DAUNORUBICIN KINETICS AND DRUG RESISTANCE IN LEUKAEMIA

By

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B. Sc. (Hons)

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Abstract

The aims of this thesis were to examine: (1) plasma and cellular pharmacokinetics of daunorubicin and its major metabolite daunorubicinol in patients with acute leukaemia, and the relationships between pharmacokinetics, patient response and the presence of P glycoprotein; (2) actions of the multidrug resistance reversing agents cyclosporin A and trifluoperazine, at clinically achievable concentrations, on daunorubicin accumulation and retention in human leukaemia cell lines and patients with acute leukaemia; and (3) effect of daunorubicin on the cell membrane of both sensitive and resistant cell lines, with and without the multidrug resistance reversing agents.

Twenty-seven patients with acute leukaemia received daunorubicin as part of induction therapy. The plasma and cellular levels of daunorubicin and its metabolite daunorubicinol were determined using HPLC. There were no significant differences between patients who went into complete remission (12/23) compared to those who did not respond for any of the plasma pharmacokinetic parameters. There was a significant difference in the cellular daunorubicin and daunorubicinol area under the concentration-time curve between responders and non responders ($p < 0.02$), as well as in cellular Cmax, cellular clearance and cellular volume of distribution. Eleven patients were P glycoprotein positive and 10 P glycoprotein negative (no sample available for 2 patients). There was no correlation between patient response and the presence of P glycoprotein; nor a correlation between the cellular concentration of daunorubicin or daunorubicinol and P glycoprotein. Patients responding to chemotherapy had higher cellular daunorubicin and daunorubicinol compared to non responders. In contrast to *in vitro* studies, overexpression of P glycoprotein was not the reason for the lower cellular daunorubicin levels.

Cyclosporin A was capable of increasing both cellular accumulation and retention in the drug resistant CEM/VLB and HL 60/ADR cell lines, but not in the drug sensitive CEM and HL 60 cell lines. Trifluoperazine had no effect in any of the four cell lines. In contrast to the cell line findings, only the combination of cyclosporin A and trifluoperazine were able to increase both accumulation and retention in the blast cells of patients at initial presentation. The multidrug resistant reversing agents alone had no effect in increasing accumulation or retention in the blast cells of P glycoprotein positive patients, nor patients in relapse. The cell line studies show that at clinically relevant concentrations only cyclosporin A is capable of increasing daunorubicin accumulation in both the drug resistant P glycoprotein positive (VLB) and P glycoprotein negative (ADR) cell lines. Thus, cyclosporin A does not work only by inhibiting the actions of P glycoprotein. Trifluoperazine

was unable to reverse drug resistance at clinically relevant concentrations in either cell lines or patient blast cells. However, the combination of cyclosporin A and trifluoperazine increased accumulation in patient blast cells at initial presentation, suggesting that these agents may be more useful in patients at initial presentation than relapse.

Daunorubicin was immobilised by linking it to poly vinyl alcohol and the effect of immobilised-daunorubicin was studied on the four cell lines above. The immobilised-daunorubicin was able to decrease cell growth in the drug sensitive HL 60 cell line but not in the drug resistant VLB or ADR cell lines. Poly vinyl alcohol itself was cytotoxic to the CEM cell line. The multidrug resistance reversing agents cyclosporin A and trifluoperazine were only capable of increasing cytotoxicity in the HL 60 cell line, with no effect in the drug resistant VLB or ADR cell lines.

Publications supporting this thesis

1. P. Galettis, J. Boutagy and D.D.F. Ma. (1994) Daunorubicin pharmacokinetics and the correlation with p-glycoprotein and treatment response in patients with acute leukaemia. *Br. J. Cancer* **70**, 324 - 329

Publications in preparation

1. P. Galettis, J. Boutagy and D.D.F. Ma. (1996) Effects of the MDR reversing agents, cyclosporin A and trifluoperazine, on daunorubicin accumulation and retention in patient leukaemic cells.

In addition, some of the work contained in this thesis has been presented at Scientific Meetings as follows:

1. P. Galettis, D.D.F. Ma and J. Boutagy. (1990) Pharmacokinetics of daunorubicin and correlation with treatment outcome in acute leukaemia. Annual Scientific Meeting of the Haematology Society of Australia, Christchurch.

2. P. Galettis, D.D.F. Ma and J. Boutagy. (1990) Accumulation and retention of daunorubicin in the blasts from patients with leukaemia. Annual Scientific Meeting of the Haematology Society of Australia, Christchurch.

3. P. Galettis, D.D.F. Ma and J. Boutagy. (1991) Pharmacokinetics of daunorubicin and correlation with treatment outcome in acute leukaemia. AACR Special Conference in Cancer Research. Membrane Transport in Multidrug Resistance, Development and Disease, Banff.

4. D.D.F. Ma, P. Galettis, J. McLachlan and J. Boutagy. (1991) Clinical significance of in-vitro daunorubicin accumulation and retention in human leukaemias. VII Congress, Asian-Pacific Division, International Society of Haematology.

5. P. Galettis, J. Boutagy and D.D.F. Ma. (1992) Effects of MDR reversing agents

on cell lines and patient leukaemic cells. Vth World Conference on Clinical Pharmacology and Therapeutics, Yokohama.

6. J. Boutagy, P. Galettis and D.D.F. Ma. (1992) Relationship between treatment outcome, p-glycoprotein and daunorubicin pharmacokinetics. Vth World Conference on Clinical Pharmacology and Therapeutics, Yokohama.

7. P. Galettis, J. Boutagy and D.D.F. Ma. (1992) Effects of MDR reversing agents on cell lines and patient leukaemic cells. Annual Scientific Meeting of the Australasian Society of Clinical and Experimental Pharmacology and Toxicology, Sydney.

8. P. Galettis, J. Boutagy and D.D.F. Ma. (1993) Reversal of drug resistance by cyclosporin A via a non p-glycoprotein mechanism. Annual Scientific Meeting of the Australasian Society of Clinical and Experimental Pharmacology and Toxicology, Brisbane.

Preface

The work described in this thesis was carried out in the Departments of Haematology and Clinical Pharmacology, Royal North Shore Hospital, under the supervision of Dr David Ma, Dr John Boutagy and Dr Anita Piper. This thesis has not been submitted for a degree at any other university. Full acknowledgement has been made where the work of others has been cited and used. A list of publications in support of this thesis is included.

Peter Galettis

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Glossary of Abbreviations

ACDA	acid citrate dextrose A
ADR	HL 60/ADR, doxorubicin resistant HL 60 subline
ALL	acute lymphocytic leukaemia
AML	acute myeloid leukaemia
ANLL	acute nonlymphocytic leukaemia
Ara C	cytosine arabinoside
at-MDR	atypical multidrug resistance
AUC	area under the curve
AUMC	area under the first moment curve
CEM	T cell lymphoblastic leukaemia cell line
CL	clearance
CL-PVA	cross linked polyvinyl alcohol
Cmax	maximum drug concentration
CR	complete remission
Cy A	cyclosporin A
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNR	daunorubicin
DOL	daunorubicinol
DOX	doxorubicin
DSIM	double strength iscoves medium
EPI	epirubicin

FCS	foetal calf serum
FE	Fisher exact test
Fr	Friedman two-way analysis of variance
GSH	glutathione
HL 60	acute myeloid leukaemia cell line
HPLC	high performance liquid chromatography
IC 50	inhibitory dose at 50% cell death
IDA	idarubicin
Imm-DNR	immobilized-daunorubicin
KW	Kruskal-Wallis one way analysis of variance
MDR	multidrug resistance
mdr1	multidrug resistance gene
mRNA	messenger ribonucleic acid
MRP	multidrug resistance-associated protein
MRT	mean residence time
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MW	Mann-Whitney U test
n	number
NR	non responders
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pgp	P-glycoprotein
PKC	protein kinase C
PR	partial remission

PVA	polyvinyl alcohol
RNA	ribonucleic acid
SD	standard deviation
SOD	superoxide dismutase
Tm	transition temperature
Tmax	time at maximum drug concentration
topo II	topoisomerase II
Tri	trifluoperazine
Vd	volume of distribution
VLB	VLB 100, drug resistant CEM subline
W	Wilcoxon signed rank test

CHAPTER 1

INTRODUCTION

This thesis involves the examination of the chemotherapeutic agent daunorubicin in the treatment of acute leukaemia, and the relationships between daunorubicin, acute leukaemia and drug resistance.

In 1961 an Italian group isolated from cultures of *Streptomyces sp.* a preparation with antitumour activity that was active against Ehrlich carcinoma and sarcoma 180 (Arcamone *et al.*, 1961). The active compound in a preparation of *Streptomyces peucetius* was isolated and characterized in 1963 and given the name daunomycin (Cassinelli & Orezzi, 1963). At a similar stage both a French and a Russian group isolated the same substance and gave it different names, rubidomycin (Dubost *et al.*, 1964) and rubomycin (Gauze, 1964). This compound is now known universally as daunorubicin (in the remainder of this thesis it will be abbreviated to DNR). A closely related active compound, doxorubicin (abbreviated as DOX, also commonly called adriamycin), was later isolated from a mutant of *Streptomyces peucetius* (Arcamone *et al.*, 1969).

Clinically, DNR was shown to be very effective for leukaemia while DOX showed a wider spectrum of antitumour activity, having applications in the treatment of several solid tumours as well as leukaemias (Young *et al.*, 1981). Both DNR and DOX lead to dose dependent cardiotoxicity and the search for new analogues with fewer side effects has been extensive (Brown, 1983; Arcamone, 1981). Recently two semi-synthetic analogs

of DNR have been approved for use in patients: epirubicin (EPI) and idarubicin (IDA). DNR, DOX, EPI and IDA all belong to a class of compounds known as the anthracyclines. The structures of these four anthracyclines, shown in Figure 1.1, are all very similar with only minor differences between each of them and the parent compound DNR. They are all red lyophilized powders composed of two parts, an amino sugar and an aglycone. The amino sugar of DNR is termed daunosamine while the aglycone is termed daunomycinone. The same amino sugar is also attached to the aglycone of DOX and IDA. The numbering of the carbon atoms of DNR is indicated in Figure 1.1.

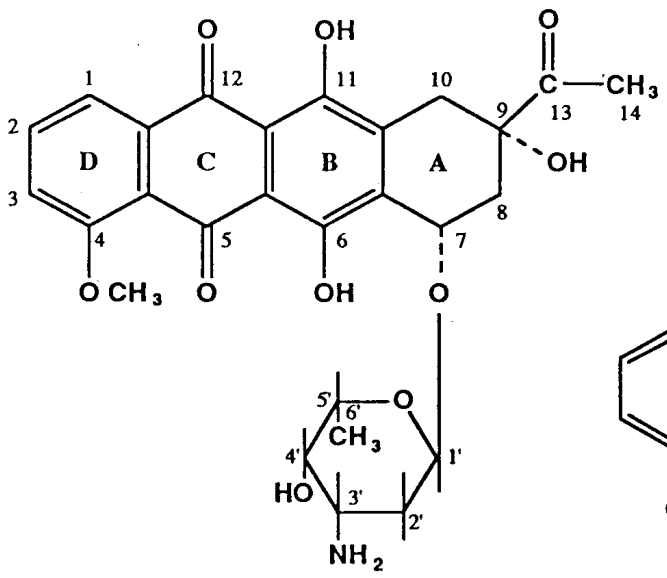
1.1. Mechanisms of Action of Anthracyclines

The anthracyclines have a number of actions that include intercalation with deoxyribonucleic acid (DNA), the formation of free radicals as well as actions on the cell membrane. These actions, with reference to the two major anthracyclines DNR and DOX, are discussed in the sections below.

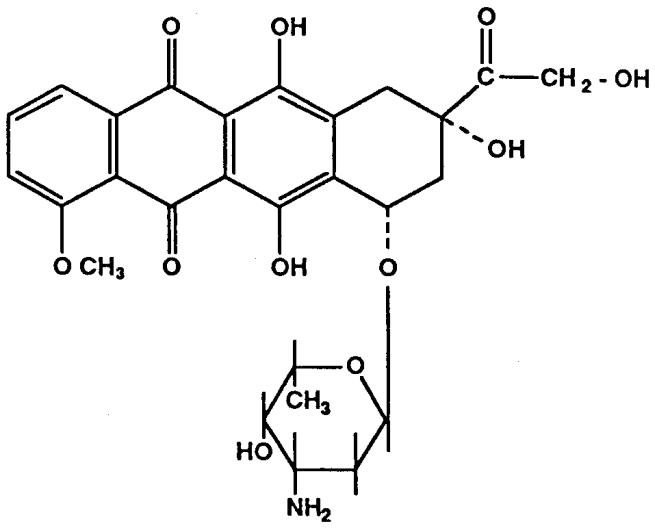
1.1.1. DNA Intercalation

A major action of both DOX and DNR is their ability to intercalate with DNA and this is believed to be the major cause of their cytotoxicity. Calendi *et al.* (1965) showed the addition of DNA from various sources to DNR in solution caused changes in the visible and ultraviolet absorption spectrum of DNR, and the quenching of the DNR fluorescence. This indicated the chemical binding between DNA and DNR. Calendi *et al.* (1965) postulated two chemical groups could be responsible for the linkage between DNR and DNA: the hydroxyl groups present in the chromophore (hydroxy groups at C6 and C11, see Figure 1.1) and the amino group on the sugar. As well as changes to DNR itself, the DNR-DNA complex caused the following changes in the physical properties of DNA: sedimentation, viscosity, thermal denaturation-renaturation behaviour and optical activity.

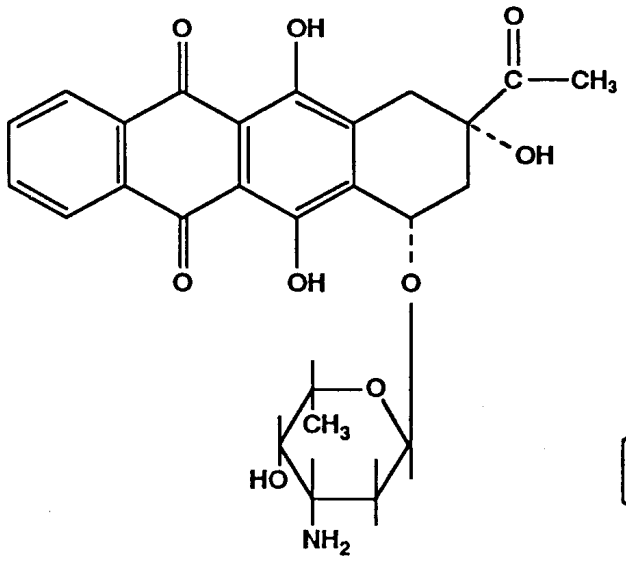
Since that study a number of spectroscopic methods have been used widely to characterize the interactions of anthracyclines and DNA in solution (reviewed by Neidle & Sanderson, 1983 and Chaires, 1990). Scatchard plot analysis has been used to obtain association constants (K) and the number of binding sites per nucleotide (n). Data from several studies (Zunino *et al.*, 1972; Chaires *et al.*, 1982; Plumbridge & Brown, 1978; Gray & Phillips, 1976; Schneider *et al.*, 1979), employing a number of different methods to calculate K and n found an average n value of 0.17 and K ranging from 0.7-7.2 x 10^6 M^{-1}



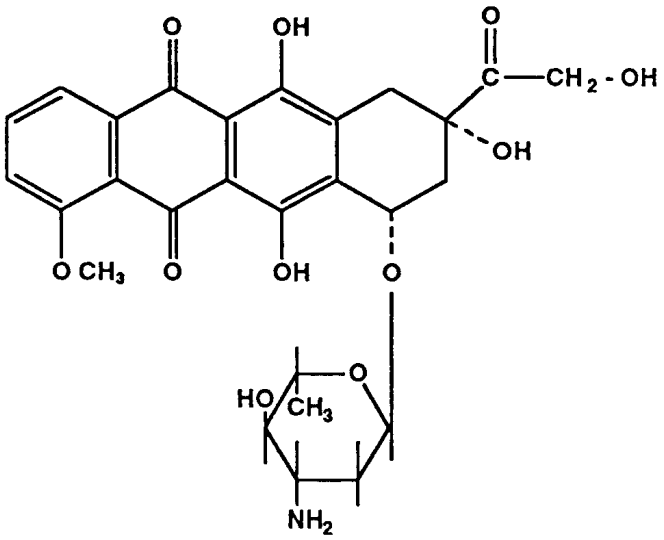
Daunorubicin (DNR)



Doxorubicin (DOX)



Idarubicin (IDA)



Epirubicin (EPI)

Figure 1.1. Structure of Anthracylines

for DNR. This corresponds to a maximal binding of one drug molecule per six nucleotides (three base pairs). The variation of K can be accounted for by the minor differences in experimental conditions, such as buffer type, ionic strength and the extent of DNA deproteinisation.

The first model proposed for the intercalation of DNR with DNA was by Pigram *et al.*(1972) who proposed that the amino-sugar of DNR lies in the large groove of the DNA molecule. The hydrophobic faces of the base pairs and the drug overlap extensively. The amino sugar is at the side of the groove close to a sugar-phosphate chain enabling the ionized amino group to interact strongly with a second DNA phosphate remote from the intercalation site. A possible additional interaction may be a hydrogen bond between the first phosphate and the hydroxyl group attached to the saturated ring of the DNR chromophore (hydroxyl group at C9 in Figure 1.1). Intercalation of the drug would separate two base-pairs by an extra 3.4 Å but since the sugar-phosphate chain in B-DNA is fully extended, the helix would also have to untwist at the point of intercalation to accommodate the DNR.

After determining the crystal structure of DNR, Neidle & Taylor (1977) proposed a model similar to that of Pigram *et al.* (1972), in which binding was in the major groove of the double helix, with the amino sugar situated in the groove such that it was in close contact with a phosphate oxygen atom. They added that DOX can participate in an additional interaction, with its C14 hydroxyl group being capable of hydrogen bonding to one of the phosphate oxygens at the intercalation site.

A complex between DNR and the hexanucleoside pentaphosphate d(CpGpTpApCpG) has been crystalised, and its structure determined by X-ray crystallography (Quigley *et al.*, 1980) and later refined at high resolution (Wang *et al.*, 1987). Two drug molecules are bound per self-complimentary oligonucleotide duplex. Each drug is intercalated between the terminal CG base pairs (Figure 1.2). A striking feature of the complex is that the drug molecules show only minor groove intercalation, in contrast to the other molecular models proposed. The amino sugar fits snugly into the minor groove; this position excludes any interaction between the charged amino group and backbone phosphates. The DNR chromophore (i.e. the tetracyclic nucleus) lies skew to the CG base-pairs, and it is notable that only rings A and D protrude from them (Figure 1.3), in complete agreement with NMR observations (Patel *et al.*, 1981). The planar B and C rings of the drug are sandwiched between adjacent base pairs and their overlap with the base pairs is small, although the hydroquinone oxygen atoms (at C5 and C12) on each side of the drug molecule clearly play a stabilizing role. The DNR-hexamer structure reveals several specific hydrogen-bonding interactions that serve to provide additional stabilization for the binding.

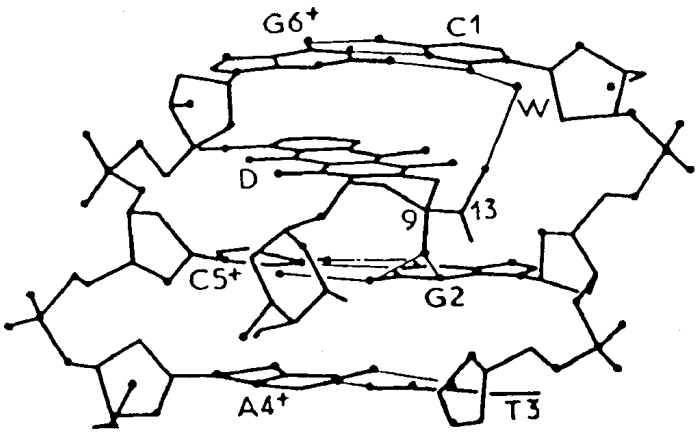


Figure 1.2. Diagram of DNR intercalated into d(CpGpTpApCpGp), showing intermolecular attractions.

Note two hydrogen bonds between O9 of DNR and N2 and N3 of G2. In addition, water forms a hydrogen-bond bridge between O13 of DNR and O2 of C1. (From Quigley *et al.*, 1980)

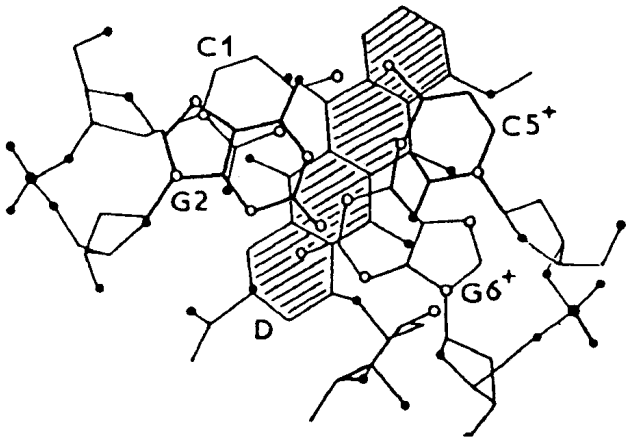


Figure 1.3. View of the intercalator perpendicular to the base plane.

The DNR ring system is stippled. The adjacent G2-C5+ base pair closer to the reader is shown by thick lines and the C1-G6+ base pair further away is shown by thin lines. The two nucleotide backbones are different. Also note that the centre of the G2-C5+ base pair has moved up, toward the major groove relative to the C1-G6+ pair. (From Quigley *et al.*, 1980)

+ designates complementary sequence

The positioning of the sugar in the minor groove of DNA provides a likely explanation for the lack of intercalation with double-stranded ribonucleic acid (RNA), since the minor groove in RNA is very shallow and would be unable to accommodate the bulky sugar group. The high resolution proton NMR study of the DNR poly(dA-dT) poly (dA-dT) complex (Patel *et al.*, 1981) has enabled a direct comparison between the solution and crystallographic complexes. The overlap of the DNR B and C rings with adjacent base pairs, and the non-overlap of ring D were very similar in both techniques.

DNA intercalation by the anthracyclines has been reported to alter a variety of DNA functions. Zunino *et al.* (1975a) demonstrated that both DOX and DNR inhibit both DNA synthesis and RNA synthesis by a similar mechanism of action: the inhibition of DNA polymerase and RNA polymerase. Later, Zunino *et al.* (1975b) showed that the reaction with DNA polymerase is competitive with respect to DNA. That finding was consistent with a direct interaction of DNR with DNA, with the DNA template being the predominant factor involved in the inhibition. In addition, the finding of a reversal of DNR-induced inhibition of the DNA polymerase by an increase in Mg^{2+} concentration is consistent with the proposed mechanism, since Mg^{2+} decreases the total binding of DNR to DNA (Calendi *et al.*, 1965). The ability of the intercalated anthracyclines to inhibit RNA and DNA polymerases has been proposed as the major mechanism by which DNA intercalation kills tumour cells.

DOX, as well as many other DNA intercalators, have been reported to trigger topoisomerase II mediated DNA damage (Tewey *et al.*, 1984a, b). Mammalian topoisomerase II catalyses the crossing of two double stranded DNA helices. This crossing over is ATP-dependent and involves the formation of a double stranded break that is bridged by a covalently bound enzyme. After strand exchange, the ends are rejoined and the enzyme dissociates. Intercalators have been shown to prevent both the strand passing and rejoining reactions (Tewey *et al.*, 1984a, b). As a result of these drugs the DNA is left with a protein concealed double strand break.

