliance on a single monoclonal antibody or method will lead to false conclusions.

Correlation between expression, resistance *in vitro*, resistance *in vivo* and reversibility either *in vitro* or *in vivo* remain unclear.

Although Rhabdomyosarcoma cell lines overexpress MDR 1, they did not show reversal of resistance *in vitro*, the fact that they had come from patients with relapsed disease indicates *in vivo* resistance.

Furthermore the variable nature of reversibility with different MDR associated drugs was clear. Vincristine was most consistently modulated in all reversible cell lines. Perhaps MDR is the main single resistance mechanism for this agent, unlike those mechanisms implicated with etoposide or doxorubicin.

Contrary to assumptions made from the clinical use of verapamil, prolonged pretreatment with CSA does not seem to be necessary. *In vitro* the optimal schedule involved CSA administration at about the same time as the cytotoxic agent (within one hour).

Reversal of resistance *in vivo* was demonstrated.

The effect of CSA on the pharmacokinetics of etoposide was documented, and the feasibility of a short high dose infusion of CSA demonstrated.
There are several questions which remain unanswered about the multidrug resistant phenotype. Further investigation should provide the answers to these and with improved understanding of the mechanisms of action, offer new possibilities in the management of malignant disease.

The ideal approach to clinical studies on MDR reversal would be to retreat relapsed patients with exactly the same regimen as they responded to initially, except for the addition of a modulator. All aspects of the regimen should be identical in the courses given with and without modulator.

Assessment of drug pharmacokinetics would enable conclusions to be made on whether the effect seen was due to modulation or dose escalation. It is likely that for many drugs it is appropriate to reduce the dose of cytotoxic when given with a modulator in order to standardize the drug AUC in treatments with and without modulator.

Ultimately it is necessary to conduct large preferably randomised trials on de novo patients with tumours known to be intrinsically resistant to chemotherapy (for example, colon and kidney cancer).

In such studies there is a need to document Pgp or MDR 1 mRNA expression in the tumours prior to treatment in order to clarify the role of MDR in these patients.

The ideal modulating agent has yet to be found, it would need to be specific for Pgp, with a low degree of systemic toxicity, little (or a predictable) effect on cytotoxic pharmacokinetics and be easy to administer (perhaps oral). A modulator capable of selectively affecting tumour and not normal Pgp would be of great interest, as would a reliable marker for efficiency of Pgp modulation. Various surrogate markers have been
suggested including hyperbilirubinaemia, though none are sufficiently specific or reliable to be useful.

Future agents which may prove to be useful and to have less toxicity include non immunosuppressive analogues of cyclosporin, monoclonal antibodies (directed against P gp) and MDR 1 antisense therapy. Structurally altered analogues of cytotoxics or cytotoxics loaded into nanospheres or liposomes may provide an additional route for the circumvention of MDR.

Many changes have been demonstrated in cells developed in vitro which express the MDR 1 phenotype. These include altered expression of other membrane and cytosolic proteins, alterations in the physical state of the plasma membrane and changes in intracellular drug binding and transport. The only feature which links these alterations is that P gp is expressed in all these cells. The fact that these cells also express other proteins may be associated with the MDR 1 phenotype, or be related to other factors associated with malignant change. If there is an association between expression of MDR 1 and the other alterations in cell phenotype observed in vitro, this remains to be demonstrated in tumour samples.

Why P gp should be able to bind to and efflux such a wide variety of apparently unrelated compounds is perhaps the most puzzling question about P gp. The stereochemical interactions between P gp and drug may be less important than the intracellular localisation of either P gp or cytotoxic. If P gp is able to remove compounds directly from the cytoplasmic membrane, then those drugs which enter cells by passive diffusion will be preferentially expelled. However, those which enter the cell via a specific transport protein or carrier will not be removed by P gp. For example,
MDR cells are sensitive to methotrexate (implying that methotrexate is not transported by P gp), which enters the cell through the normal folate carrier. However trimetrexate, a folate analogue, enters the cell by passive diffusion and is apparently effluxed by P gp (Klohs et al 1986).

The normal role of the MDR 1 gene product remains unproven, but plant and chemically derived compounds which are transported by P gp are unlikely to be natural targets. Progesterone is the only compound transported by P gp in humans which is known to have a physiological role. The function of the MDR 2 gene product is even more unclear, whilst it does appear to code for a transmembrane protein this does not transport lipophilic cytotoxic drugs.

**Future Clinical Strategies**

Whilst it is generally assumed that expression of P gp by a tumour cell will confer cytotoxic resistance, this may not be the case. Assessment of 'functional P gp' would clarify this issue, and in vitro demonstration of modulation (e.g. with CSA or verapamil) might be a more helpful indicator in planning treatment. The fluorescent dye rhodamine 123 (R-123) has become a useful tool to assess functional P gp in vitro as this dye is effluxed from cells via P gp (Ludescher et al 1991).

With improved reliability in the assessment of MDR status in tumour from biopsy specimens it may be possible to recognise tumours which are MDR negative at diagnosis. It is not possible to assume that MDR negative tumours will be sensitive to agents transported by P gp, as they may exhibit other resistance mechanisms which also
involve these drugs. On the other hand a tumour expressing MDR 1 may indicate the need to have treatment which either included drugs not transported by P gp, or included effective modulation of P gp mediated efflux.

The development of transgenic mice which express MDR 1 in bone marrow (Mickisch et al 1991, 1992) has led to interest in using retroviral methods to introduce the MDR gene into the bone marrow of patients. A study is underway (Andreeff et al 1993, Diesseroth et al 1993) in which women with advanced ovarian cancer receive an autograft with bone marrow manipulated to express the MDR 1 phenotype. They are then treated in a dose escalating fashion with taxol, whose commonest dose limiting factor is usually myelosuppression.

Many aspects of the phenomenon of multidrug resistance are now understood, and it is recognised that it may play an important role in clinical resistance to treatment. Exciting ways in which this type of drug resistance may be overcome are being developed and are progressing into clinical practice. If they are to prove useful in the long term, we need to continue improving our understanding of this phenotype, its role in normal and malignant tissues and how to maximise the benefit from therapeutic options available.
REFERENCE LIST


Chambers, T., Chalikonda, I., and Eilon, G. Correlation of protein kinase C translocation, P glycoprotein phosphorylation and reduced drug accumulation in


CORRIGENDA

Page 31
Title should read:
Figure 1.4d 'Vacuum cleaner' model of P glycoprotein

Page 33
Title should read:
Figure 1.4c 'Pump' model of P glycoprotein action

Page 63
Para 3, line 2:
'2800 ng/ml' not '1800 ng/ml'

Page 163
Table 2.12a, IC 50 (ng/ml) results should read:
'40 / 40 / 600 / 700 / 100 / 100 / 3000 / 2000 / 5000 / 4000'
not
'40 / 40 / 3000 / 2000 / 100 / 100 / 600 / 700 / 5000 / 4000'

Page 174
Para 1, line 1:
'Cells were cytospun as described on page 188', not 'as described in the previous section'.

Para 1, line 3:
'described on page 188', not 'as described earlier'.

Page 227
Figure 4.2b:
Arrows to indicate time of etoposide administration should fall within the time period of CSA administration.