The anthracyclines can also cause DNA damage such as fragmentation and single strand breaks (Levin *et al.*, 1981; Kanter & Schwartz 1979a, b). Levin *et al.*(1981) showed that the semi-synthetic anthracycline N-trifluoroacetyladriamycin-14-valerate produced protein associated DNA strand breaks and DNA protein cross links similar to those of DOX. However, N-trifluoroacetyladriamycin-14-valerate was unable to intercalate with DNA. They concluded that mechanisms other than direct interaction with DNA play a role in the toxic effects of these compounds and some of these mechanisms are discussed below.

1.1.2. Free Radical Formation

Two mechanisms of anthracycline-mediated free radical formation have been described, one dependent on the formation of semiquinone radicals generated during flavoprotein-mediated redox-cycling, and the other dependent on the anthracycline-iron complex (eg DOX-iron complex). These two mechanisms and the role played by these mechanisms in the cytotoxic effects of the anthracyclines will be discussed.

Anthracyclines can be reduced by cellular flavoproteins to form a semiquinone free radical (Figure 1.4). This involves a one electron reduction in the C ring of anthracycline and can be achieved by several flavoproteins; NADPH cytochrome P450 reductase (Berlin & Haseltine, 1981), NADH dehydrogenase (Doroshow, 1983) and xanthine oxidase (Bates & Winterbourn, 1982) as well as by intact cells (Sato *et al.*, 1977). In the presence of oxygen the semiquinone free radical is oxidized back into the parental quinone, with the formation of superoxide free radicals (Doroshow, 1983; Bachur *et al.*,1982). In the absence of oxygen the semiquinone is unstable; it loses its sugar moiety and an intermediate C7 free radical can be formed. This radical can bind covalently to cellular macromolecules or become reduced again, forming a relatively stable product, the C7-deoxyaglycone, which is in fact a doubly reduced anthracycline molecule that has lost its sugar moiety (Sinha *et al.*, 1984; Sinha & Gregory, 1981). A tautomer of C7-deoxyaglycone is the C7-quinone methide, which is a potent DNA alkylating species and potentially toxic for tumour cells (Sinha *et al.*, 1984).

Formation of an oxidised semiquinone in ring B of DOX is known to occur in the presence of iron (Zweier, 1985) when no reducing system is present. The DOX-iron II complex can react with molecular oxygen or hydrogen peroxide leading to the formation of superoxide or hydroxyl radicals, respectively, while the complex is oxidized to DOX-iron III (Eliot *et al.*, 1984). The DOX-iron complex can support free radical formation by two mechanisms, one dependent on a reducing system and the other from the complex itself without the presence of a reducing system (Figure 1.5). DOX-iron III can be reduced enzymatically by cytochrome P450 reductase (Sugioka & Nakano, 1982) or nonenzymatically via a reaction with reduced glutathione (Zweier, 1985), reforming DOX-iron II, which can again react with oxygen and hydrogen peroxide. In the presence of a reducing system no metabolites are formed and free radical production can proceed indefinitely. In the absence of a reducing system, DOX-iron III can reduce its iron intramolecularly by oxidising its hydroquinone moiety leading to the formation of an oxidized DOX semiquinone free radical (Gianni *et al.*, 1985) or by oxidising its C9 side chain. Further oxidation of the C9 side chain leads to the formation of 9-dehydroxyacetyl-9-carboxyl DOX, a DOX metabolite (Gianni *et al.*, 1988).

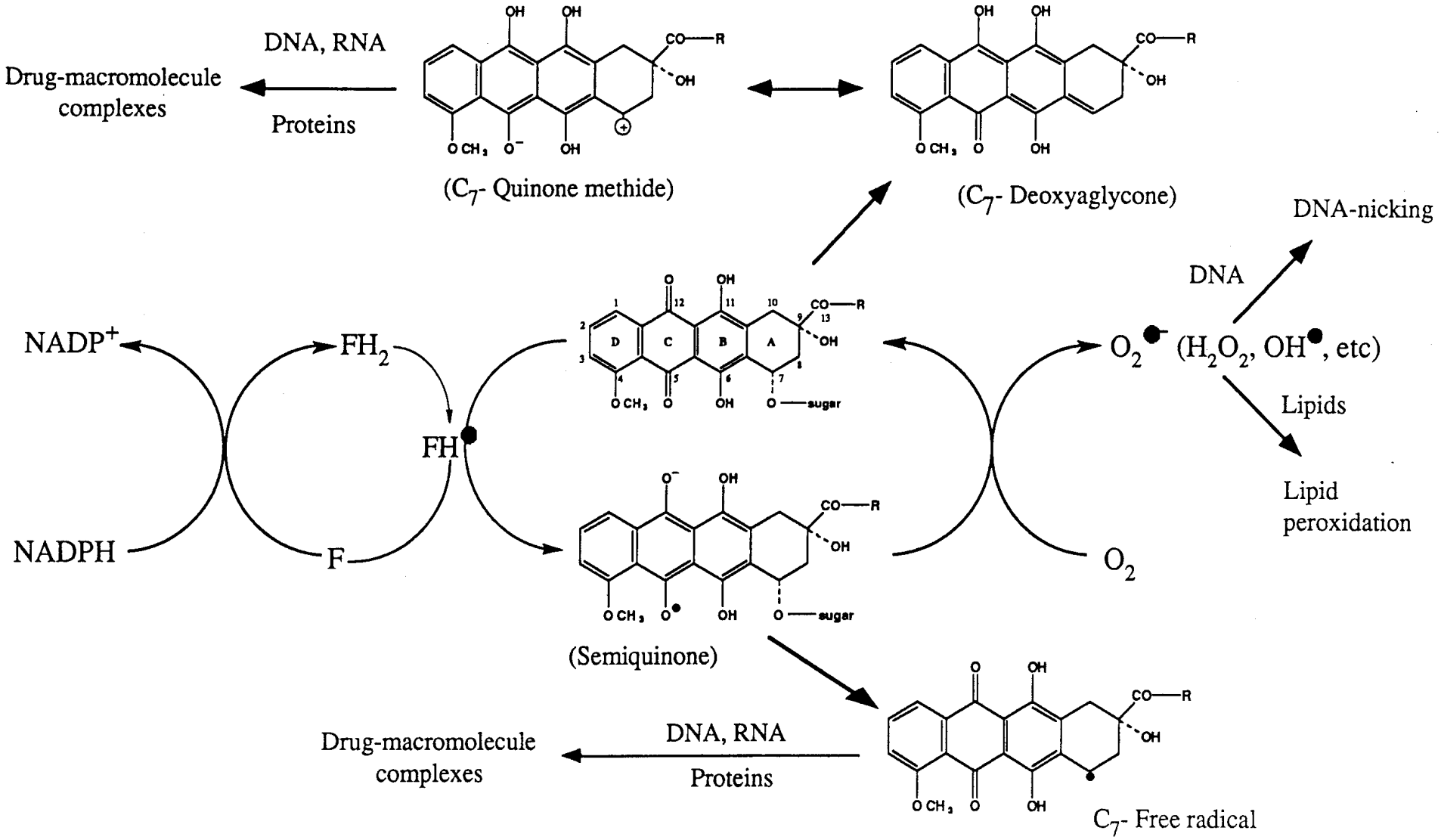


Figure 1.4. Free radical and alkylating intermediates produced by the anthracyclines. From Sinha *et al.* 1984

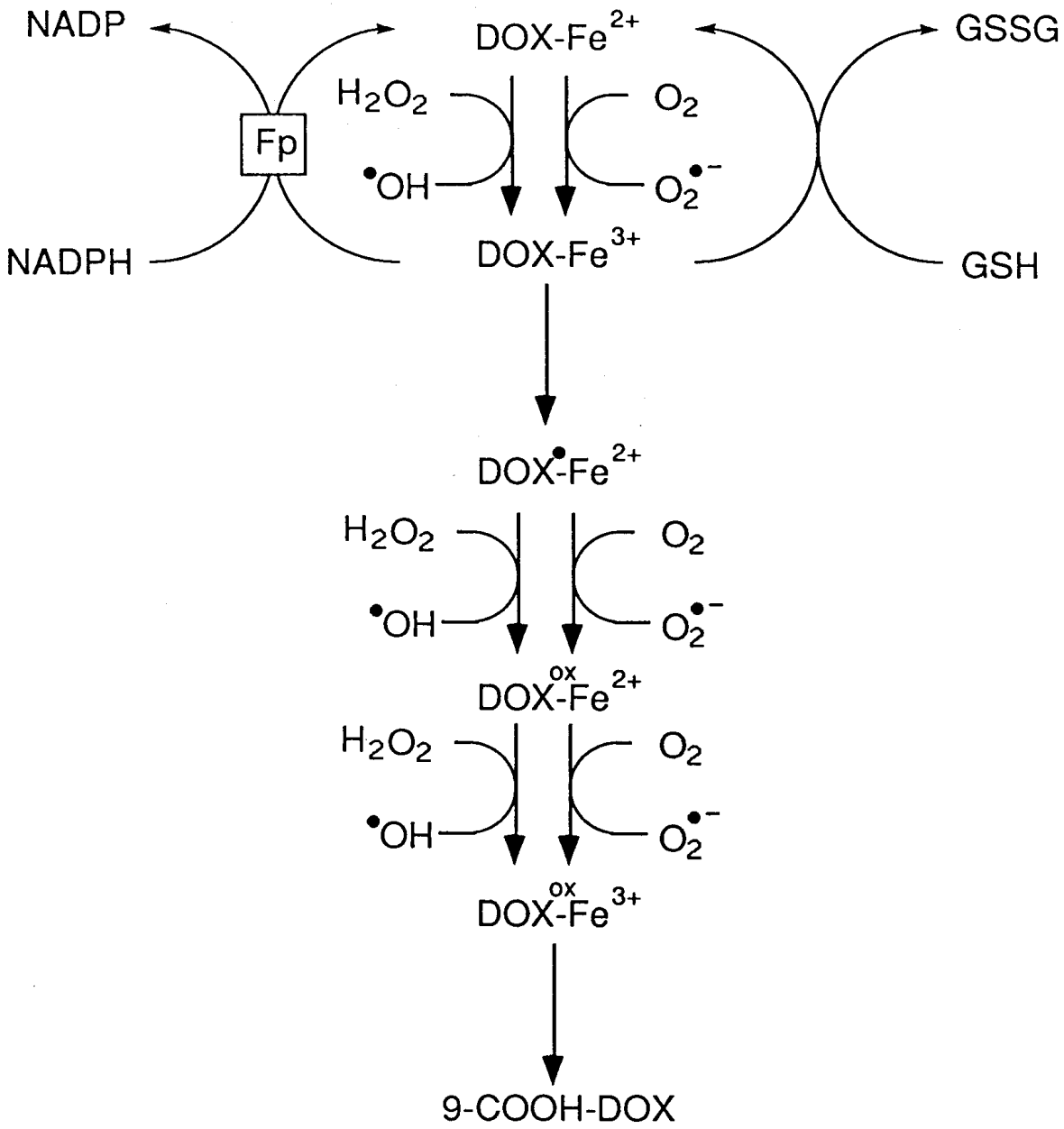


Figure 1.5. Free radical production from the DOX-iron complex

The hydroxyl radical is presumed to be the damaging species in free radical reactions. Therefore investigators have attempted to detect hydroxyl radicals in intact cells. Sinha *et al.* (1987a, b) detected hydroxyl radical formation in MCF7 human breast cancer cells and Doroshow (1986) obtained similar data for intact Ehrlich ascites tumour cells. Catalase completely inhibited this effect suggesting that the hydroxyl radicals were detected outside the cells. The extracellular addition of superoxide dismutase (SOD) or catalase can protect cells against cytotoxicity (Doroshow, 1986; Sinha *et al.*, 1987a), although SOD and catalase did not protect A2780 cells (Cervantes *et al.*, 1988). This suggests a non-universal mechanism of cytotoxicity.

Potmesil *et al.* (1984) observed that DOX causes two types of DNA strand breaks, and at concentrations of up to 2.8 μM, only protein associated strand breaks occurred. At higher concentrations, direct strand breakage increased only in the presence of oxygen and was inhibited by extracellularly added SOD or catalase (Potmesil *et al.*, 1983). Therefore, free radicals generated at the cell surface have the potential to damage cellular DNA. Since the ability to kill L1210 cells was almost 100% at concentrations higher than those clinically achievable, a free radical mechanism is not important in L1210 cells (Potmesil *et al.*, 1984) although it may play a more important role in MCF7 cells and Ehrlich ascites. Scavengers of hydroxyl radicals protect cells against the cytotoxicity of anthracyclines. However, protection is found only at extremely high concentrations of scavengers. In the same concentration range as these scavengers, NaCl also protected against cytotoxicity (Iliakis & Lazar, 1987). Therefore doubts exist over the use of hydroxyl radical scavengers at this concentration.

These results suggest that in the clinical situation only cells that are well oxygenated and exposed to relative high concentrations of anthracyclines, or have a relative lack of antioxidant defence capacity, can be damaged by a free radical dependent mechanism. This will probably include only a minor fraction of tumour cells. However, in heart tissue such conditions are met during treatment with a single high dose and cardiotoxicity is a major problem associated with the anthracyclines. In mice, lipid peroxidation of heart tissues was observed (Myers *et al.*, 1977) even though the process could not be demonstrated in tumour cells, suggesting that cardiac tissue is damaged by a free radical dependent mechanism causing lipid peroxidation.

1.1.3. Cell Membrane Effects

One of the main actions of the cell membrane is to act as a permeability barrier.

Therefore anything that disrupts the cell membrane may cause cell death. Murphree *et al.* (1976, 1981) found that there was a correspondence between the concentration of DOX required to induce agglutination changes and that required to induce cytotoxicity. An enhanced rate of agglutination by concanavalian A of Sarcoma 180 cells was produced after exposure to DOX and the increase in agglutination rate caused by the anthracycline was concentration dependent. The changes in agglutination rate were not due to either alterations in the number of lectin-recognised residues on the cell surface, or to the rate of occupancy of the sugars by the lectin, implying that direct membrane perturbation by the drug may be the underling effect.

Protein kinase C (PKC) also has been studied as a target for DOX action (Posada *et al.*, 1989). Drug treatment produces an increase in turnover of phosphatidylinositol with a consequent production of diacylglycerol which acts as the intracellular activator of PKC. In fact, PKC activity is elevated in drug-treated cells. Experiments with phorbol esters suggest that PKC activity is linked with DOX action, since activation of the enzyme with TPA (12-O-tetradecanoylphorbol-13-acetate) enhances DOX's cytotoxicity as well as its ability to provoke DNA damage. Thus the ability of cells to be injured by DOX appears to be correlated with the activity of PKC.

DOX loses its cytotoxic ability at temperatures below 20°C (Lane *et al.*, 1987). This effect is not due to uptake, metabolism or intracellular localisation differences. The temperature profile of cytotoxicity is identical to the temperature profile for topoisomerase II-mediated DNA damage (Vichi *et al.*, 1989), so there appears to be some control exerted over the activity of this enzyme by endogenous factors. Vichi & Tritton (1992) have shown that the presence of DOX in the nucleus, regardless of the concentration, does not induce cytotoxicity or DNA damage; only when the drug is present also in the extracellular fluid and capable of interacting with the plasma membrane is the cytotoxic cascade set in motion. Thus the surface actions appear capable of modulating nuclear activities like topoisomerase II.

Tritton & Yee (1982) developed the technique of immobilizing DOX by covalently linking it to agarose beads with a mean diameter of 100μm. The DOX was attached to the agarose by activating the agarose with 1,1'-carbonyldiimidazole, which could then react with a free amino group (the amino group attached to the 3'C of DOX). The cytotoxicity of the conjugate was assessed by exposing it to L1210 (murine leukaemia cell line) cells in suspension and then the surviving fraction was determined by colony formation. The results showed that the immobilised-DOX was cytotoxic, with no free DOX accumulated in the cells. The finding that the immobilised-DOX could actively kill the cells

under conditions where it was not accumulated, strongly suggests that interaction at the cell surface was sufficient to induce cytotoxicity. Wingard *et al.* (1985) extended the study of Tritton & Yee (1982) by immobilising DOX using cross linked polyvinyl alcohol (CL-PVA). The CL-PVA was activated with cyanuric chloride that allowed the attachment of the amine on the 3'C of DOX to be bound. They also attached another anthracycline, carminomycin, to CL-PVA through the 3C atom of carminomycin, thereby leaving the amino group of carminomycin free. Wingard *et al.* (1985) found that the exact orientation of the anthracycline when in contact with the cell surface was not crucial for initiating cytotoxicity. Rather, the mere presence of the drug in the lipid bilayer was sufficient to cause cell death. This finding argues against a specific drug receptor, and suggests that the lipid bilayer itself may act as the receptor for the drug. In studies by Tokes *et al.* (1982) and Rogers *et al.* (1983) anthracyclines were attached to polyglutaraldehyde microspheres. The bound drug retained cytotoxic activity against cultured cells without entering the cells, and was also active against cells that were normally resistant to free DOX. Tokes *et al.* (1982) suggested that the reason the immobilised drug is so active is that it causes multiple and repetitive interactions with the cell that results in continuous perturbation of the plasma membrane. To test this hypothesis, Rogers & Tokes (1984) immobilised a non cytotoxic analog of DNR (4-demethoxy-7,9-di-epi-daunorubicin). This immobilisation caused the analog to become significantly cytotoxic, providing further evidence for the notion that continuous membrane perturbation may enhance anthracycline cytotoxic activity.

Anthracyclines also have been covalently immobilised on dextran (Bernstein *et al.*, 1978), N-(2-hydroxypropyl)metacrylamide copolymers (O'Hare *et al.*, 1989: Seymour *et al.*, 1990), poly-L-aspartic acid (Zunino *et al.*, 1982) and tumour selective antibodies (Hurwitz *et al.*, 1975, 1978). These preparations have cytotoxic activity, but detailed mechanistic studies and delineation of the target site is not available.

Since the anthracyclines interact with the membrane to provide a range of biological responses, the nature of the association between the drug and the lipid bilayer has been studied predominantly in liposomal membranes. However, isolated plasma membrane preparations and whole cells also have been studied. A property of the phospholipid bilayer is to undergo a gel-liquid phase transition. Alterations in the transition temperature (Tm) is expected to be wrought by molecules that bind to the bilayer and perturb the packing of the phospholipid molecules. If Tm is increased it is associated with a decrease in fluidity and if Tm is decreased there is an increase in membrane fluidity. Tritton *et al.* (1978) found a Tm of 34.6°C for liposomes prepared from dipalmitoylphosphatidyl choline (DPPC) alone. This was decreased to 33.3°C in the presence of DOX indicating an increase in membrane fluidity. Using other positively and negatively charged phospholipids, an increase in

membrane fluidity was also observed. However, on the incorporation of cardiolipin (a negatively charged phospholipid) in membranes treated with DOX the Tm increased, indicating a decrease in membrane fluidity and the opposite effect to all other phospholipids. Thus DOX has a unique effect on membranes that contain cardiolipin. Cardiolipin is normally confined to the mitochondrial membrane, but upon malignant transformation this phospholipid occurs in all other membrane locations including the plasma membrane. Because of the differential structural response to DOX of membranes that contain cardiolipin, cancer cells with cardiolipin on the surface would be expected to respond differently to this drug than normal cells lacking the lipid, and this may rationalise why the anthracyclines are useful antitumour agents with some specificity to diseased cells. In addition this concept may help to explain the cardiotoxicity of the anthracyclines (Goormaghtigh *et al.*, 1980a, b) since heart muscle is rich in respiring mitochondria, and mitochondrial membranes are the major repository of cardiolipin in normal cells.

Proposing that the anthracyclines interact with the cell membrane does not indicate how they interact with the lipid bilayer, or whether they are in or on the bilayer. Burke & Tritton (1985) using fluorescence quenching with I^-, showed that free anthracyclines are quenched by both static (results from when a fluorophore is immediately adjacent to the quenching molecule at the instant of excitation) and dynamic (collisional) mechanisms, whereas membrane bound drugs are predominately quenched by the dynamic mechanism. Ferrer-Montiel *et al.*(1988) found that in experiments with low drug/lipid ratios the anthracycline appeared to be predominantly located in domains near the hydrophilic surface, although a second more deeply embedded drug location arises at higher drug/lipid ratios. Circular dichroism studies by Henry *et al.* (1985) and Henry-Toulme *et al.* (1988) provide evidence for two distinct types of membrane binding sites for the drug. They suggest that in site I the amino sugar of DOX is bound to a charged phosphate with the chromophore lying outside the bilayer, while in site II, the sugar phosphate interaction persists, but the chromophore is embedded in the bilayer. Adler & Tritton (1988) developed a technique for determining the angular rotation of molecules in membranes. Their results showed that most membrane components take on an angle that is either parallel or perpendicular to the bilayer. DOX on the other hand, adopted an angle of about 55°, which could be very disruptive to orderly bilayer packing.

Burke *et al.* (1987) have also studied how substitution of the amine (at the 3'C of DNR or DOX) simultaneously affects both cellular accumulation and cytotoxicity. The net cellular accumulation of the anthracyclines was greatly enhanced by an ionizable amino group and reduced by a non-basic amino group. However, cytotoxicity studies showed that drugs with a non-basic amino group had increased cytotoxicity over anthracyclines with an

ionizable amino group. The data demonstrated an inverse correlation between uptake and potency. It was postulated that the cell surface, rather than intracellular sites, is a sensitive target for anthracycline action since the analogs that were taken up the least were the most active.

Tritton (1991) has proposed a mechanism of action for DOX that involves both DNA and the membrane as targets for drug action. DOX first interacts with the cell membrane to produce a disruption of membrane structure, manifested by fluidity changes. This leads to an increase in phosphoinositide turnover, one consequence of which is the activation of PKC. This enzyme may have several important substrates for phosphorylation, such as topoisomerase II. The activity of topoisomerase II is controlled by phosphorylation but the enzyme can malfunction, perhaps requiring a drug induced distortion of the double helix, to introduce stable DNA-protein cross links that lead to cell death. This pathway offers great promise in the understanding of how the anthracyclines kill cancer cells.

1.2. Multidrug Resistance

Multidrug resistance (MDR) is a unique phenomenon in the study of cellular drug resistance. Cell lines exhibiting this phenotype have been selected for resistance to a single cytotoxic agent, yet display a broad unpredictable cross-resistance to a variety of unrelated cytotoxic drugs, many of which are used clinically in cancer treatment. The drugs involved in MDR are alkaloids or antibiotics of fungal or plant origin, including vinblastine, vincristine, the anthracyclines DNR and DOX, colchicine and dactinomycin.

Initial studies in MDR mutant cell lines revealed that the mechanism of resistance involves reduced accumulation of drugs within the cell (Kessel *et al.*, 1968). This reduced accumulation was initially attributed to either increased drug efflux (Dano, 1973) or decreased cell permeability (Ling & Thompson, 1974). In most cell lines, both increased efflux and decreased influx can be demonstrated (Dano, 1973; Skovsgaard, 1978a, b; Inaba, 1979; Fojo, 1985). One important feature of this resistance is the requirement for a source of cellular energy. Analysis of the biochemistry of the MDR cell lines suggested that protein alterations of the plasma membrane existed. One of these alterations was an increased expression of a cell surface glycoprotein, termed P-glycoprotein (Pgp) by Ling and his colleagues (Juliano & Ling 1976; Riordan & Ling, 1979). Pgp was shown to be associated with MDR cell lines, and it was suggested that Pgp was responsible for the both the increased drug efflux and decreased influx seen in MDR cells.

With increased studies in the area of MDR other examples of drug resistance that were not mediated by Pgp became evident. These include alterations in glutathione metabolism, especially glutathione S-transferase (Morrow & Cowan, 1990), alterations in topoisomerase II (Beck *et al.*, 1987) and another membrane transporter multidrug resistance-associated protein (MRP) (Cole *et al.*, 1992). Pgp and non-Pgp mechanisms of MDR will be discussed in further detail below.

1.2.1. P-glycoprotein Mediated Resistance

The human mdr1 gene encodes Pgp, which is a 1280 amino acid protein with a molecular mass of 141kDa, as predicted from the cDNA sequence (Chen *et al.*, 1986). The predicted primary structure of Pgp has not yet been confirmed by analysis of the amino acid sequence of the purified protein. The predicted sequence agrees with the estimate for unglycosylated Pgp, expressed in glycosylation-deficient mutants of Chinese hamster cells (Ling *et al.*, 1983). In MDR KB cells, a single Pgp precursor of 140 kDa is observed that undergoes maturation to a final molecular size of 170 kDa through N-linked glycosylation (Richert *et al.*, 1988).

The most notable characteristic of Pgp is the homology between the N-terminal and C-terminal halves of the protein. The similarity of the two halves is pronounced in the comparison of their hydrophobicity profiles, which look identical. Analysis of the hydrophobicity profiles showed that each half of Pgp consisted of a short hydrophilic N-terminal region, a long hydrophobic region and a long, relatively hydrophilic C-terminal region. The protein sequence was analyzed for the presence of potential a-helical transmembrane segments and six transmembrane segments within each of the two hydrophobic regions were found (Roninson, 1991). The proposed structure of Pgp based on predicted positions is shown in Figure 1.6.

The C-terminal region in each half of Pgp contains a sequence that is believed to form two folds constituting nucleotide-binding sites (Roninson, 1991). These nucleotide binding folds are probably responsible for the observed ATP binding and hydrolysis by Pgp (Hamada & Tsuruo, 1988). The orientation of Pgp across the plasma membrane is known from studies using monoclonal antibodies, which have localised the C-terminus to the cytoplasm (Kartner *et al.*, 1985).

The exact function of Pgp is unknown although it is believed to act as a transport pump. Pgp pumps cytotoxic agents out of the cells, decreasing the effective cellular drug

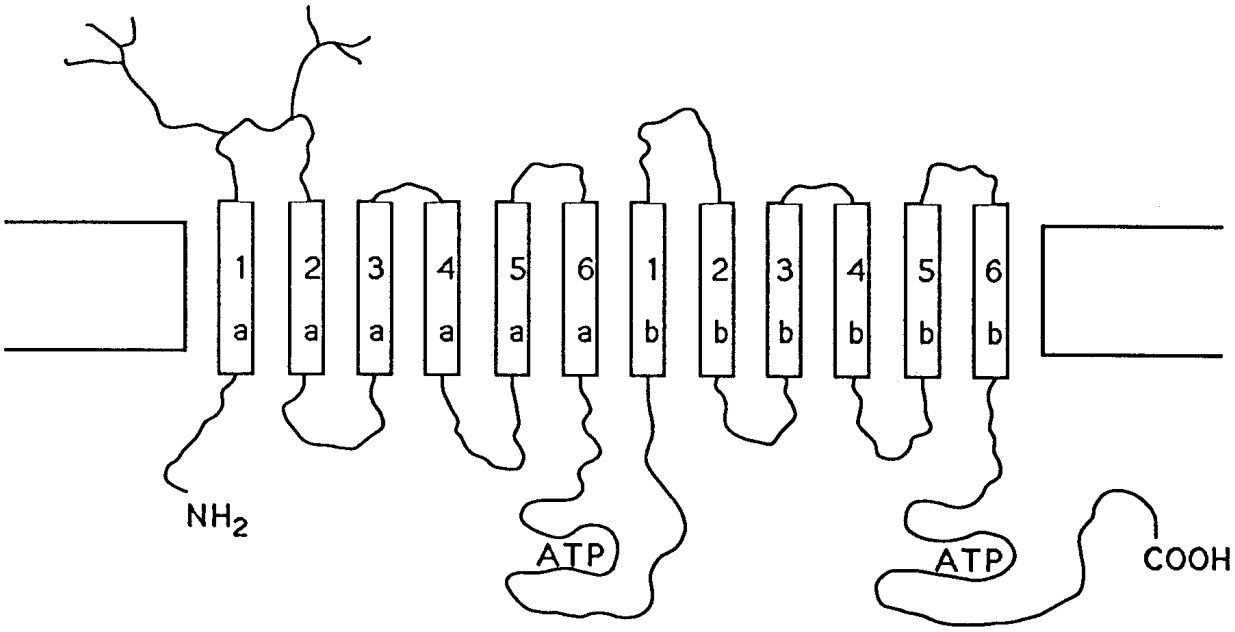


Figure 1.6. A diagrammatic representation of the proposed orientation of P-glycoprotein in the plasma membrane. The twelve a-helices are shown as rectangles, with the homologous halves labelled 'a' and 'b'. The putative position of N-linked carbohydrate is symbolised as a branched structure on the extracellular loop between the 1a and 2a transmembrane segments. The two cytoplasmic, nucleotide-binding domains are labeled 'ATP'.

concentration and thereby causing drug resistance. Several groups have proposed models for the action of Pgp based on the structural information and biochemical studies. Bradley *et al.* (1988) proposed a model for Pgp based on the information obtained from the amino acid sequence analysis of Pgp: Pgp forms a channel in the plasma membrane and transports drugs out of cells using energy derived from ATP hydrolysis. The number of Pgp molecules required to form one channel is not known. In one version of this model it was suggested that Pgp binds drugs directly and then removes them from the cell. The binding of drugs must be reversible and Pgp must have binding sites for a diverse group of drugs. In a second version of the model for Pgp function (Bradley *et al.*, 1988), a drug binding protein is transported out of the cells by the Pgp pump analogous to the export of hemolysin by Hly in E.Coli. Drugs may bind irreversibly to this protein and the entire drug protein complex is removed from the cell. The hypothetical drug binding protein may be a normally expressed cellular constituent, but it must be produced in sufficiently large quantities as it is continuously exported by Pgp.

Gottesman & Pastan (1993) have proposed an alternate model for Pgp function. Their model of Pgp function has two major features. The first is that drugs can be expelled as they enter the plasma membrane in the manner of a hydrophobic "vacuum cleaner" (i.e. drugs can be expelled by Pgp as they pass through the cell membrane, before they have entered the cytosol): this accounts for the decreased accumulation in the cytosol. The second feature is that transport occurs through a single barrel of the transporter.

Other proposed models of Pgp function are: (1) the transporter is essentially a flippase that detects drug within the inner leaflet of the plasma membrane and flips it into the outer leaflet or directly into the extracellular space (Higgins & Gottesman, 1992); and (2) another speculative model integrates the putative proton pump and chloride channel activity of the transporter to provide motive force for the hydrophobic vacuum cleaner (Gottesman & Pastan, 1993).

1.2.2. Reversal of Multidrug Resistance

An important mechanism of MDR is the enhanced drug efflux in resistant cells. In 1982 Tsuruo *et al.* found that calcium channel blockers such as verapamil and calmodulin inhibitors such as trifluoperazine (Tri) enhanced intracellular concentrations of vincristine and DOX in both vincristine and DOX resistant cells by inhibiting their outward transport. The higher intracellular drug accumulation was directly related to the enhancement of the cytotoxicity of the antitumour agents and resistance was circumvented in these cells by the

addition of either verapamil or Tri (Tsuruo *et al.*, 1982). Other researchers also showed that MDR could be reversed in a variety of cell lines with verapamil and Tri (Ganapathi *et al.*, 1984; Akiyama *et al.*, 1986; Kessel & Wilberding, 1985). Since the findings of Tsuruo *et al.* (1982) many compounds have been examined for their ability to reverse drug resistance and these have been extensively reviewed by Ford & Hait (1990) and Stewart & Evans (1989). Due to the large number of MDR reversing agents available, only the two MDR reversing agents used in this work (i.e. Cy A and Tri) will be discussed in greater detail below. The MDR reversing agents are also commonly referred to as either MDR modulating agents or MDR modifiers.

The most promising of these agents appears to be cyclosporin A (Cy A) that was first shown by Slater *et al.* (1986) to reverse drug resistance to both vincristine and DNR in a drug resistant subline of human T cell acute lymphatic leukaemia. Cy A is widely used for its ability to prevent graft rejection following organ transplantation and is used as an immunosuppressant. The immunosuppresive action of Cy A is based on its inhibition of T-cell activation. The actual mechanism by which Cy A inhibits T-cell activation remains elusive (Schreiber & Crabtree, 1992; Halloran & Mandrenas, 1991). Apart from its immunosuppresive action, Cy A has been shown to have a number of effects on the cell membrane. O'Leary *et al.* (1986) showed that Cy A perturbs single component dipalmitoyl phosphatidylcholine membranes, Cy A incorporation into these membranes caused no alteration in the arrangement of the lipids in the membrane and in the conformational arrangement of Cy A itself. However, the temperature and the maximum heat capacity of the lipid bilayers gel to liquid crystalline phase transition changed significantly. Thus, Cy A can perturb phospholipid membranes but not cause functional changes in cell membranes. Binding studies with liposomes of different chemical composition and human lymphocytes revealed that there is no specific cell membrane lipid receptor for Cy A (LeGrue *et al.*, 1983). Cy A is also capable of binding to the cell membrane and either directly or indirectly decreases transmembrane potential (Matyus *et al.*, 1986). Although Cy A does not bind to any specific membrane lipid it is capable of binding to a number of proteins including cyclophilin (Handschumacher *et al.*, 1984), calmodulin (Colombani *et al.*, 1985) and Pgp (Foxwell *et al.*, 1989; Tamai & Safa, 1990).

The use of photoactive, labelled analogues of vinblastine enabled two different groups to demonstrate that verapamil and cytotoxic drugs involved in the MDR phenotype bind competitively to Pgp (Cornwell *et al.*, 1986; Safa *et al.*, 1986). A similar technique was used to demonstrate interaction between Cy A and Pgp (Tamai & Safa, 1991). A more direct confirmation was obtained in a study that used a photoactive analogue of Cy A to photolabel viable MDR Chinese hamster ovary cells (Foxwell *et al.*, 1989). These studies indicate that

the MDR reversing agents work by competitive inhibition, binding directly to Pgp and not allowing the removal of cytotoxic agents. A study by Boscoboinik *et al.*, (1990) found that MDR cells had consistently higher intracellular pH values than their corresponding parental cell lines and Cy A was able to reverse both resistance and lower intracellular pH in one resistant cell line, but had no effect on either resistance or intracellular pH in another cell line.

Tri was one of the first drugs shown to be able to reverse multidrug resistance (Tsuruo *et al.*, 1982) and is used clinically as an antipsychotic. Tri is one of the most potent inhibitors of calmodulin, by binding selectively to calmodulin in a calcium-dependent manner. The binding of Tri is believed to involve two types of attachment: one is a hydrophobic interaction between the nucleus of Tri and a nonpolar region of calmodulin and the other is an electrostatic interaction between the positive charged amino group and a negatively charged residue on calmodulin (Hickie *et al.*, 1983). The actions of Tri for calmodulin binding have been clearly established (Roufogalis, 1982; Roufogalis *et al.*, 1983), however, very little is known about how Tri reverses drug resistance. Studies with Tri to date have predominately shown an association between reversal of drug resistance and cytotoxicity (Tsuruo *et al.*, 1982; Ganapathi *et al.*, 1984; Akiyama *et al.*, 1986; Kessel & Wilberding, 1985; Ganapathi *et al.*, 1988), with some showing an increase in drug accumulation in the drug resistant cell lines with increased reversal (Tsuruo *et al.*, 1982; Ganapathi *et al.*, 1984; Akiyama *et al.*, 1986) and others not showing a relationship between cellular accumulation and cytotoxicity (Kessel & Wilberding, 1985; Ganapathi *et al.*, 1988). In contrast to Cy A no studies have examined the binding of Tri to Pgp, thus whether Tri binds to Pgp remains to be determined. A recent study examined the possible role of intracellular calcium and cyclic adenosine monophosphate in mediating the reversal of drug resistance. They found Tri had no effect on these two parameters and ruled out any implication of calcium or cyclic adenosine monophosphate levels in the reversal of drug resistance (Mestdagh *et al.*, 1994).

1.2.3. Non P-glycoprotein Mediated Resistance

Topoisomerases are essential nuclear enzymes that catalyse the interconversion of topological forms of single and double stranded DNA (Liu, 1989). Cell lines expressing resistance to drugs that act on topoisomerase II (topo II) have been described (Beran & Anderson, 1987; Danks *et al.*, 1987). Cells selected for resistance to one inhibitor of topo II express cross resistance to other topo II inhibitors. Cells expressing this phenotype have been termed at-MDR, since the phenotype appears to be mediated by alterations in topo II (Danks *et al.*, 1988). Cells expressing at-MDR are generally unaltered in drug accumulation

and retention (Danks *et al.*, 1987) and do not overexpress Pgp (Beck *et al.*, 1987). Cells exhibiting at-MDR display a decrease in topo II activity, but how this is achieved is unknown. The general features of at-MDR include: (1) decreased cytotoxicity of many natural product drugs excluding vinca-alkaloids; (2) no enhancement of drug cytotoxicity by membrane active compounds; (3) no alteration in drug accumulation or retention; (4) no expression of mdr1 gene or Pgp; (5) decreased activity or amount of topoisomerase II or its mRNA; (6) phenotype expressed recessively and (7) the MDR reversing agents verapamil and Cy A are unable to reverse at-MDR.

Some MDR cell lines selected for DOX resistance display alterations in glutathione (GSH) levels (Yusa *et al.*, 1988) and GSH distribution (Hindenburg *et al.*, 1989) as well as changes in GSH metabolising enzymes (Bellamy *et al.*, 1989; Cowan *et al.*, 1986). The GSH biosynthesis inhibitor, butithionine sulfoximine (BSO) was shown to have some ability to modulate certain forms of MDR (Kramer *et al.*, 1988). Therefore it was thought that GSH may play a role in MDR, by acting as a deactivating agent for cytotoxic drugs, but this mechanism remains to be elucidated.

Cole *et al.* (1992) described an ATP-binding protein present in a drug resistant lung cancer cell line H69AR. This protein was termed MRP or multidrug resistance-associated protein, and was expressed in H69AR cells 100-200 fold more than in the drug sensitive H69 cell line. The drug resistance was reversed with the loss of gene amplification and a marked decrease in mRNA expression. MRP appears to be a member of the ATP-binding cassette transmembrane transporter superfamily, to which Pgp also belongs. Krishnamachary & Center (1993) also have shown that this protein is present in the drug resistant HL60/ADR cell line which has previously demonstrated decreased accumulation and retention of cytotoxic agents in the absence of Pgp. Cole *et al.*, (1994) and Breuninger *et al.*, (1995) have both transfected MRP into cell lines and were able to show a decrease in drug accumulation in the transfected cell lines.

1.3. Acute Leukaemia

Acute leukaemia is not a single disease but a group of neoplastic disorders characterised by the proliferation and accumulation in the bone marrow and peripheral blood of immature haematopoietic cells. These malignant cells gradually replace and inhibit the growth and maturation of the normal haematopoietic cells. If untreated, acute leukaemia is usually fatal within weeks to months from the time of diagnosis. Acute

leukaemia is divided into acute lymphocytic leukaemia (ALL) and acute nonlymphocytic leukaemia (ANLL, which is also more commonly referred to as AML (acute myeloid leukaemia)). The acute leukaemias are classified on the basis of the presumed cell origin (Table 1.1). A group of international investigators developed the FAB classification system for acute leukaemia's in 1976 based on morphological observations (Bennett *et al.*, 1976). Subsequently, several modifications have since been introduced (Miller *et al.*, 1981; Bennett *et al.*, 1981; Bennett *et al.*, 1985) into the system that has been widely adopted to permit comparisons of treatment results and to take advantage of differences between morphological subtypes. There are three subtypes of ALL (L1, L2, L3) and these are distinguished based on cell size, nuclear shape, number and prominence of nucleoli and the relative amount and appearance of the cytoplasm. The FAB classification defines eight subtypes of ANLL (M0 - M7) that differ with respect to cell lineage and degree of differentiation as described in Table 1.1. An experienced morphologist can classify 70% of acute leukaemia's accurately and this is increased to 99% when morphological criteria is supplemented with cytochemistry and immunophenotypic information (Browman *et al.*, 1986).

1.3.1. Treatment

Treatment for both ALL and ANLL is divided into two phases: (1) remission induction therapy and (2) postinduction therapy. Postinduction therapy can be subdivided into consolidation, intensification and maintenance therapy. The objective of induction therapy is the rapid eradication of detectable leukaemia, as determined by morphological methods, and the restoration of bone marrow function. The postinduction phase is required for the removal of clinically undetectable residual leukaemia after induction chemotherapy to prevent relapse, as well as the emergence of drug-resistant cells. Although the strategies for treating ALL and ANLL are the same, differences exist in the protocols and types of drugs used. Therefore the treatment for ALL and ANLL will be discussed separately.

The drugs used in the treatment of ALL include the anthracyclines DNR and DOX, vincristine, prednisone, dexamethasone and L-asparaginase. These agents are generally used in combination for induction therapy. There have been very few controlled or randomized trials for the treatment of adult ALL, and therefore the relative efficacy of the regimens cannot be assessed. A randomized trial compared the combination of vincristine, prednisone and L-asparaginase with or without DNR (Gottlieb *et al.*, 1984) and found that the addition of DNR markedly increased the complete remission rate from 47% to 83%. In other clinical trials involving adult ALL the complete remission rate is generally 70% to 80% with vincristine, prednisone and an anthracycline (Ruggero *et al.*, 1979;

Table 1.1. Classification of Acute Leukaemia

- Acute Lymphocytic Leukaemia (ALL)
  - Early pre-B cell ALL
  - Pre-B cell ALL
  - B cell ALL
  - T cell ALL
- Acute Nonlymphoblastic Leukaemia (ANLL)
  - Acute myelocytic leukaemia (AML, M0, M1, M2)
  - Acute promyelocytic leukaemia (APL, M3)
  - Acute myelomonocytic leukaemia (AMMoL, M4)
  - Acute monocytic leukaemia (AMoL, M5)
  - Acute erythroleukaemia (AEL, M6)
  - Acute megakaryocytic leukaemia (AMegL, M7)
- Acute Undifferentiated Leukaemia

Blacklock *et al.*, 1981; Gingrich *et al.*, 1985; Radford *et al.*, 1989; Linker *et al.*, 1987; Hussein *et al.*, 1989; Hoelzer *et al.*, 1984; Schauer *et al.*, 1983; Clarkson *et al.*, 1985); the addition of other drugs does not significantly increase the remission rate and their effects on remission duration is unknown (Hoelzer & Gale, 1987). The two most successful treatment protocols are those developed by Hoelzer *et al.* (1984) and Schauer *et al.* (1983). These protocols are more intensive than most, but the median duration of remission is generally better (21 to 51 months). Most patients free of disease at 50 months will remain in remission. Consolidation and maintenance involves the use of several drugs including methotrexate, cytosine arabinoside (Ara C), 6-mercaptopurine, as well as the agents used at remission induction.

The most common regimens in ANLL involve the use of Ara C and an anthracycline with or without 6-thioguanine. Ara C is given as an infusion over 7 days and DNR is given by intravenous push on the first three days of treatment, this combination is designated 7-3. The optimal schedules for ANLL have been evaluated and the 7-3 regimen is regarded as superior to most (Preisler *et al.*, 1987; Rai *et al.*, 1981). The addition of etoposide does not improve complete remission but did improve survival in patients younger than 55 years (Bishop *et al.*, 1990). It also appears that the addition of 6-thioguanine does not enhance remission rates (Preisler *et al.*, 1987; Toronto Leukaemia Study Group, 1986). Post remission therapy for ANLL involves the use of the agents used at induction. In a study by Cassileth *et al.* (1988) patients were randomized to receive postinduction treatment or not. A significant prolongation in remission duration was seen in those patients that had postinduction chemotherapy. Postremission therapy can be divided into three types: maintenance therapy defined as the postremission therapy which minimises severe bone marrow suppression by using low doses of chemotherapeutic agents; consolidation therapy, which consists of regimens that are essentially the same as induction regimens, in terms of both drugs and doses; and intensification therapy, defined as therapy using the same drugs as for induction but at significantly higher doses or with myelosuppressive doses of active agents other than those administered in induction (Bloomfield, 1985). With maintenance chemotherapy 15% of patients experience longer than 8 years remission and therefore are probably cured (Preisler *et al.*, 1989). In a study by Preisler *et al.* (1987) patients were randomized between two arms, high dose Ara C and regular Ara C with amsacrine. The two arms were equivalent regarding overall remission duration.

Several trials (Berman *et al.*, 1991; Vogler *et al.*, 1992; Vogler *et al.*, 1989; Wiernik *et al.*, 1989; Wiernik *et al.*, 1992) have compared the effectiveness of DNR and Ara C against IDA and Ara C and these studies have shown that complete remission rates have increased from 58% with DNR and Ara C to between 67%-80% with the use of IDA and

Ara C, indicating that IDA plus Ara C may be a more effective regimen than DNR plus Ara C.

The aim of induction therapy in patients is to achieve complete remission. Other possible outcomes include a partial remission or no response to treatment. These terms are defined as follows: Complete remission should be present for at least 4 weeks with near normalisation of neutrophils (≥ 1.5) and platelets (≥100), and is documented by a marrow biopsy that demonstrates at least 20% cellularity, less than 5% blasts, and no Auer rods. Partial remission requires that all the criteria for complete remission be satisfied except the bone marrow may contain between 5-25% blasts (Cheson *et al.*, 1990). Treatment failure is classified into 5 types according to Preisler (1978): (1) Failure to produce marrow hypocellularity at any time during chemotherapy, i.e. the leukaemic cells are drug resistant. (2) Attainment of marrow hypocellularity but regrowth of leukaemic cells within four weeks of attainment of hypocellularity. A majority of the leukaemic cells are drug sensitive but early regrowth of leukaemia occurs. (3) Patient survives for more than four weeks with a hypocellular marrow and peripheral blood cytopenia without detectable leukaemia. These patients are drug sensitive but have inadequate normal stem cell reserves to recover complete normal marrow function. (4) Patient expires with a hypocellular marrow without evidence of residual disease. (5) Inadequate trial, patient expires less than seven days after cessation of induction therapy.

1.3.2. Problems Associated with Treatment

Relapse results from regrowth of the original clone of malignant cells (Raghavachar *et al.*, 1987). Those that occur during treatment are thought to result from the emergence of drug resistance, some of which is mediated by the overexpression of the MDR gene (Rothenberg *et al.*, 1989). The treatment and management of relapse is dependent on the duration of the first remission. If a patient's first remission is less than 18 months, there is rarely a second remission lasting more than a few months. Those who relapse while receiving therapy more than 18 months after diagnosis and those who relapse after chemotherapy are often treated effectively with reinstitution of conventional therapy. The site of relapse is also important in determining treatment success. Bone marrow relapse is a worse prognosis than an isolated extramedullary relapse, and patients with isolated CNS relapse are treated more successfully than those estimated with testicular relapse (Bleyer *et al.*, 1986). Second remissions can be achieved in 50-75% of adults with ALL who have a bone marrow relapse (Gaynor *et al.*, 1988; Miliped *et al.*, 1990). Treatment of relapsed ALL is generally with the same agents as used in induction and postremission therapy. Single agent high dose methotrexate, high dose Ara C and mitoxantrone result in a second

remission in up to 30% of patients (Hoelzer & Gale, 1987). By using either the same combination of drugs that achieved initial remission or other drug combinations instead of single agents, 50-75% of relapsed adult ALL achieve a second remission. The most effective regimens appear to be (1) moderate to high dose methotrexate with L-asparaginase or folinic acid rescue; (2) the combination of teniposide and Ara C and (3) high dose Ara C in combination with amsacrine, an anthracycline or other agents. Median remission after treatment of refractory or relapsed ALL is usually less than six months and disease free survival is less than 5%.

About 10-20 % of patients with ANLL fail to enter complete remission with conventional chemotherapeutic regimens. In addition to this approximately 50 -70% of patients who achieve complete remission will eventually suffer a clinical relapse (Tricot, 1991). The majority of early relapses probably represent the regrowth of leukaemic cells that were present at the time of initial diagnosis and have proven drug resistance. A study by Kantarjian *et al.*(1988) showed a 17% complete second remission rate in patients relapsing within 12 months, with 59% having drug resistant disease. In patients who relapsed after 18 months complete second remission was achieved in 66%, with only 19% having drug resistant disease. The median overall survival was better in late relapse patients (18 months vs 3 months) and the 3 year remission duration was 25% in late relapse patients and 5% in early relapse. High dose Ara C is probably the single most active agent in refractory or relapsed ANLL. The probability of cure in patients with refractory, second and later relapse and those in first relapse after a remission duration of less than one year are extremely low.

1.3.3. Pgp and Acute Leukaemia

As discussed above (1.2), the presence of Pgp leads to a decrease in cellular concentrations of drugs, thus causing drug resistance. This resistance is clearly defined for cell lines, however, it has not been proven whether Pgp is responsible for resistance to chemotherapy in patients with acute leukaemia, since a number of studies have examined the presence of Pgp and resistance in patients with acute leukaemia and have found conflicting results. Ma *et al.* (1987) were one of the first to report the detection of Pgp in two patients with relapsed ANLL. Campos *et al.* (1992) have shown that in ANLL patients, complete remission rates are significantly lower in Pgp positive patients (23/71, 32%) compared to Pgp negative patients (64/79, 81%). Marie *et al.* (1991) and Pirker *et al.* (1991) also found a correlation between Pgp (mdr1 gene expression) and patient response. Marie *et al.* (1991) observed a complete remission of 67% in patients with undetectable mdr1 expression, versus 29% in patients with increased expression. Pirker *et al.* (1991) found the

complete remission rate to be 89% in mdr1 RNA negative patients and 53% in mdr1 positive patients. Kuwazuru *et al.* (1990a) also found a correlation between Pgp and patient response. In 36 acute leukaemic patients, 17 were Pgp positive with only 23.5% of patients responding to treatment, whereas 14/19 (73.7%) Pgp negative patients responded to treatment. Wood *et al.* (1994) showed that in 54 newly diagnosed patients with AML complete remission rates were lower in Pgp positive patients (18/30, 60%) than in Pgp negative patients (22/24, 92%). The overall survival for Pgp positive patients (329 days) was also shorter than for Pgp negative patients (522 days). Studies by Zhou *et al.* in 1992 and 1995 also confirmed the above findings. They found that the expression of the mdr1 gene correlated significantly with clinical drug resistance, 62% of patients positive for mdr1 RNA eventually developed resistance while only 24% of patients that were negative for mdr1 RNA developed resistance (Zhou *et al.* 1992). Later they showed an overexpression of the mdr1 gene in 18% (9/51) of newly diagnosed AML patients and 42% (8/19) in resistant AML patients (Zhou *et al.* 1995).

In contrast to the above findings, Holmes *et al.* (1989) established that the amplification (i.e. increase in gene copy number) of the Pgp gene was not an important mechanism in previously untreated AML. In that study elevated levels of Pgp mRNA were seen in two out of eight cases of untreated AML, five out of eight refractory AML and four of five cases of secondary AML. Kuwazuru *et al.* (1990b) also found 8/20 patients at initial presentation were Pgp positive while 6/6 patients at relapse were Pgp positive. In a study by Ito *et al.* (1989), 14 patients at initial presentation and 18 patients at relapse were all Pgp negative. Rothenberg *et al.* (1989) observed that 8/9 patients with ALL at presentation had low levels of mdr1 mRNA. In 5 patients at primary relapse, none had evidence of mdr1 overexpression and 3/15 patients with multiple relapses had elevated mdr1 expression. They concluded that Pgp might play a role in some cases of drug resistance, but that other mechanisms of resistance must also exist. Wattel *et al.* (1995) demonstrated that 32 of 50 ALL patients were Pgp positive, but found no correlation between Pgp expression and complete remission, actuarial disease-free survival or survival. Tiirikainen *et al.* (1993) showed that in 46 AML and ALL patients, after two induction cycles, 10/12 (83%) Pgp positive patients achieved complete remission and 30/34 (88%) Pgp negative patients achieved complete remission, as well as this there was no correlation between remission duration or survival and Pgp expression. Gruber *et al.* (1992) found in a group of 46 AML and ALL patients, 23 of 28 (82%) patients leukaemic cells without detectable mdr1 RNA entered complete remission compared to 12 of 18 (67%) patients with leukaemic cells where mdr1 RNA was detected (p=0.40). Schneider *et al.* (1995) used quantitative reverse transcriptase polymerase chain reaction to detect relative levels of transcripts for mdr1 in 43 AML and 19 ALL patients samples. In 27 of the samples mdr1 expression was too low

to be quantified in the remaining 35 samples, relative mdr1 expression ranged from 0.05 fold to 3.7 fold the level expressed in the SW620 cell line which expresses low levels of mdr1. They found no correlation between mdr1 expression and leukaemia type, response to therapy or duration of remission.

There is no doubt that Pgp is found in the cells of patients with acute leukaemia. However, the relationship between the presence of Pgp and patient response remains unclear. There has also been no study examining the relationship between Pgp and drug accumulation in the cells of patients with acute leukaemia.

Reversal of drug resistance is achieved *in vitro* by the use of agents such as verapamil, Cy A and Tri. Few studies however, have examined the effects of these reversing agents in patient leukaemic cells either *in vitro* or *in vivo*. Studies by Kessel *et al.* (1984), Ross *et al.* (1986) and Andersson *et al.* (1987) have shown that verapamil had no effect on increasing cellular drug accumulation in the blast cells of patients with acute leukaemia. Nooter *et al.*, (1990) has examined the effects of an MDR reversing agent and its relationship with Pgp in acute leukaemia. In that study Cy A was able to increase DNR accumulation in leukaemic cells from patients overexpressing the mdr1 gene. Studies by Sonneveld *et al.*(1992) and Dalton *et al.*(1989) have shown that Cy A and verapamil may be of beneficial use to patients with drug resistant multiple myeloma. Therefore the potential does exist for these MDR reversing agents to be used clinically.

1.4. Pharmacokinetics

Pharmacokinetics is the study of the time course of action of drugs in biological systems as well as the mathematical relationships required to develop models to represent the time-dependent changes in the concentrations of drugs in such systems to interpret the data (Peng & Chiou, 1990). Pharmacokinetics involves the principles of drug absorption, distribution and elimination, and contributes to the understanding of those factors that determine the intensity and duration of an individual's response to a drug. It has provided a basis for rational rather than empirical dosing of drugs.

1.4.1. Principles

There are three different approaches to describing the pharmacokinetics of a drug in the body: compartmental analysis, noncompartmental analysis and physiological

pharmacokinetic modelling (Gibaldi, 1991; Rowland & Tozer, 1989). The traditional approach of compartmental analysis considers the rates of absorption, distribution and elimination as described by rate constants usually of the first order. Knowledge of the values of the rate constants and the equations of which they are part allows one to reproduce the observed plasma drug concentration-time curve and predict concentrations that would arise from differing dosage routes or schedules. The strength of compartmental analysis then is the ability to simulate plasma drug concentrations and to predict drug-concentration time profiles in altered physiological or pathological conditions and during chronic medication. Disadvantages of this approach are the mathematical complexity of the equations and the difficulty of obtaining reliable estimates of values of the rate constants.

In noncompartmental analysis the parameters characterizing the pharmacokinetics of a drug are very easily obtained; most of them can be derived from relationships involving the area under the plasma drug concentration-time curve (AUC) and the first moment of the plasma drug concentration-time curve (AUMC). The advantage of this approach is the ease of derivation of parameters using in most cases simple algebraic equations. Noncompartmental analysis of the data will be used in this thesis and the parameters AUC, AUMC, clearance, volume of distribution and mean residence time will be calculated as described below.

AUC is determined by dividing the observed drug concentration-time curve into trapezoids, and using the equation below to estimate the area of each trapezoid where C_1 and C_2 are the observed drug concentrations at times T_1 and T_2.

$$AUC_{1\text{-}2} = \frac{(C_1 + C_2)}{2} X (T_2 - T_1)$$

AUMC is determined by measuring the area under the product of time and concentration versus time plot (tC vs t) using the trapezoidal rule described above. Clearance (CL) is the fundamental measure of drug elimination and can be calculated by dividing the intravenous bolus dose by AUC. The basic measure of the extent of drug distribution is the apparent volume of distribution, Vd. For an intravenous dose this parameter is calculated by

$$Vd = \frac{\text{Dose x AUMC}}{AUC^2}$$

The mean residence time (MRT) is a measure of the length of time a drug

remains in the body, and is similar to the half life obtained by compartmental analysis. MRT for a drug administered by intravenous bolus injection is

$$MRT = \frac{AUMC}{AUC}$$

1.4.2. Pharmacokinetics of Anthracyclines

The earliest pharmacokinetics of DNR in humans were performed by Alberts *et al.* (1971). In that study DNR was given by IV infusion to 11 cancer patients and it was shown that the plasma concentrations of DNR followed biphasic kinetics with a short (or α phase) plasma half life of around 40 min and a long (or β phase) plasma half life of 55 hr. The apparent volume of distribution was determined to be about 1000L and the relative volume of distribution to be 580 L/m^2. The pharmacokinetics of DOX were first examined by Benjamin *et al.*(1973). They found the long plasma half life of DOX and its metabolites similar to that of DNR and its metabolites. They also found that the half life of the metabolites (31.7 hr) of DOX was longer than the half life of DOX (16.7 hr).

Since those two early pharmacokinetic studies only a few pharmacokinetic studies for anthracyclines have been reported. Benjamin *et al.* (1977) examined the plasma pharmacokinetics of DOX and a range of DOX metabolites in cancer patients with normal hepatic and renal function. Chan *et al.*(1980) showed no differences in the pharmacokinetic parameters of DOX in patients with hepatoma compared to non-hepatoma patients. However, impairment of both formation and elimination of the metabolite, doxorubicinol (see section 1.4.3)was observed in the hepatoma patients. Speth and colleagues have examined the pharmacokinetics of DOX in myeloma patients (Speth *et al.*, 1987a), DNR (Speth *et al.*, 1987b) and IDA (Speth *et al.*, 1986). Other pharmacokinetic studies performed include a study on EPI (Tjuljandin *et al.*, 1990) and IDA (Gillies *et al.*, 1987). Two studies have examined the pharmacokinetics of the anthracycline, iodo-doxorubicin (Mross *et al.*, 1990) and the chronopharmacokinetics of DOX in patients with breast cancer (Canal *et al.*, 1991).

Three studies have examined the pharmacokinetics of DNR in both plasma and leukaemic cells from patients with ANLL (Paul *et al.*, 1989; Kokenberg *et al.*, 1988; Speth *et al.*, 1987b). The study by Speth *et al.* (1987b) examined the duration of DNR infusion on leukaemic cell drug concentrations. The areas under the cellular concentration-time curve were similar and independent of the duration of the DNR infusion. DNR concentrations in nucleated blood and bone marrow cells correlated well. Paul *et al.*(1989) examined the

pharmacokinetics of DNR and DOX in plasma and leukaemic cells from patients with acute leukaemia and found that both clearance and volume of distribution decreased when the drugs were administered as DNA-conjugates. They showed that DNR reached higher intracellular peak concentrations than DOX, but DOX was retained by the cells much longer. This more pronounced intracellular retention of DOX could explain the broader activity spectrum of DOX compared with DNR; because solid tumour cells grow more slowly than leukaemic cells DOX can be retained longer in the solid tumours (Paul *et al.*, 1989). Kokenberg *et al.*(1988) published a study examining the cellular kinetics of DNR and the relationship with the response to treatment in patients with acute myeloid leukaemia. They found (1) plasma DNR concentrations did not correlate with DNR concentrations in bone marrow nucleated cells; (2) plasma AUC values of DNR correlated inversely with AUC values of DNR in white blood cells; (3) concentrations of DNR in white blood cells correlated positively with DNR concentrations in bone marrow nucleated cells; and (4) the concentrations of DNR in white blood cells showed a negative correlation with the number of peripheral blast cells at diagnosis. Kokenberg *et al.*(1988) tested whether the pharmacokinetic parameters had predictive value for the clinical outcome of therapy, but none of the plasma levels or white blood cell and bone marrow concentrations of DNR predicted treatment outcome.

1.4.3. Metabolism of Anthracyclines

Anthracycline metabolism has been investigated in both animal models as well as humans (Loveless *et al.*, 1978). In leukaemic patients who received a single IV dose of DNR, the plasma levels of the metabolite daunorubicinol (DOL) exceeded the parent drug within one hour (Huffman *et al.*, 1971). This rapid and specific biotransformation was explained by the presence of DNR carbonyl reductase (an enzyme belonging to a group of enzymes known as either aldo/keto reductases or carbonyl reductases). The transformation involves the conversion of the carbonyl group at C13 (Figure 1.1) to a hydroxy group. DNR reductase was present in nearly all tissues of the rat with highest activity in the small intestine, liver and kidney (Bachur & Gee, 1971). DNR has also been metabolised to the DNR aglycone, and DOL to its aglycone in rats (Bachur & Gee, 1971) but, these metabolites have not been detected in human plasma.

Metabolism of the other anthracyclines is similar to that of DNR. DOX is metabolised to doxorubicinol, and a number of aglycones (Benjamin *et al.*, 1977). In contrast to DNR however, aglycone metabolites of DOX have been detected in human plasma (Benjamin *et al.*, 1977). IDA is metabolised to idarubicinol, and no aglycones have been detected in human samples (Speth *et al.*, 1986; Gillies *et al.*, 1987). Metabolism of EPI

is slightly different to that of the other anthracyclines in that as well as the formation of epirubicinol and the aglycones that are detectable in human plasma, EPI also undergoes glucuronidation (Maessen *et al.*, 1987). The glucuronides being the major metabolites of EPI in man.

Successful chemotherapy remains largely a matter of individual patient response and variations in metabolism, therefore, will have a profound influence on the ultimate outcome of DNR treatment. A study by Huffman & Bachur (1972) found the response of patients with AML to DNR was correlated with the level of DNR reductase in peripheral myeloblasts. The levels of DNR reductase in responding patients were significantly higher than in non-responding patients or those who died.

1.5. Aims of this Thesis

The aims of this thesis are to examine the relationships between the plasma and cellular pharmacokinetics of DNR and its major metabolite DOL, with both patient response and the role played by Pgp, in patients with acute leukaemia. The actions of Pgp have been well defined *in vitro* in cell lines, in that Pgp presence leads to a decrease in the intracellular concentration of DNR in drug resistant cells. Therefore, the first hypothesis to be tested is that Pgp is responsible for a decrease in intracellular drug concentrations in patients with acute leukaemia, and that this decrease in intracellular DNR concentration is responsible for the patients' poor response to chemotherapy.

Several agents have been shown recently to reverse drug resistance *in vitro*, predominately in cell lines with a few studies using human leukaemic cells. However, nearly all the studies performed to date use either high concentrations of DNR, high concentrations of the MDR reversing agents, or both. Therefore the actions of these MDR reversing agents will be examined firstly in four acute leukaemic cell lines (CEM, VLB, HL 60 and ADR) at clinically achievable drug concentrations of DNR and the MDR reversing agents Cy A and Tri, and then in the blast cells of patients with acute leukaemia. This will test a second hypothesis that MDR reversing agents can increase DNR accumulation and retention in Pgp positive patient cells.

Pgp is associated with drug resistance in some cell lines. Is this drug resistance associated with the ability of Pgp to act as an efflux pump, or is it related to the presence of Pgp in the cell membrane? DOX has previously being shown to have cytotoxic actions

without entering the cell (Chapter 1.1.3), indicating that intracellular drug is not required for cytotoxicity. To test this third hypothesis, DNR will be immobilised by covalently coupling the amine (at the 3'C) of DNR to activated CL-PVA, so that DNR is unable to enter the cell and its cytotoxic actions on drug sensitive and Pgp positive drug resistant cell lines will be examined. The MDR reversing agents have been shown to reverse drug resistance in Pgp positive cells. Therefore, in an attempt to increase our understanding of the actions of Pgp, the effect of the MDR reversing agents on the cytotoxicity of immobilised DNR will also be examined.

CHAPTER 2

Materials and Methods

2.1. Materials

2.1.1. Cell Culture

RPMI 1640 Medium (RPMI), Penicillin/Streptomycin (5000 IU/ml, 5 mg/ml), Sodium bicarbonate ($NaHCO_3$) and Phosphate Buffered Saline (PBS) tablets were obtained from ICN Biomedicals, Costa Mesa, CA, U.S.A. Foetal Calf Serum (FCS) and Glutamine (200 mM) were obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. FCS was heat inactivated at 56 °C for 30 minutes before being used.

Trypan blue and MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were purchased from Sigma Chemical Company, St. Louis, Mo, U.S.A. Dimethyl sulphoxide (DMSO) was obtained from Ajax Chemicals, Auburn, N.S.W., Australia.

Daunorubicin hydrochloride was obtained from David Bull Laboratories Pty. Ltd., Mulgrave, Victoria, Australia. Cyclosporin (Cy A) was obtained from Sandoz Australia Pty. Ltd., North Ryde, N.S.W., Australia, and trifluoperazine (Tri) was obtained from Smith Kline and French Laboratories (Aust.) Ltd., Sydney, N.S.W., Australia.

Iscoves medium was from Gibco Laboratories, Life Technology Inc., Grand Island, New York, U.S.A. L-Asparagine from Sigma Chemical Company, St. Louis, Mo, U.S.A., DEAE-Dextran was obtained from Pharmacia LKB Biotechnology AB, Uppsala, Sweden, 2-mercaptoethanol, BDH Chemicals Ltd., Poole, England. Agar from Difco Laboratories, Detroit, Michigan, U.S.A.

Acid citrate dextrose A (ACDA) was obtained from Baxter Healthcare Pty. Ltd., Old Toongabbie, N.S.W., Australia, Ficoll-Paque from Pharmacia LKB Biotechnology AB, Uppsala, Sweden, water was Milli Q grade (Millipore, Lane Cove, N.S.W., Australia).

2.1.2. HPLC Assay

Daunorubicin hydrochloride (DNR) and daunorubicinol hydrochloride (DOL) HPLC standards were obtained from Rhone-Poulenc, Centre De Resherches, De Vitry, France. Adriamycin (doxorubicin hydrochloride) was purchased from Farmatalia Carlo Erba, Hawthorn, Victoria, Australia. Concentrations of DNR are expressed as ng/ml of the hydrochloride (527.5 g daunorubicin base = 563.5 g daunorubicin hydrochloride = 1 mole of daunorubicin). Acetonitrile, methanol, dichloromethane and isopropanol were all HPLC grade solvents and obtained from Ajax Chemicals, Auburn, N.S.W., Australia. Glass double-distilled water, prefiltered through a 0.45 μm filter, was used in the preparation of HPLC mobile phases.

The aglycone from daunorubicin (daunomycinone) was prepared by acid hydrolysis of daunorubicin (i.e. daunorubicin was dissolved in 3M HCl in ethanol to a concentration of 25 μg/ml and incubated at room temperature for 48 hrs). The daunomycinone was then kept at 4° C until required for use.

Normal human frozen fresh plasma (drug free) was obtained from the blood transfusion service at Royal North Shore Hospital, St. Leonards, N.S.W., Australia.

2.1.3. P glycoprotein assay

C 219 was purchased from Centecor Corporation, Malvern, PA, U.S.A. and JSB 1 was from Boehringer Mannheim GmbH Biochemica, Mannheim, Germany. Human Serum Group AB was obtained from Commonwealth Serum Laboratories, Melbourne,

Australia and monoclonal mouse anti-human cytokeratin (non-specific mouse IgG1) was obtained from Dakopatts a/s, Glostrup, Denmark.

2.1.4. Immobilisation

Cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) was purchased from Sigma Chemical Company, St. Louis, Mo, U.S.A. Terephthalaldehyde (terephthaldicarboxaldehyde) was obtained from Aldrich Chemical Company Inc., Milwaukee, Wis, U.S.A. and polyvinyl alcohol 22000 (PVA) was obtained from Fluka, Buchs, Switzerland.

All the remaining reagents were AR grade.

2.1.5. Cell Lines

Throughout this project four leukaemia cell lines were used. These were CEM and VLB 100 (obtained from Dr D. Bell, Department of Clinical Oncology, Royal North Shore Hospital, Australia) and HL 60 and HL 60/ADR (obtained from Dr. M. Center, Division of Biology, Kansas State University, USA). The CEM cell line is a T-cell lymphoblastic leukaemia cell line isolated from a patient (Foley *et al*, 1965). VLB 100 is a drug resistant subline of CEM which has been grown in the presence of increasing concentrations of vinblastine up to 100 ng/ml (Beck *et al*, 1979). HL 60 is a cell line derived from a patient with acute myeloid leukaemia (Gallagher *et al*, 1979), HL 60/ADR (this cell line will be abbreviated to ADR in the remainder of this thesis) is a drug resistant subline of HL 60 grown in the presence of doxorubicin (Marsh *et al*, 1986).

2.1.6. Patients

Peripheral blood or bone marrow was collected from patients (details of sample collection are given in a separate section of the methods 2.2.6) with either Acute Myeloid Leukaemia (AML) or Acute Lymphoblastic Leukaemia (ALL). Details of patient characteristics are given in the thesis chapters which refer to these patients. Informed consent was obtained from the patients before samples were collected and ethics approval was obtained from the Royal North Shore Hospital Medical Ethics Committee. Patience diagnosed with acute leukaemia, that were to be treated with DNR and gave informed consent were considered eligible.

2.2. Methods

2.2.1. Buffers

RPMI media was prepared as follows: One packet of RPMI 1640 Medium was made up to one litre with the addition of 3.3 ml of $NaHCO_3$ (7.5% w/v) and Millipore Milli Q water. This was then filtered through a 0.2 μm filter (Millipore) before being used.

PBS was prepared by the dissolution of one PBS tablet in 100 ml of Millipore Milli Q water which was then autoclaved at 15psi, 110 °C for 15 minutes.

DSIM - double strength Iscoves medium was prepared as follows: One packet of Iscoves medium, 0.2g of L-asparagine, 4.68g of $NaHCO_3$, 20 ml of penicillin/streptomycin (5000 IU/ml, 5 mg/ml), 1.5ml of DEAE-Dextran (50μg/ml) 5.44 μl of 2-mercaptoethanol were added to 370ml of Millipore Milli Q water. The reagents were mixed and then filtered through a 0.2 μm filter (Millipore) before use.

Potassium phosphate buffer was prepared by adding 0.2M KH_2PO_4 solution to 500ml of 0.2M K_2HPO_4 until the pH was 7.4. This was then diluted 1:10 with water to give 0.02M potassium phosphate buffer.

Carbonate buffer was prepared by the addition of 0.2M Na_2CO_3 solution to 100ml of 0.2M $NaHCO_3$ until the pH was 8.9.

2.2.2. Culturing of Cells

All cell lines were cultured in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin (final concentrations of 50IU/ml/ 50μg/ml) and glutamine (final concentration, 2mM). The cell lines were grown as suspension cultures in an incubator (Hotpack, Selby Anax, Lidcome, N.S.W., Australia) at 37° C in an atmosphere of 95% air 5% CO_2 and 99% relative humidity. The cells were subcultured every 2 or 3 days. Cell lines were tested every six months for mycoplasma (Department of Microbiology, Westmead Hospital, Sydney) and only mycoplasma free cells were used.

2.2.3. Cell Viability

Cell viability of all cells was determined prior to their use by the trypan blue exclusion method. Cells suspended in media were added to an equal volume of 0.2% (w/v) trypan blue in PBS, and loaded onto a Neubauer haemocytometer chamber for counting. Viability was calculated as follows:-

$$\% \text{ viability} = \frac{\text{No. of viable cells (cells which did not stain blue)}}{\text{Total No. of cells}}$$

Cells with a viability greater than 90% were used in all experiments.

2.2.4. MTT assay

A modified method of that used by Mosmann (1983) was used. Cells were taken from culture and washed once with RPMI. The cells were then resuspended at a concentration of 5 x 10^5 cells/ml in RPMI + 10 % FCS. To a 96 well microtitre plate (Linbro) was added 100µl of RPMI + 10 % FCS containing DNR. The concentration of DNR ranged from 2 ng/ml to 20 µg/ml (Table 2.1). The first and last columns of the microtitre plate were not used, due to high evaporation rates from these wells. The second column of wells contained 200µl of RPMI + 10 % FCS (blank), the third column contained 100µl of RPMI + 10 % FCS and 100µl of cells (this was the control column) and the remaining columns contained the varying concentration of DNR (Table 2.1) and 100µl of cells. When the modifiers Cy A, Tri and the combination of Cy A + Tri were used, column four contained 100µl of cells and 100µl of the modifiers. The final concentration of the modifiers was Cy A 1.5 µg/ml and Tri 150 ng/ml and the concentrations of DNR added are given in Table 2.2. The final concentration of cells in each well was 2.5 x 10^5 cells/ml. The final concentrations of DNR ranged from 1 ng/ml to 10µg/ml (Table 2.1, 2.2). The final volume in the wells was 200 µl. The microtitre plate was then incubated at 37° C for 72 hrs. After the 72 hr incubation, 20µl of MTT was added to each well and the plate was incubated for a further 4 hrs at 37° C. MTT was dissolved in PBS at a concentration of 5mg/ml and then filtered through a 0.2µm filter (Advantec). The microtitre plate was then centrifuged at 700 x g for 5 min. The supernatant was taken off using a well washer (Denley, Well Wash 2) and the crystals formed were resuspended in 100µl of DMSO. The optical density (OD) was then read at 540 nm using a microtitre plate reader (BioRad Model 3550).

Table 2.1. Concentrations of DNR in the MTT assays

Column	Concentration of DNR added (ng/ml)	Final DNR concentration (ng/ml)
1	-	-
2	RPMI alone	0
3	Cells alone	0
4	2	1
5	20	10
6	100	50
7	200	100
8	1000	500
9	2000	1000
10	10000	5000
11	20000	10000
12	-	-

Table 2.2. Concentrations of DNR in the MTT assays with modifiers

Column	Concentration of DNR added (ng/ml)	Final DNR concentration (ng/ml)
1	-	-
2	RPMI alone	0
3	Cells alone	0
4	Cells + modifier	0
5	2	1
6	20	10
7	100	50
8	200	100
9	1000	500
10	2000	1000
11	10000	5000
12	-	-

The same procedure as above was followed for the cytotoxicity of DOL, using the same drug concentrations as those described above.

The results were calculated as percentage viability of the control cell line without the addition of drug as follows

$$\% \text{ viability} = \frac{\text{mean OD}_{\text{sample}}}{\text{mean OD}_{\text{control}}} \times 100$$

The concentration of DNR which inhibited cell growth by 50% (IC 50) was calculated using the following non linear regression equation

$$\% \text{ viability} = \frac{E_{max} \times C^N}{IC_{50}^N + C^N}$$

were E max is the maximum response, C is the drug concentration (ng/ml) and N is the Hill coefficient. The equation was solved using the MK Model (Biosoft, Cambridge, U.K.) program on an IBM PC computer.

2.2.5. Clonogenic Assay

Cells were made to a concentration of 5×10^4 cells/ml in DSIM/PBS (1:1 v/v) for the HL 60, ADR and VLB cell lines. A cell concentration of 1×10^5 cells/ml of the CEM cell line was used. To each tube was added 1.35ml of DSIM/FCS (1:1), 0.3 ml of cells and 1.35 ml of 0.66% (w/v) agar. The cells were mixed and then 0.5ml added to each of 4 wells in a 4 well plate (Nunc). The agar was allowed to set and then placed in an incubator at 37° C. The number of colonies formed after 10 days was counted using an inverted microscope (Nikon). A colony was the cluster of 50 or more cells together for the HL 60, ADR and VLB cell lines and 20 or more cells for the CEM cell line. Preliminary observations of up to 10 days for CEM cells found cells formed colonies of 20 cells. The plating efficiency was determined by dividing the number of colonies formed by the concentration of cells added to the wells.

2.2.6. Collection of blood and sample preparations for pharmacokinetics

Peripheral blood samples from AML and ALL patients (for patient details and treatment protocols see section 4.2) were collected through a central venous catheter, into glass tubes containing ACDA (acid citrate dextrose A). Each 10ml blood sample collected was immediately placed on ice. Samples were taken before DNR infusion then at 15min, 30min, 1hr, 1.5hr, 2hr, 4hr, 6hr, 8hr, 10hr, 12hr and 24 hrs post-infusion and then daily for seven days. Blood samples were centrifuged at 500 x g for 5 min, the plasma removed and stored at -80° C. The red cells were then removed by the addition of hypotonic lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, 100μM EDTA). The remaining white cells were immediately washed twice with cold PBS and then resuspended in 1.3 ml PBS of which 0.3ml was counted electronically using a Coulter STKS (Coulter Corporation, Florida, U.S.A.) and the remainder stored at -80° C.

2.2.7. Accumulation/ Retention Experiments

2.2.7.1. Isolation of Cells

(1) Patients' cells were extracted from either a peripheral blood sample or a bone marrow sample (see Chapter 5). Each sample was diluted 1:1 with PBS and then layered onto Ficoll-Paque. The sample was then centrifuged at 400xg for 20min and the cells remaining at the interface were removed. The cells were then washed with lysis buffer to remove any remaining red blood cells. The cells were then washed a further two times with PBS and then resuspended in RPMI + 10 % FCS.

(2) Cell lines were washed once with RPMI and then resuspended in RPMI + 10 % FCS.

2.2.7.2. Accumulation

Four tubes were set up each containing 19 ml of RPMI + 10 % FCS, to one tube was added 8 μl of DNR (500 μg/ml) to give a final concentration of 200 ng/ml of DNR. The second tube contained a final concentration of 200 ng/ml DNR + 1.5μg/ml Cy A (6 μl of 5 mg/ml), the third contained 200 ng/ml of DNR + 150 ng/ml of Tri (3 μl of 1 mg/ml) and the fourth contained 200 ng/ml DNR + 1.5μg/ml Cy A + 150 ng/ml of Tri. One ml (≈ 20 x

10^6) of cells was then added to each of the tubes which were then incubated at 37° C. Immediately, and then after 30 min, 1 hr, 2 hr, 3 hr and 4 hr a sample (3.3 ml) was removed from each of the tubes. The samples were centrifuged at 500 x g for 5 min and washed twice with cold PBS. The cells were then resuspended in 1.3 ml of PBS. One ml was used to assay the amount of DNR present in the cells as described below (2.2.8.4.) and the remainder was used to obtain a cell count, using a Coulter STKS. Accumulation was expressed as the AUC (0-4hr) of DNR, obtained from a plot of DNR concentration versus time.

Accumulation of DOL, was performed exactly as described above, except DNR was replaced by DOL.

2.2.7.3. Retention

Another four tubes identical to those in the accumulation study were set up. These tubes were incubated for 2 hrs at 37° C. After incubation the tubes were centrifuged at 500 x g for 5 min and washed twice with 20 ml cold PBS. The cells were then resuspended in 12 ml fresh (drug free) RPMI + 10 % FCS. The cells were then incubated at 37° C and samples removed at the following time points, immediately, 30 min, 1 hr, 2 hr, 3 hr and 4 hr after the incubation. The removed cells were centrifuged at 500 x g for 5 min and resuspended in 1.3 ml of PBS. One ml was used to assay the amount of DNR present in the cells as described below and the remainder was used to obtain a cell count, using a Coulter STKS. Retention was expressed as the AUC (0-4hr) of DNR, obtained from a plot of DNR concentration versus time.

Retention of DOL, was performed exactly as described above, except DNR was replaced by DOL.

2.2.7.4. Retention with the addition of MDR reversing agents.

Four tubes identical to those in the accumulation study were set up. These tubes were incubated for 2 hrs at 37° C. After the incubation the tubes were centrifuged at 500 x g for 5 min and washed twice with 20 ml of cold PBS. The cells were then resuspended as follows: (1) the DNR was resuspended in 12 ml of fresh RPMI (drug free) + 10 % FCS; (2) the DNR + Cy A was resuspended in 12 ml of fresh RPMI + 10 % FCS + Cy A (3.6 µl of 5 mg/ml); (3) the DNR + Tri was resuspended in 12 ml of fresh RPMI + 10 % FCS + Tri (1.8 µl of 1 mg/ml); (4) the DNR + Cy A + Tri was resuspended in 12 ml of fresh RPMI +

10 % FCS + Cy A (3.6 µl of 5 mg/ml) + Tri (1.8 µl of 1 mg/ml). The remainder of the procedure was as described above (2.2.7.3.).

2.2.8. HPLC assay

2.2.8.1. Chromatographic Conditions

Analysis of DNR and DOL was performed using a system comprising a Waters Model 6000 pump, an automated injector (Wisp Model 710B, Waters Associates), a reversed phase C-18 column (Waters Novapak 3.9 x 150 mm, 4µm) and a Hitachi 5100 or Shimadzu F500 fluorescence spectrophotometer for detection (excitation of 480 nm and emission of 560 nm). The detector was connected to either a Servogor 120 or an Omniscribe chart recorder. The mobile phase consisted (by volume) of 27% acetonitrile and 73% potassium dihydrogen phosphate (80mM). The mobile phase (pH 5.03) was pumped at a flow rate of 1 ml/min and the chromatography was performed at ambient temperature.

2.2.8.2. Standard Solutions for Assay Calibration

Stock solutions of DNR and DOL in methanol containing 5 mg/ 100 ml were prepared and stored at 4° C. A stock solution of the internal standard DOX was prepared in water at a concentration of 2 mg/ 100ml. Working solutions of DNR, DOL and DOX were prepared at a concentration of 1 µg/ ml in 0.1M HCl and these solutions were used to prepare the calibration curve.

2.2.8.3. Plasma Samples

To 1ml of fresh frozen plasma was added 50µl of KOH, and 50µl of DOX (1µg/ ml) as an internal standard. The plasma was extracted with 10ml of dichloromethane: isopropanol (9:1) by vortex mixing for 1 min. The samples were then centrifuged at 1600 x g for 5 min and the aqueous phase was removed. The organic phase was transferred to a clean glass tube and evaporated to dryness under reduced pressure using a Savant Speed Vac (Savant Instruments Inc., U.S.A.). The dried extract was reconstituted in 150µl of mobile phase and 50µl injected onto the HPLC system. The chromatograms obtained from 3 time points from a single patient are shown in Figure 2.1. The plasma calibration curve ranged from 5-120 ng/ml for both DNR and DOL. The intraassay and interassay coefficients of variation for DNR at 25 ng/ml were 13% and 14%, and at 100 ng/ml were 6% and 14%, respectively. A 25 ng/ml and 100 ng/ml plasma samples were used as quality controls, an

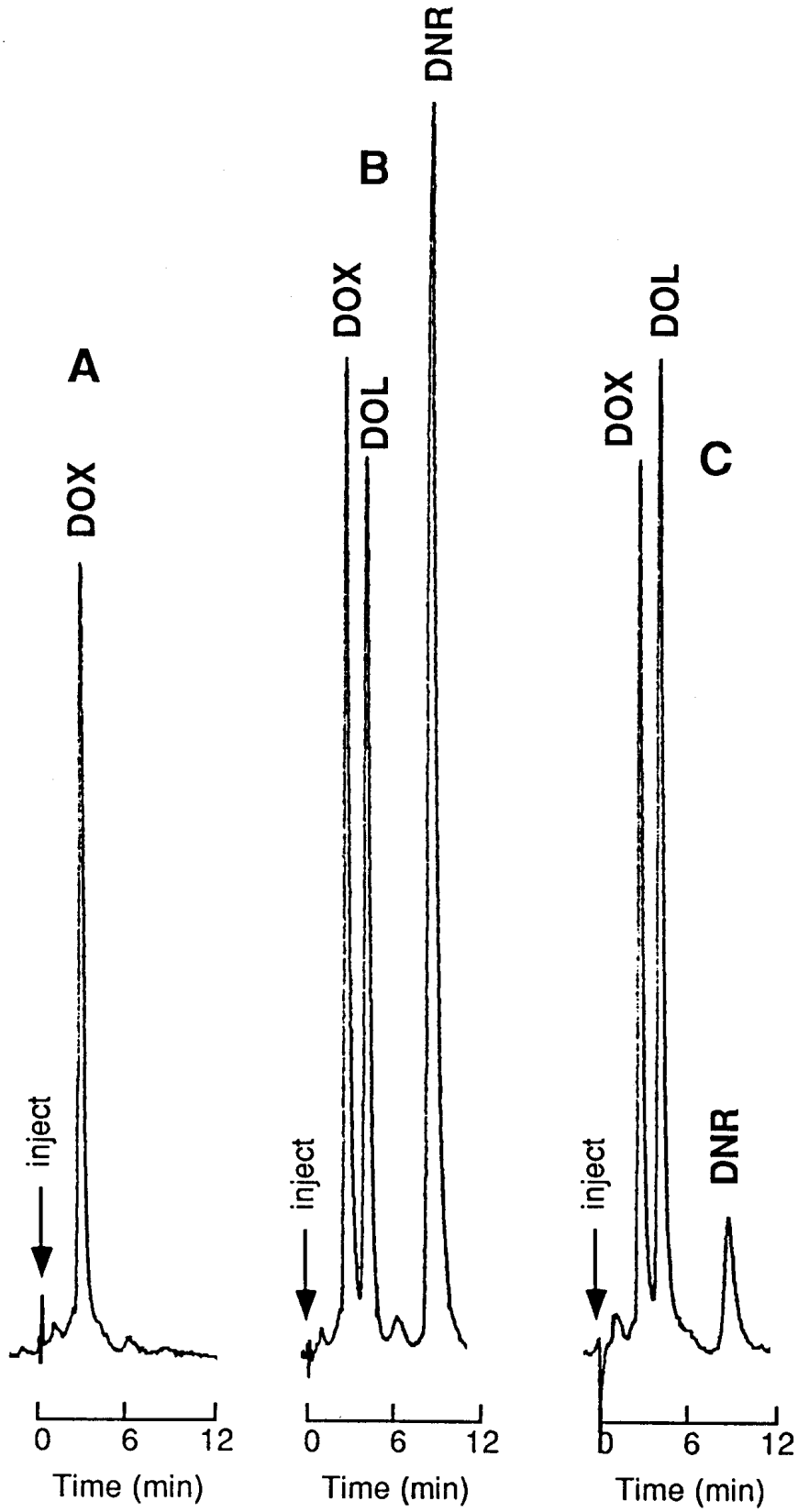


Figure 2.1. Chromatograms obtained from patient plasma samples: (A) a pre-infusion sample, in which only the internal standard DOX is present; (B) a sample taken 15 min after DNR infusion; (C) a sample taken 12 hr after DNR infusion.

acceptable run was a standard curve with a correlation coefficent > 0.9 and less than 15% variation in the quality control samples. The interassay variation at the 5 ng/ml standard was 17%. Samples that did not fit between the range of the standard curve were rerun or considered as below assay limits, therefore the limit of quantitation for this assay was 5 ng/ml.

2.2.8.4. Cellular Samples

Intracellular DNR and DOL were analysed by taking a known number of leukaemic cells (0.5-30 x 10^6 cells) in 1ml PBS. To this was added 100µl of 3M HCl in ethanol and the internal standard DOX (50ng, same as for plasma). The cells were subjected to sonication (Unisonics Pty. Ltd.) for 5 min and extracted as described above. Standard curves were prepared using cell concentrations of untreated leukaemic cells similar to those being assayed. The cellular calibration curve ranged from 5-200 ng DNR or DOL in one ml of PBS. The interassay coefficient of variation was 12%, and the intraassay coefficient of variation at 25 ng/ml was 3.1%, and at 150 ng/ml was 3.7%. A 25 ng/ml and 150 ng/ml cellular samples were used as quality controls, an acceptable run was a standard curve with a correlation coefficent > 0.9 and less than 15% variation in the quality control samples. The interassay variation at the 5 ng/ml standard was 12%. Samples that did not fit between the range of the standard curve were rerun or considered as below assay limits.

2.2.9. Calculation of Pharmacokinetic Parameters

The pharmacokinetic parameters Cmax (maximum drug concentration), Tmax (time at maximum drug concentration), area under the concentration-time curve (AUC), area under the first moments concentration-time curve (AUMC), mean residence time (MRT), plasma clearance (CL), and apparent volume of distribution (Vd) were calculated using the MK Model program (Biosoft, Cambridge, U.K.) on an IBM PC computer. To compare different doses of DNR the pharmacokinetic parameters were corrected for dose, by dividing the parameter by the dose of DNR given.

2.2.10. P glycoprotein assay

The Pgp assay performed for the experiments described in this thesis were carried out by Janet McLachlan at the Department of Haematology, Royal North Shore Hospital, Sydney. Pgp was detected by an immuno-alkaline phosphatase method, using the α-P-glycoprotein antibodys JSB1 and C219 as described by Gala *et al.* (1994). In brief,

cytospins of patients' cells were prepared from either blood or bone marrow samples. The cells were fixed in acetone:ethanol (1:1) for 90 sec, and the antibodies JSB 1 (13.3 μg/ml) or C219 (10 μg/ml) were applied and incubated overnight at 4° C. Normal human serum was used to block non specific binding. An inappropriate mouse IgG1 and the CEM cell line were used as negative controls and the drug resistant cell line VLB 100 as a positive control. After washing, the slides were incubated in rabbit anti-mouse Ig (30 min) followed by alkaline phosphatase-anti-alkaline phosphatase complex. The staining intensity was enhanced by a further 10 min incubation with each of the second and third layer antibodies. The colour reaction was realized by a 20 min incubation with substrate containing Naphthol AS-MX phosphate and fast red TR salt, endogenous alkaline phosphatase activity was blocked by the addition of levamisole. Slides were counterstained using Harris' haematoxylin and mounted in aqueous mounting medium. Slides were assessed by light microscopy, red colour product indicating a positive result. The results are reported as either positive, ≥ 5% of blast cells producing a red colour or negative, < 5% of blast cells producing a red colour.

2.2.11. Immobilisation of DNR

2.2.11.1. Preparation of immobilised DNR

DNR was immobilised as described by Wingard *et al.* (1985) and shown in Figure 2.2. Cross linked poly-vinyl alcohol (CL-PVA) was prepared by the reaction of terephthalaldehyde with poly-vinyl alcohol (PVA). PVA (22g) was dissolved in 1L of water (warm, ≈ 75° C). To it was then added 3.35g of terephthalaldehyde and 10ml of 32% (w/w) HCl and the mixture stirred for 72hrs. The CL-PVA was filtered and washed with distilled water (≈ 3L) and then methanol (≈ 3L). The CL-PVA was dried in a dessicator under reduced pressure and then ground and sieved. The fraction of particle size between 88 and 250 μm was used for the attachment of DNR or for the control with cultured cells.

The CL-PVA (3.5g) was suspended and stirred in 50ml of 10% (w/v) NaOH. The CL-PVA suspension was heated slowly to 80-90° C (1.5 hr required to reach this temperature), then cooled to room temperature (1hr required to cool), and drained of excess liquid. A solution of 10g of cyanuric chloride in 93g of acetone (118 ml) was added to the CL-PVA. After 10 min, the coupling was stopped by the addition of 40ml of 20% (v/v) acetic acid. The activated support was washed with acetone (≈ 500 ml) to remove unattached cyanuric chloride. Four ml of 5mg/ml DNR (in normal saline) was added to 25ml 0.2M sodium bicarbonate-carbonate buffer (pH 8.9). The DNR solution was then added to 2.7g (wet weight) of activated CL-PVA. The solution was stirred gently for 4hrs at room

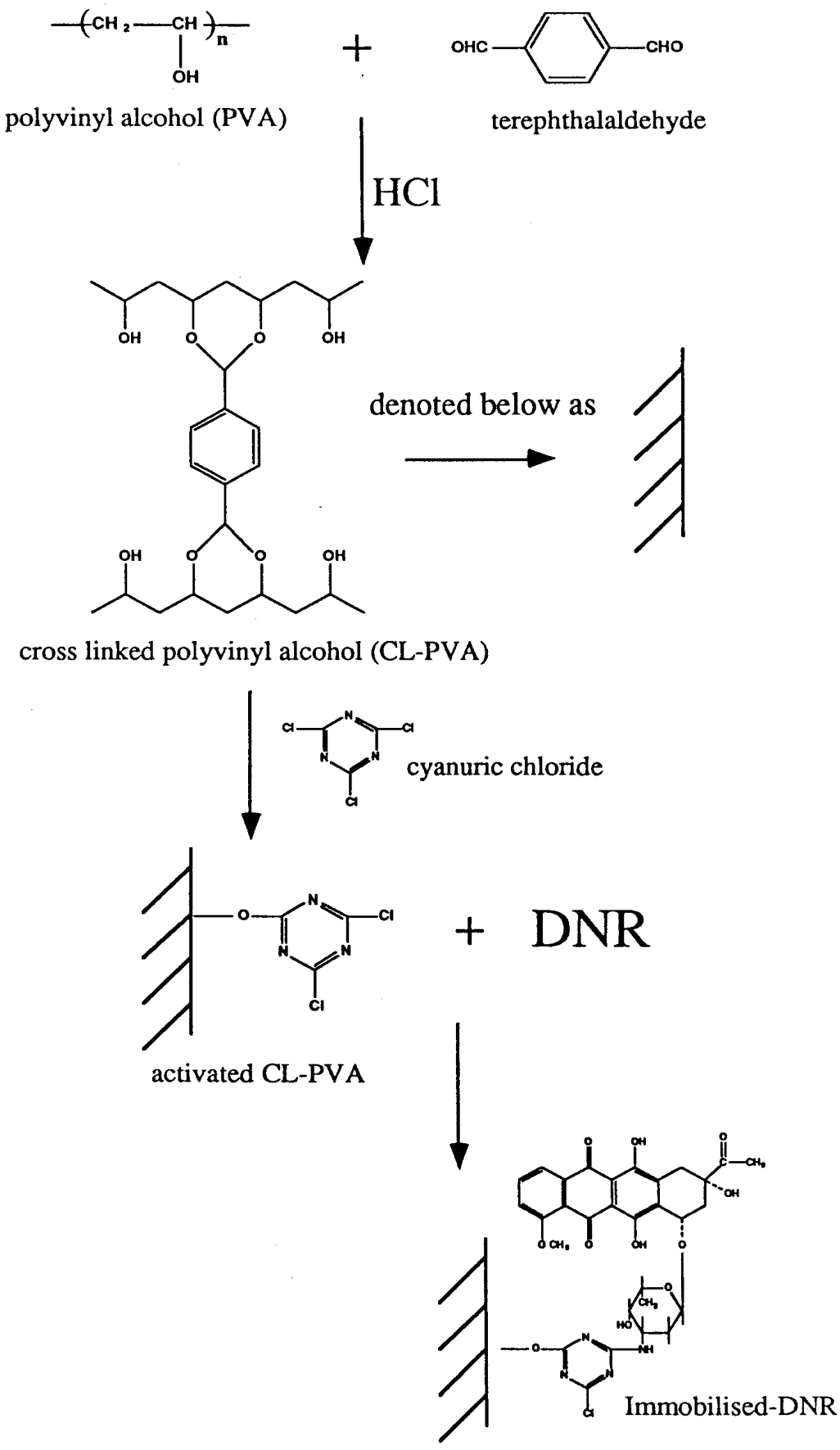


Figure 2.2. Preparation of Immobilised-DNR

temperature using a magnetic stirrer. The immobilised DNR was then extensively washed to remove unattached DNR (see below).

2.2.11.2. Washing of immobilised DNR

The immobilised DNR was extensively washed until the amount of DNR being released, was insufficient to have cytotoxic effects on cell lines by the MTT assay. The washing procedure was as follows:-

The immobilised-DNR was first placed on filter paper (Whatman No. 1) in a buchner funnel and washed with 200 ml methanol. The immobilised-DNR was then air dried and, using a spatula, placed in a 1.5 cm x 7.5 cm plastic column (PD 10 column, Pharmacia, with the sephadex removed). The immobilised-DNR was then washed firstly with another 200 ml methanol followed by 200 ml acetonitrile, 500 ml 0.02M potassium phosphate buffer, 2.5 L 3M NaCl, 1 L double distilled water and then another 200 ml methanol.

The immobilised-DNR was air dried in the column and then removed by spatula to a plastic tube. The immobilised-DNR was washed twice with 20 ml RPMI at 37°C and then returned back to the column where it was washed with another 150 ml RPMI and 1.3 L methanol. The immobilised-DNR was then washed again, using batch washing with 200 ml methanol. A total of 3 L methanol was used with the batch washing. The immobilised DNR was air dried and used for the experiments described below.

The release of free DNR or DNR aglycone from immobilised-DNR was measured firstly by UV spectrophotometry monitoring at 485 nm, (Shimadzu Model UV-240 spectrophotometer) and, when there was no detectable DNR by UV spectrophotometry the HPLC method described above (2.2.8.1) was used to detect free DNR or DNR aglycone.

2.2.11.3. Amount of DNR bound to PVA

To determine the amount of DNR that was bound as immobilised-DNR, 91.1 mg dried immobilised-DNR was incubated with 3M HCl (6 ml) in ethanol and allowed to stand at room temperature for 48 hr. The mixture was then centrifuged and the supernatant was placed in a 50 ml volumetric flask. The beads were then washed twice with mobile phase (27:73 acetonitrile-80 mM potassium dihydrogen phosphate) and the washings were added to the volumetric flask. The volumetric flask was made up to volume with mobile

phase and the amount of DNR aglycone (daunomycinone) was measured using HPLC, with the same conditions as described in section 2.2.8.1. The retention time of the DNR aglycone was 18 min.

2.2.11.4. Sterilization of immobilised-DNR

The following procedure was performed entirely in a laminar flow hood. Immobilised-DNR was sterilized by taking a known amount (≈ 10 mg or ≈ 100 mg) of immobilised-DNR and adding 5 ml of 70% ethanol. The mixture was then placed on a laboratory suspension mixer (Clements) for 4 hr. After the 4 hr the immobilised-DNR was then washed with 5 ml of sterile PBS and then 5 ml of sterile RPMI.

2.2.11.5. Experiments using immobilised DNR

An amount of immobilised DNR was added to RPMI (≈ 10 ml) to give a final concentration of either 2 μg/ml DNR or 20 μg/ml DNR. The cell lines CEM, VLB, HL 60 or ADR were added at a concentration of 5×10^5 cells/ml. The combination of experiments included the cells alone, the addition of CL-PVA alone, 1.5 μg/ml Cy A, 150 ng/ml of Tri, immobilised-DNR, immobilised-DNR plus Cy A and immobilised-DNR plus Tri. The cells were then placed in a laboratory suspension mixer (Clements) and incubated for 72 hrs at 37° C. After incubation the cells and immobilised-DNR were centrifuged and the supernatant removed (assayed for free DNR). The cells and immobilised DNR were layered onto a Ficoll gradient and separated from each other. The cells were washed with PBS and then a clonogenic assay was performed as described above (2.2.5). The recovered immobilised-DNR was washed with distilled water and methanol and then reused.

2.2.12. Statistics

All data are presented as mean ± standard deviation unless stated otherwise. The statistical significance of the data was determined by the appropriate test using the Abacus Concepts, Statview program (Abacus Concepts, Inc., Berkeley, CA, U.S.A.) on a Macintosh computer. $P < 0.05$ was considered statistically significant for all tests.

The statistical tests used in this thesis are as follows: (1) the Mann-Whitney U test (MW), when comparing two groups that are independent and ordinal; (2) the Wilcoxon signed rank test (W), when comparing two groups that are related and ordinal; (3) the Kruskal-Wallis one-way analysis of variance (KW), when comparing multiple groups that

are independent and ordinal; (4) the Friedman two-way analysis of variance (Fr), when comparing multiple groups that are related and ordinal; (5) the Fisher exact test (FE), when comparing two independent groups that are categorical and (6) the Spearman rank correlation (S), when comparing measures of association that are ordinal. For a more detailed description of these statistical methods the reader is referred to "Nonparametric Statistics for the Behavioural Sciences" by Siegel & Castellan (1988). Where p values are given in the thesis the number is followed by the abbreviation for the test used. For example ($p < 0.05$, MW) means that the result is significant as determined by the Mann-Whitney U test.

CHAPTER 3

Actions of DNR and MDR Reversing Agents on Leukaemic Cell lines

3.1. Introduction

DNR is known to have cytotoxic actions on a variety of tumours including leukaemia, lymphoma and several other soft tissue tumours (Young *et al.*, 1984). In this chapter the cytotoxic action of DNR on four different acute leukaemic cell lines is examined. The cell lines include: CEM, a T-cell lymphoblastic cell line; VLB, a drug resistant subline of CEM grown in the presence of vinblastine; HL 60, a myeloid cell line and ADR, a drug resistant subline of HL 60 grown in the presence of DOX.

High concentrations of MDR reversing agents have been shown to increase the cytotoxicity of DNR in drug resistant cell lines (Ganapathi *et al.*, 1984; Tsuruo *et al.*, 1983). These high doses of MDR reversing agents are toxic to patients. Therefore, here, the effects on DNR cytotoxicity of the MDR reversing agents Cy A and Tri were examined at concentrations of 1.5 μg/ml and 150 ng/ml respectively, which are clinically achievable doses of these drugs (Kaye, 1990).

As well as examining the cytotoxicity of DNR, the accumulation and retention of DNR at a clinically achievable concentration (200 ng/ml) was also studied. Previous studies have used high concentrations (10 μg/ml, Dano, 1973 or 2 μg/ml, Marsh *et al.*, 1986) of DNR when examining the accumulation and retention of DNR. These high concentrations make it difficult to extrapolate findings to clinical settings. The accumulation and retention of DNR was studied in the four leukaemic cell lines. This chapter also contains results of studies into the actions of Cy A and Tri at concentrations of 1.5 μg/ml and 150 ng/ml respectively on DNR accumulation and retention in an attempt to explain the actions of the MDR reversing agents *in vivo*.

3.2. Cytotoxicity of DNR on Cell lines

The cytotoxic effect of DNR on the cell lines CEM and VLB is shown in Figure 3.1. The VLB cell line requires a significantly ($p < 0.01$, MW) higher concentration of DNR to achieve an equivalent cell kill. The concentration of DNR that leads to 50% cytotoxicity is defined as the IC 50. It is this criteria that will give a comparison of resistance between cell lines. The IC 50 for VLB is 571 ± 371 ng/ml compared to the CEM line that has an IC 50 of 49 ± 11 ng/ml. Therefore by this comparison the VLB cell line was approximately 12 times more resistant to DNR than the CEM cell line (Table 3.1).

The effect of DNR on the cell lines HL 60 and ADR is shown in Figure 3.2. Significantly ($p < 0.0001$, MW) higher concentrations of DNR are required to kill the ADR cells as compared to the HL 60 cell line. The HL60 cell line is a drug sensitive cell line with an IC 50 of 35 ± 11 ng/ml, compared to the drug resistant ADR with an IC 50 of 975 ± 743 ng/ml (Table 3.2). Thus ADR was approximately 28 times more resistant than the sensitive HL 60.

3.3. Effects of MDR reversing agents on DNR cytotoxicity

Cell lines were incubated with DNR alone, or in combination with Cy A, Tri or both. To determine the resistance of each cell line and combination of treatment the parent cell line was assigned a relative resistance of one. The effect of the MDR reversing agents on the four cell lines is given in Tables 3.1 and 3.2. CyA was able to reverse the resistance to DNR in both VLB (Figure 3.3) and ADR (Figure 3.4). Cy A was more effective in reversing resistance in the ADR cell line, in which the IC 50 was decreased 39 fold to that

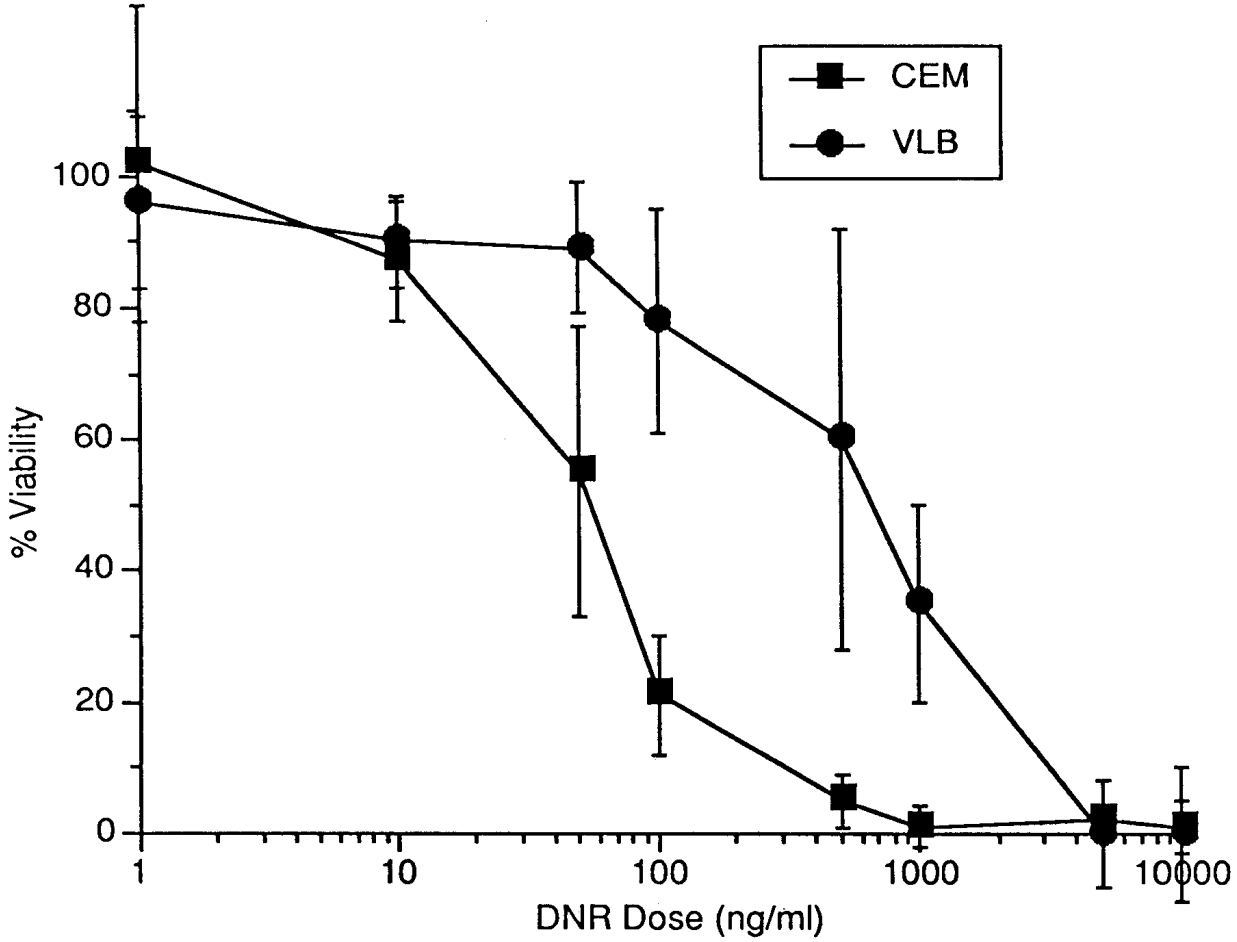


Figure 3.1. Dose response curve for the cytotoxic actions of DNR on the drug sensitive CEM cell line and drug resistant Pgp positive VLB cell line. Each point is shown as the mean ± SD (n=6).

Table 3.1. IC 50 values for DNR with or without the MDR reversing agents in the CEM and VLB cell lines, expressed as mean ± SD (n)

Cell Line	IC 50 (ng/ml)	Relative Resistance*
CEM	49 ± 11 (6)	1
CEM + Cy-A	34 ± 13 (5)	0.7
CEM + Tri	110 ± 74 (5)	2
CEM + Cy A + Tri	44 ± 2 (5)	0.9
VLB	571 ± 371 (5)	12
VLB + Cy-A	245 ± 113 (5)	5
VLB + Tri	945 ± 494 (5)	19
VLB + Cy A + Tri	130 ± 47 (5)	3

* relative resistance is the IC 50 divided by the IC 50 of the parent CEM cell line

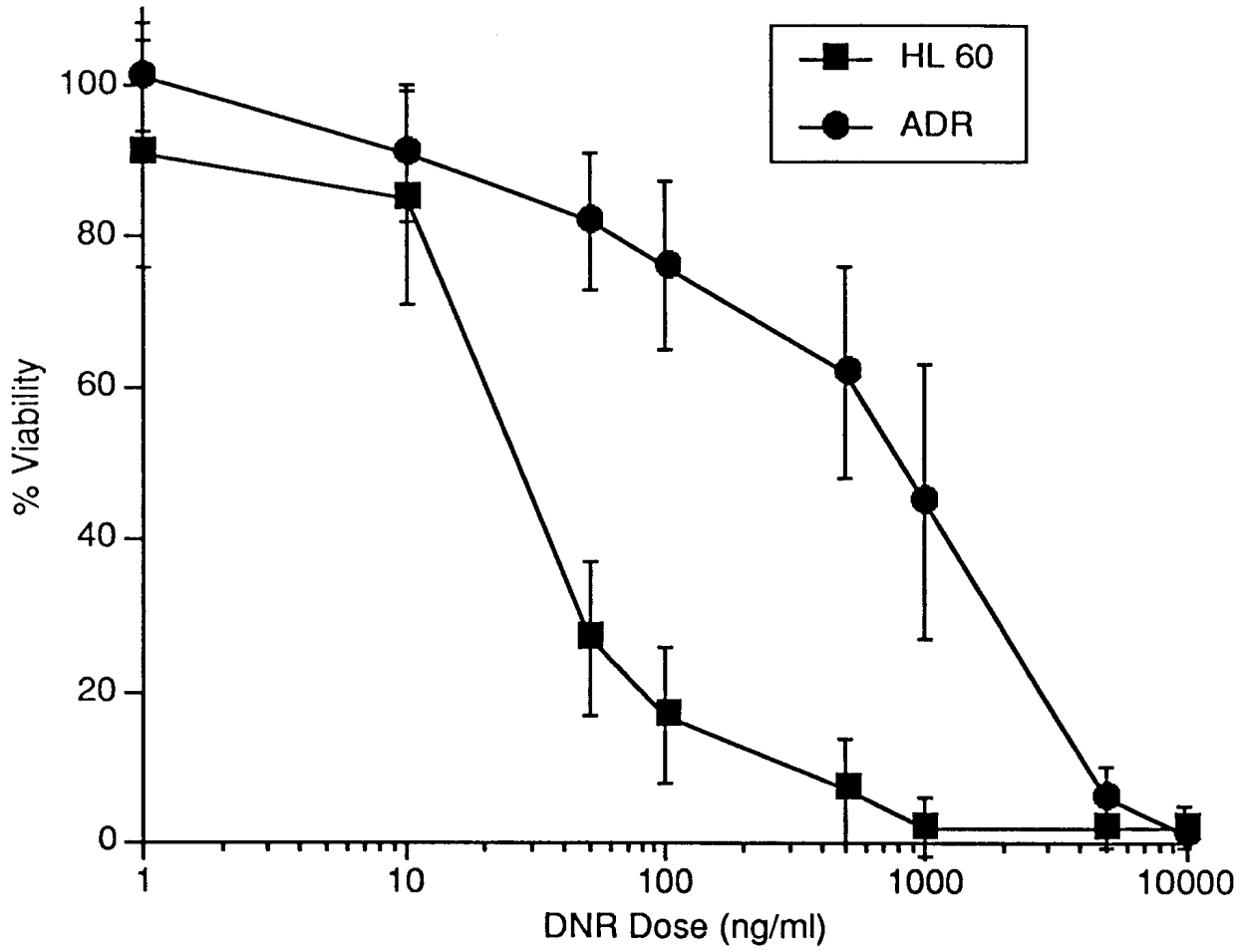


Figure 3.2. Dose response curve for the cytotoxic actions of DNR on the drug sensitive HL 60 cell line and drug resistant Pgp negative ADR cell line. Each point is shown as the mean ± SD (n=12).

Table 3.2. IC 50 values for DNR with or without the MDR reversing agents in the HL 60 and ADR cell lines, expressed as mean ± SD (n)

Cell Line	IC 50 (ng/ml)	Relative Resistance*
HL 60	35 ± 11 (12)	1
HL 60 + Cy-A	20 ± 7 (8)	0.6
HL 60 + Tri	44 ± 18 (8)	1.3
HL 60 + Cy A + Tri	29 ± 6 (8)	0.8
ADR	975 ± 743 (12)	28
ADR + Cy-A	25 ± 9 (8)	0.7
ADR + Tri	446 ± 234 (8)	13
ADR + Cy A + Tri	134 ± 107 (8)	4

* relative resistance is the IC 50 divided by the IC 50 of the parent CEM cell line

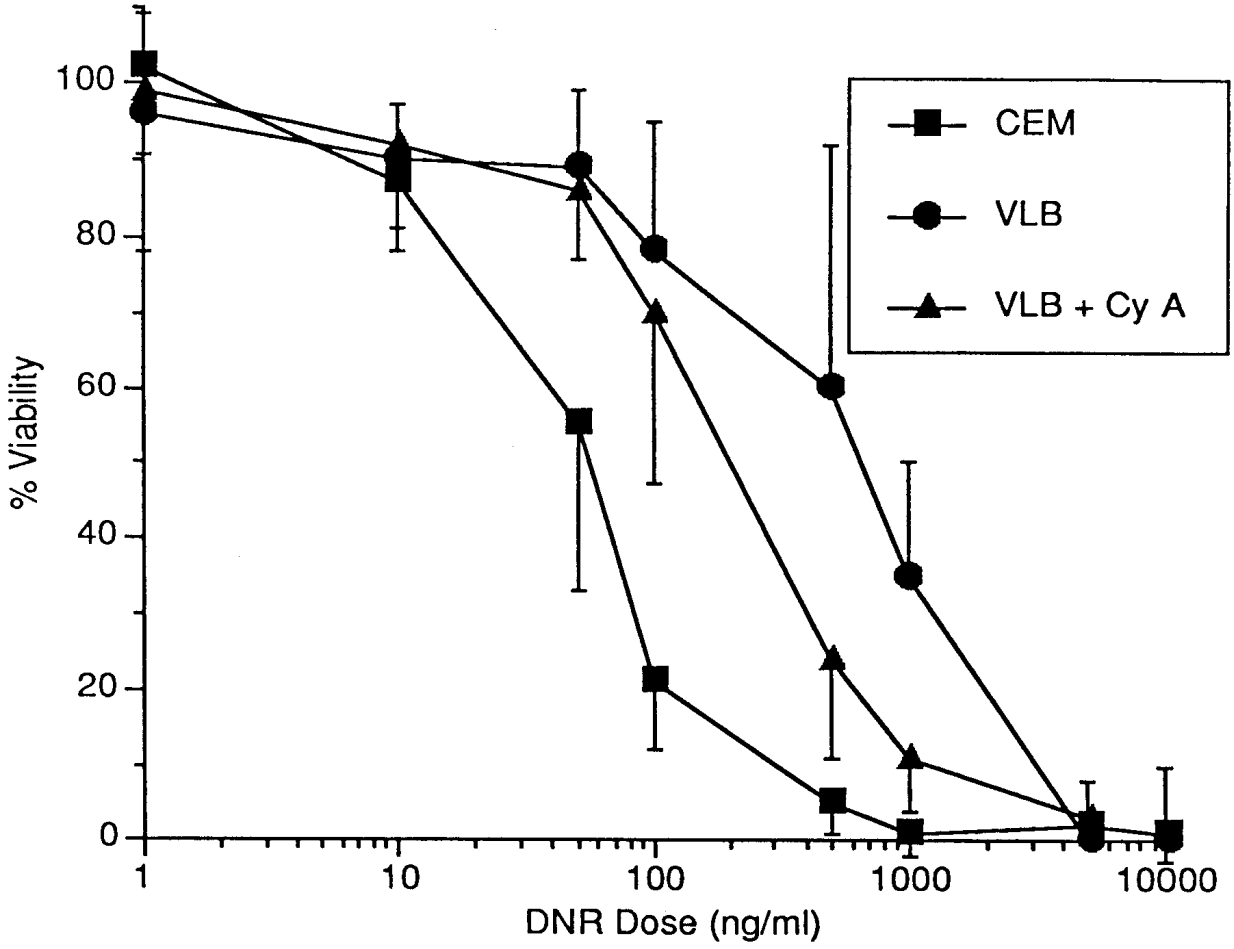


Figure 3.3. Dose response curve for the cytotoxic actions of DNR on the drug sensitive CEM cell line and the drug resistant Pgp positive VLB cell line. Each point is shown as the mean ± SD (n=5 or 6).

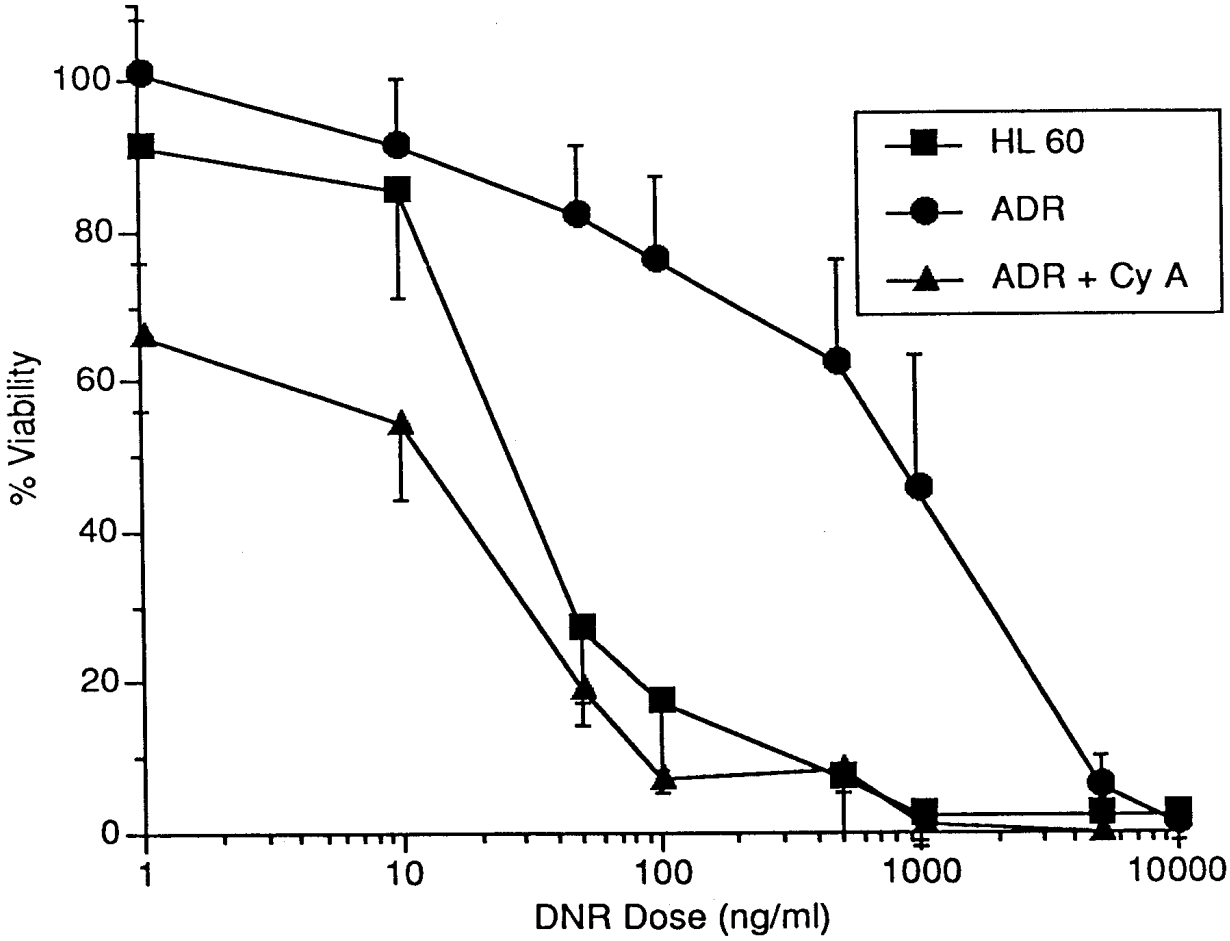


Figure 3.4. Dose response curve for the cytotoxic actions of DNR on the drug sensitive HL 60 cell line and the drug resistant Pgp negative ADR cell line. Each point is shown as the mean ± SD (n=8 or 12).

of the sensitive parent HL 60 cell line. Cy A was only able to partially reverse resistance in the VLB cell line reducing the IC 50 by two fold. This was still 5 times more resistant than the drug sensitive parent CEM cell line. Cy A was able to slightly increase the cytotoxicity of DNR both drug sensitive cell lines. In the CEM line the IC 50 decreased from 49 ± 11 ng/ml to 34 ± 13 ng/ml and in the HL 60 line the IC 50 decreased from 35 ± 11 ng/ml to 20 ± 7 ng/ml.

Tri displayed no consistency in the four cell lines. In the CEM and VLB cell lines (Table 3.1) the changes in IC 50 were minimal and in fact Tri slightly increased the resistance in both cell lines. In contrast, Tri was able to reverse resistance by 54% in the ADR cell line, reducing the IC 50 from 975 ± 743 ng/ml to 446 ± 234 ng/ml. Tri had no effect on the drug sensitive HL 60 cell line (Table 3.2). The combination of both Cy A and Tri had no effect in the CEM cell line (Table 3.1). The combination was able to increase the reversing effects of Cy A alone in the VLB cell line, reducing the relative resistance from 5 to 3. Cy A and Tri together had no additional effect on the HL 60 and ADR cell lines over that of the agents individually (Table 3.2).

3.4. Cytotoxicity of DOL on Cell lines

In vivo DNR is rapidly metabolised to DOL as discussed in Chapter 1.4.3. The enzyme responsible for metabolism, DNR reductase, is predominately found in the liver but is also present in several other tissues including leukaemic cells (Huffman & Bachur, 1972). Therefore the cytotoxic actions of DOL were examined and were found to have one fifth of the cytotoxicity of DNR in the CEM cell line. The IC 50 for DOL was 253 ± 82 ng/ml compared to that of 49 ± 11 ng/ml for DNR. The IC 50 for DOL was also much greater in the drug resistant VLB line with an IC 50 of 36,771 ± 43,329 ng/ml compared to the IC 50 of 571 ± 371 ng/ml for DNR. Therefore the metabolite DOL is much less cytotoxic than its parent compound DNR in these cell lines.

3.5. Accumulation of DNR in Cell lines with or without MDR reversing agents

Pgp has been proposed to act as an efflux pump, removing cytotoxic agents from within the cell and a number of agents such as Cy A and Tri have been shown to inhibit

this action of Pgp (Chapter 1.2.1). Therefore to examine whether Pgp was responsible for reduced accumulation in drug resistant cells and to determine the effects of Cy A and Tri in drug resistant cells, experiments examining the accumulation and retention of DNR in the cell lines were performed.

3.5.1. CEM

The amount of DNR accumulated in the CEM cells steadily increased up to a mean level of 125 ng.10^{-6} cells at 3 hrs of incubation, when the drug concentration plateaued (Figure 3.5). To compare the effects of the MDR reversing agents the area under the concentration time curve (AUC) (Figure 3.5) was used. The AUC (0-4 hr) are given in Table 3.3 for the accumulation of DNR in CEM cells, which accumulated 378 ± 69 ng.hr.10^{-6} cells. The addition of the MDR reversing agents Cy A and Tri had no effect on the accumulation of DNR in the CEM cell line (Table 3.3).

3.5.2. VLB

The drug resistant Pgp positive VLB cell line accumulated 33 ± 9 ng.hr.10^{-6} cells. This was less than 10% of DNR accumulated by the drug sensitive Pgp negative CEM cell line. The addition of Cy A significantly increased the accumulation of DNR in the VLB cell line to 176 ± 21 ng.hr.10^{-6}. This was greater than five fold increase compared to DNR alone. Although the accumulation of DNR was significantly increased by Cy A, the actual DNR AUC was still only half of the DNR accumulated by the CEM cell line. Therefore although Cy A was able to increase accumulation in the VLB cell line it was not able to increase it to the same levels as that obtained by the parent CEM cell line (Figure 3.6). The addition of Tri to the VLB cell line had no effect on DNR accumulation (Table 3.3). The combination of Cy A and Tri was also able to significantly increase the amount of DNR accumulated but was no greater than that done by the addition of Cy A alone (Table 3.3).

3.5.3. HL 60

The concentration time curve for the accumulation of DNR in the HL 60 cell line is shown in Figure 3.7. There was a steady increase in the DNR concentration with time reaching a plateau concentration of 133 ± 94 ng.10^{-6} cells after 3 hr. The amount of DNR accumulated over the 4 hour period is given in Table 3.3. The HL 60 cells accumulated 397 ± 253 ng.hr.10^{-6} cells. The DNR accumulation in the drug sensitive HL 60 cell line was

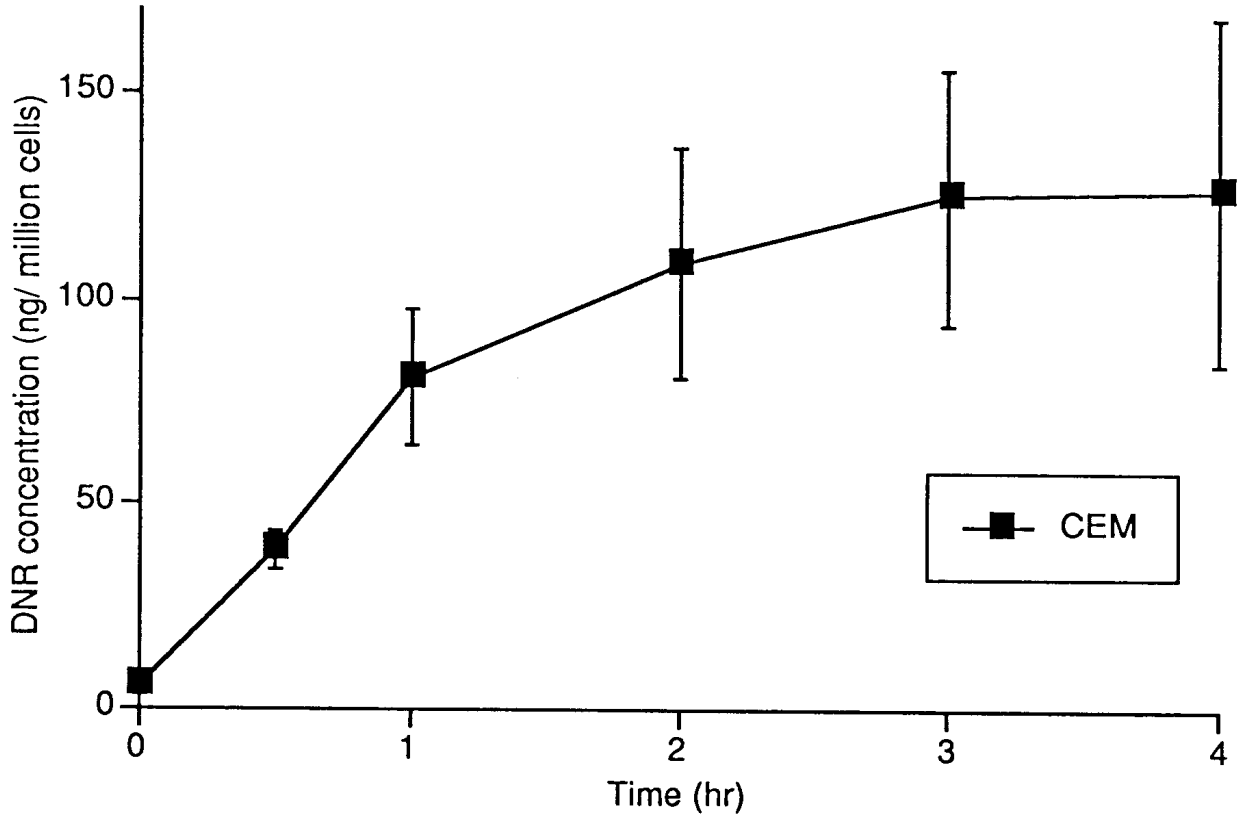


Figure 3.5. Concentration-time curve of DNR accumulation in the drug sensitive CEM cell line. Each point represents the mean ± SD (n=4).

Table 3.3. Accumulation of DNR by Leukaemic Cell Lines, given as the mean AUC (0-4 hr) ± SD (ng.hr.10^{-6} cells).

	DNR	DNR + Cy A	DNR + Tri	DNR + Cy A + Tri
HL 60 (5)	397 ± 253	471 ± 377	395 ± 219	544 ± 492
ADR (5)	185 ± 78*^	341 ± 201^	226 ± 120	403 ± 255*
CEM (4)	378 ± 69	428 ± 148	384 ± 108	452 ± 159
VLB (5)	33 ± 9*†	176 ± 21†	41 ± 8	176 ± 42*

* $p < 0.01$ (Fr) DNR vs DNR + Cy A + Tri

† $p < 0.01$ (Fr) DNR vs DNR + Cy A

^ $p < 0.05$ (Fr) DNR vs DNR + Cy A

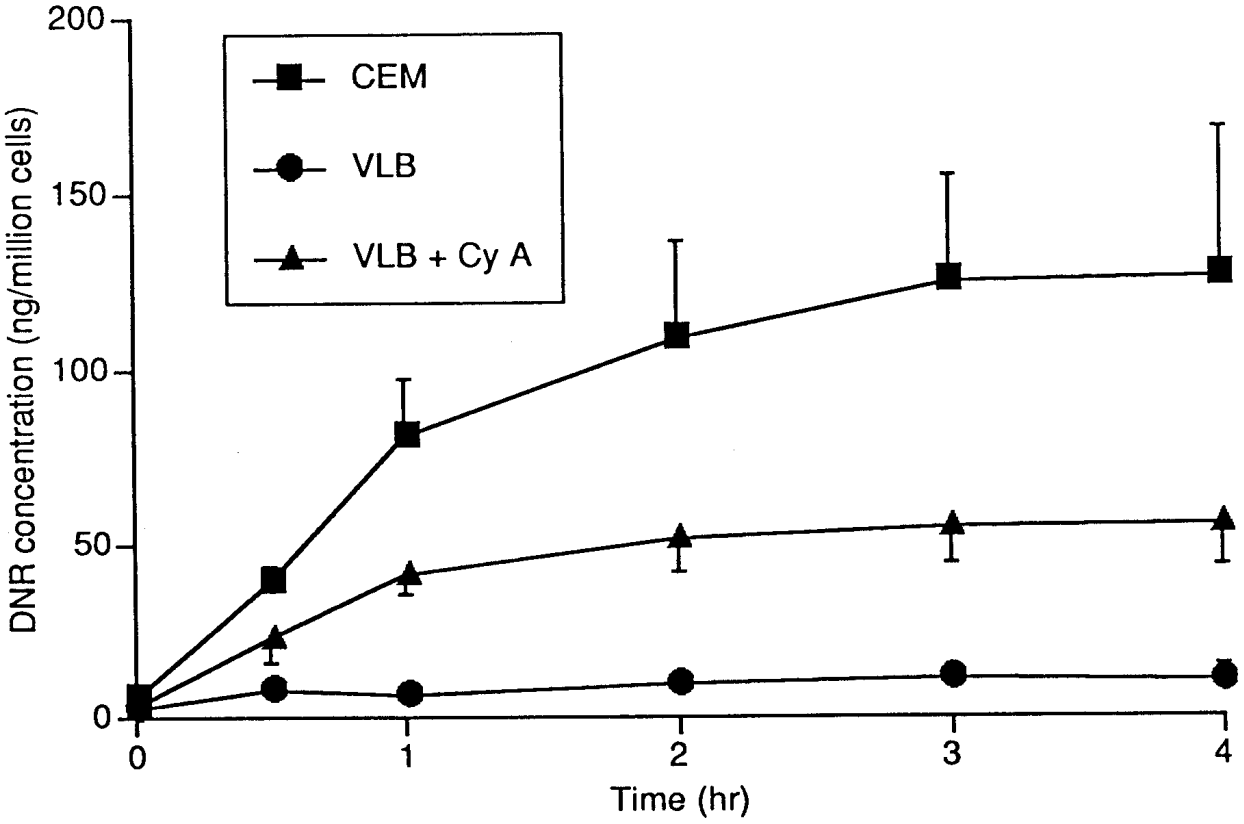


Figure 3.6. Concentration-time curve of DNR accumulation in the drug sensitive CEM cell line, drug resistant Pgp positive VLB cell line and the addition of Cy A to the VLB cell line. Each point represents the mean ± SD (n=4 or 5).

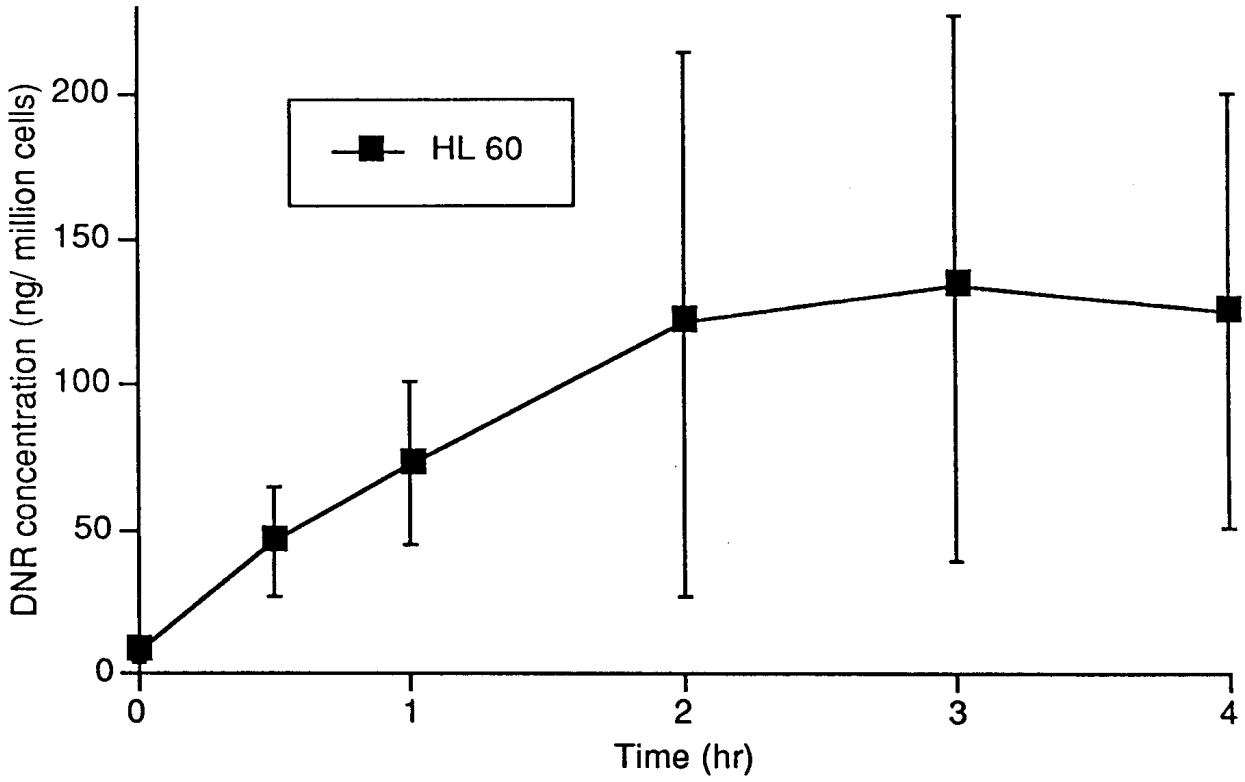


Figure 3.7. Concentration-time curve of DNR accumulation in the drug sensitive HL 60 cell line. Each point represents the mean ± SD (n=5).

similar to the amount of DNR accumulated in the drug sensitive CEM cell line. Addition of the MDR reversing agents individually or in combination had no effect on the accumulation of DNR in this cell line.

3.5.4. ADR

The drug resistant ADR cell line accumulated less DNR than its parent drug sensitive HL 60 cell line as shown in Figure 3.8. ADR accumulated 185 ± 78 ng.hr.10^{-6} cells of DNR. This was only 47% of that accumulated by HL 60. The Pgp negative drug resistant ADR accumulated significantly higher concentrations of DNR (185 ± 78 ng.hr.10^{-6} cells) compared to the Pgp positive drug resistant VLB cell line (33 ± 9 ng.hr.10^{-6} cells).

Cy A significantly increased accumulation in the drug resistant ADR cell line (Table 3.3). Tri caused a slight increase in accumulation but was not significant (Table 3.3). The combination of the MDR reversing agents Cy A and Tri was able to increase significantly the accumulation of DNR to 403 ± 255 ng.hr.10^{-6} cells. This is the same level as that obtained by the sensitive HL 60 cell line (Figure 3.8) and slightly greater than with Cy A alone.

3.6. Retention of DNR in Cell lines with or without MDR reversing agents

Retention is the amount of DNR retained by the cells after the removal of DNR from the media, that is, the amount of drug that is retained by the cells when no extracellular drug is present. After a 2 hr incubation all drugs were removed.

3.6.1. CEM

The amount of DNR retained by the cells steadily decreased over a four hour period from a mean level of 146 ng.10^{-6} cells to 81 ng.10^{-6} cells as shown in Figure 3.9. The amount of DNR retained over the four hours measured as the AUC (0-4 hr) was 388 ± 106 ng.hr.10^{-6} cells. The MDR reversing agents Cy A and Tri did not increase DNR retention (Table 3.4).

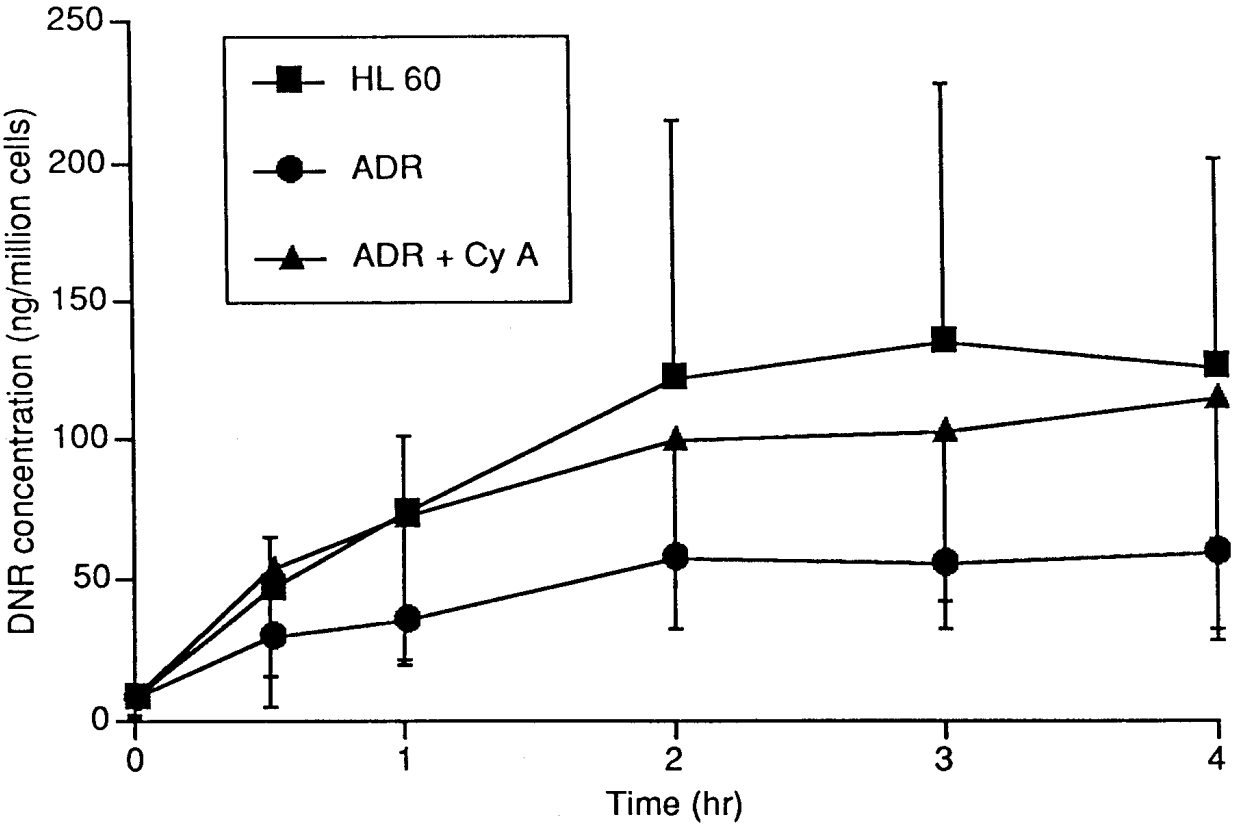


Figure 3.8. Concentration-time curve of DNR accumulation in the drug sensitive HL 60 cell line, drug resistant Pgp negative ADR cell line and the addition of Cy A to the ADR cell line. Each point represents the mean ± SD (n=5).

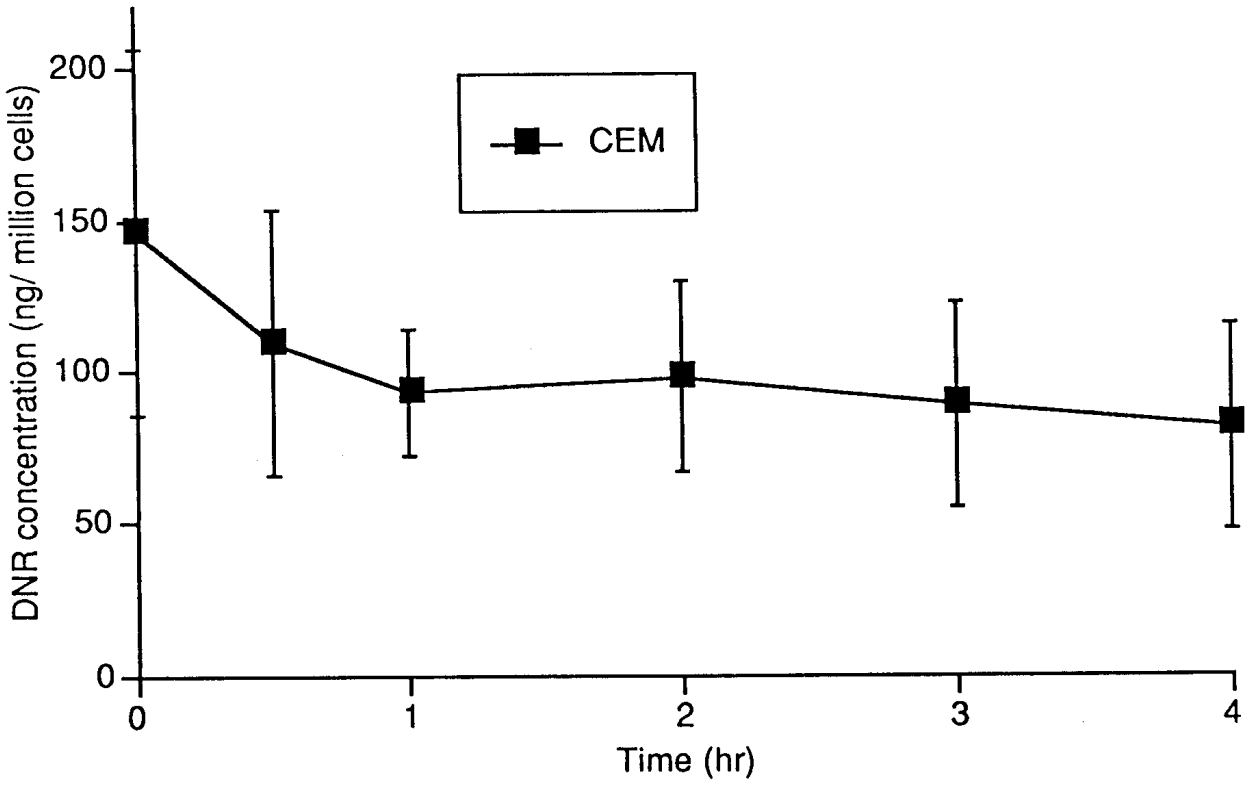


Figure 3.9. Concentration-time curve of DNR retention in the drug sensitive CEM cell line. Each point represents the mean ± SD (n=4).

Table 3.4. Retention of DNR by Leukaemic Cell Lines, given as the mean AUC (0-4 hr) ± SD (ng.hr.10^{-6} cells).

	DNR	DNR + Cy A	DNR + Tri	DNR + Cy A + Tri
HL 60 (5)	257 ± 123	267 ± 99	252 ± 101	267 ± 108
ADR (5)	66 ± 24^#	122 ± 22#	71 ± 21	129 ± 29^
CEM (4)	388 ± 106	355 ± 85	322 ± 131	276 ± 71
VLB (5)	25 ± 8^#	99 ± 34#	28 ± 13	93 ± 47^

^ $p < 0.05$ (Fr) DNR vs DNR + Cy A + Tri

# $p < 0.05$ (Fr) DNR vs DNR + Cy A

3.6.2. VLB

The drug resistant Pgp positive VLB cell line retained 25 ± 8 ng.hr.10^{-6}cells, this is only 6% of the DNR retained by the parental drug sensitive CEM cells (Figure 3.10). The addition of Cy A significantly increased the retention of DNR by the VLB cell line to 99 ± 34 ng.hr.10^{-6}cells, (a 400 % increase in retention) but did not reach the amount of DNR retained by the drug sensitive CEM (Figure 3.10). Therefore the MDR reversing agent Cy A could only partially reverse drug resistance in the VLB cell line. The addition of Tri to the drug resistant VLB had no effect on DNR retention. The combination of Cy A and Tri was also able to significantly increase DNR retention, however this was no greater than that achieved by the addition of Cy A alone (Table 3.4).

3.6.3. HL 60

The concentration time curve for retention of DNR in the HL 60 cell line is shown in Figure 3.11. The amount of DNR retained by the cells steadily decreased over a four hour period from a mean level of 84 ng.10^{-6} cells to 51 ng.10^{-6} cells. The amount of DNR retained over the four hours measured as the AUC (0-4 hr) was 257 ± 123 ng.hr.10^{-6} cells. The MDR reversing agents Cy A and Tri did not increase DNR retention (Table 3.4). The drug sensitive HL 60 retained less DNR than the drug sensitive CEM, 257 ± 123 ng.hr.10^{-6} cells and 388 ± 106 ng.hr.10^{-6} cells respectively. This is a disparity with accumulation in that CEM and HL 60 cell lines accumulated equivalent amounts of DNR.

3.6.4. ADR

The drug resistant Pgp negative ADR cell line retained less DNR than it's parent drug sensitive HL 60 cell line as shown in Figure 3.12. ADR retention of DNR was only 26 % of that achieved by HL 60, 66 ± 24 ng.hr.10^{-6} cells and 257 ± 123 ng.hr.10^{-6} cells respectively. The Pgp negative drug resistant ADR retained higher concentrations of DNR (66 ± 24 ng.hr.10^{-6} cells) compared to the Pgp positive drug resistant VLB cell line (25 ± 8 ng.hr.10^{-6} cells).

Cy A significantly increased DNR retention in the ADR cells by almost 2 fold. However, this increase was still only 46% of the amount of DNR retained by the HL 60 cell line (Figure 3.12). Tri had no effect on DNR retention and the combination of Cy A and Tri was equivalent to the actions of Cy A alone (Table 3.4).

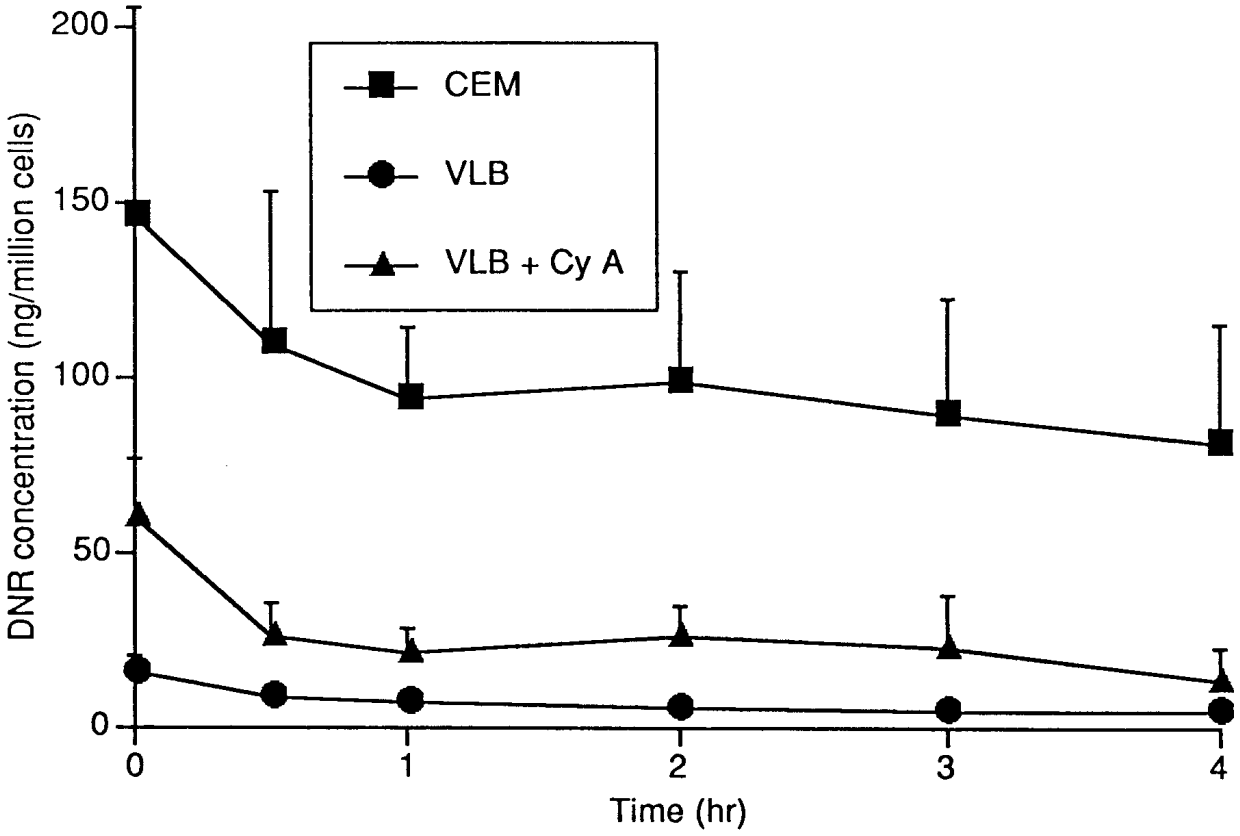


Figure 3.10. Concentration-time curve of DNR retention in the drug sensitive CEM cell line, drug resistant Pgp positive VLB cell line and the addition of Cy A to the VLB cell line. Each point represents the mean ± SD (n=4 or 5).

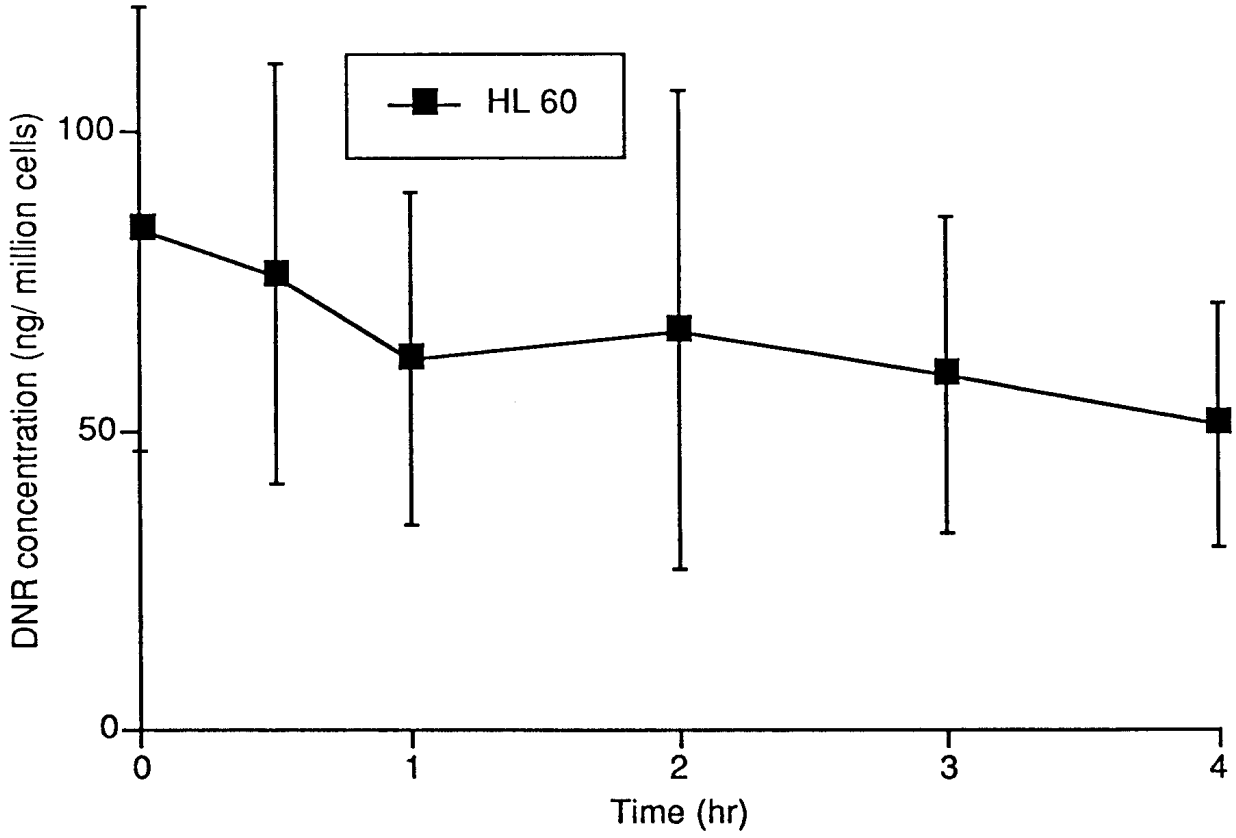


Figure 3.11. Concentration-time curve of DNR retention in the drug sensitive HL 60 cell line. Each point represents the mean ± SD (n=5).

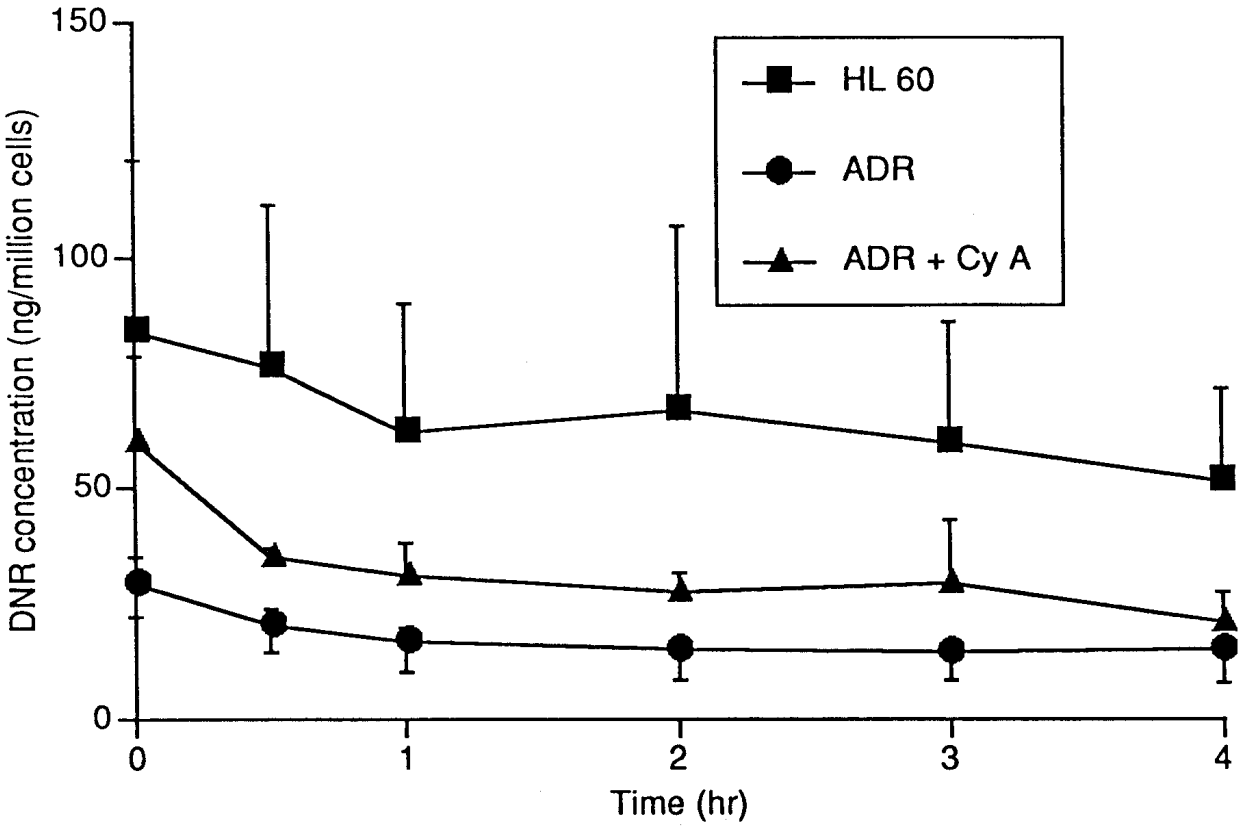


Figure 3.12. Concentration-time curve of DNR retention in the drug sensitive HL 60 cell line, drug resistant Pgp negative ADR cell line and the addition of Cy A to the ADR cell line. Each point represents the mean ± SD (n=5).

3.7. Effect of MDR reversing agents after removal of DNR

To prove that the increased retention of DNR was due to the action of the MDR reversing agents, rather than just a result of the increased accumulation in the presence of these agents, the MDR reversing agents were not removed after the two hour incubation. When the MDR reversing agents remained present the effect of the reversing agents in increasing retention of DNR in the drug resistant Pgp positive VLB cell line was more pronounced. The percentage increase of DNR retention was obtained by dividing the AUC (0-4 hr) for the retention samples by the AUC (0-4 hr) of the retention of DNR. Therefore, the % retention for DNR in the VLB cell line was 100%. For Cy A retention increased from 396% to 570%, Tri 112% to 129% and the combination from 372% to 701% (Figure 3.13). Therefore the presence of the MDR reversing agent did cause greater retention of DNR.

3.8. Accumulation and Retention of DOL by CEM

The amount of DOL accumulated by the CEM cell line was 71 ± 34 (n=8) ng.hr.10^{-6} cells and retained 33 ± 8 (n=4) ng.hr.10^{-6} cells. By comparison, the CEM cell line accumulated 378 ± 69 ng.hr.10^{-6} cells and retained 388 ± 106 ng.hr.10^{-6} of DNR. The concentration of DOL retained in the CEM cells is only 18% of the concentration of DNR accumulated and 9% of the concentration of DNR retained. Thus the metabolite DOL is not taken up by the cells as readily as the parent drug DNR. This is consistent with the decreased cytotoxicity of DOL that is only 20% of the DNR cytotoxicity in the CEM cell line.

3.9. Discussion

CEM is a drug sensitive leukaemic cell line and VLB is a subline of CEM grown in the presence of vinblastine. The VLB cell line has been shown to be drug resistant (Beck *et al.*, 1979) and this resistance is due to the presence of Pgp. Pgp is believed to act as an efflux pump removing intracellular cytotoxic agents from the cells, thereby reducing the effectiveness of the cytotoxic agent and making the cells resistant. The VLB cell line is not only resistant to vinblastine but also to several other chemotherapeutic agents such as DNR, DOX, EPI, vincristine, colchicine and etoposide. This resistance can be reversed by a number of agents, including verapamil, Cy A and Tri (Chapter 1.2.1). Marks *et al.* (1992) and Beck *et al.* (1986) have shown that the VLB line is 73 fold and 102 fold, respectively,

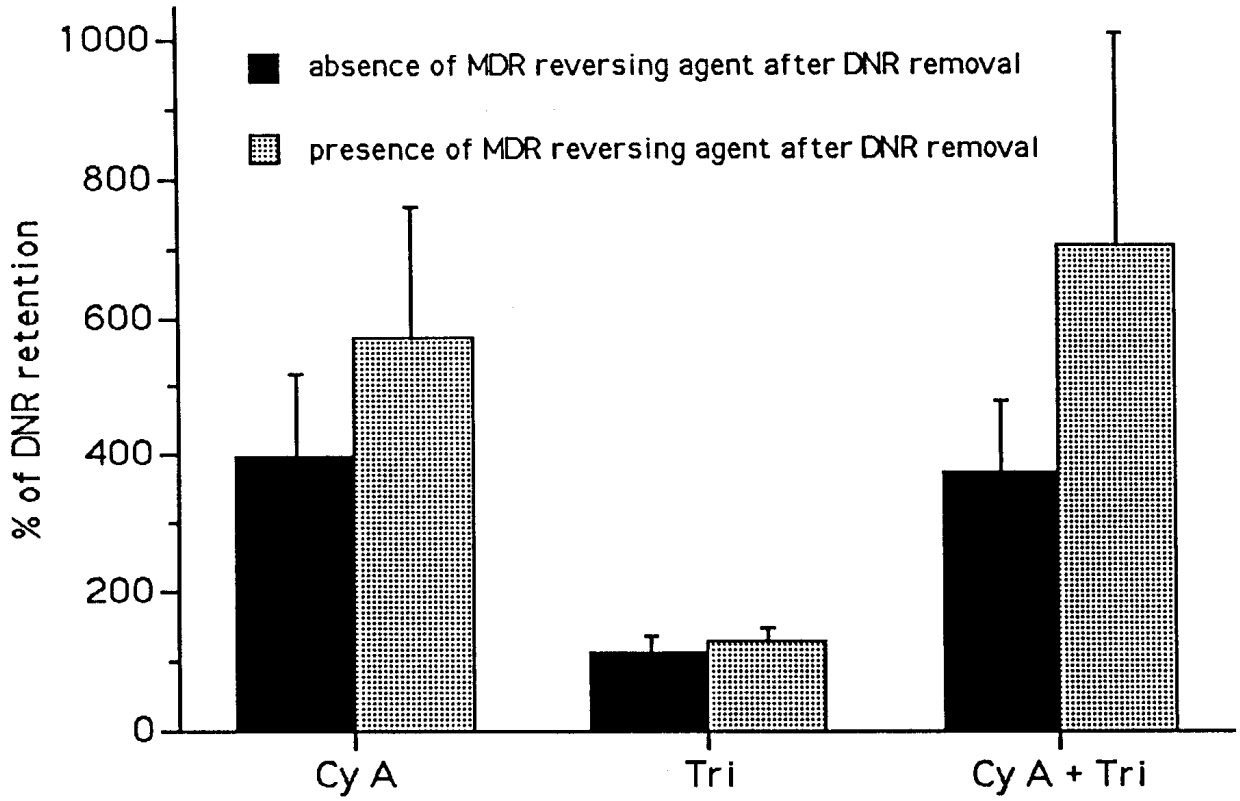


Figure 3.13. The effect of leaving the MDR reversing agent after the removal of DNR.

more resistant to DNR than the drug sensitive CEM cell line. The present work found that the VLB cell line was only 12 fold resistant (Table 3.1). As expected with bioassays in general it is difficult to compare quantitative data from one laboratory with another, because minor differences in experimental conditions can make significant differences in end point measurements. Therefore differences found could be due to the different types of methods used to test cytotoxicity. Beck *et al.* (1986) used a growth inhibitory assay, and examined the effects after 48 hr as compared to 72 hr used in the MTT method in the present study. They obtained an IC 50 of 26 nM for the CEM cell line and 2650 nM for the VLB cell line, i.e. 102 fold increased resistance. Marks *et al.* (1992) used two methods to determine IC 50's: an MTT method similar to the one used in this work and a leucine incorporation assay. They found IC 50's of 170 nM for CEM cells and 12400 nM for VLB using the MTT method (73 fold increased resistance) and IC 50's of 13 nM for CEM cells and 620 nM for VLB using the leucine incorporation assay (48 fold increased resistance). The MTT IC 50's can be compared to the values obtained in this study, which are 93 nM (49 ng/ml) for the CEM cell line and 1084 nM (571 ng/ml) for the VLB cell line. Although there are differences in the IC 50's for the CEM and VLB cell lines between laboratories, it remains evident that the CEM cell line is drug sensitive and the VLB cell line is drug resistant.

HL 60 is a drug sensitive leukaemic cell line and its subline ADR is drug resistant. In contrast to the VLB cell line that is a drug resistant cell line and Pgp positive, ADR is drug resistant and Pgp negative (McGrath & Center, 1988). Therefore in this instance a mechanism(s) other than Pgp is responsible for drug resistance. Hindenburg *et al.* (1989) found an IC 50 for DNR of 110 nM for HL 60 and 21000 nM for a drug resistant subline HL 60/AR (HL 60/AR is not the same as cell line as ADR, since they were isolated and characterised in two separate laboratories). With the same cell lines Bhalla *et al.* (1985) found IC 50 of 50 nM and 2500 nM for the sensitive and resistant lines, respectively, using DNR. This was a 190 fold and 50 fold increase in resistance for the respective authors for the HL 60/AR as compared to the parent HL 60. The reason for the large difference between the study of Hindenburg *et al.* (1989) and Bhalla *et al.* (1985) in the IC 50 of the drug resistant HL 60/AR may be that Hindenburg *et al.* (1989) incubated the cells in the presence of DNR for only one hour compared to a 72 hr incubation used by Bhalla *et al.* (1985). McGrath & Center (1988) have also shown an 80 fold difference in the resistance for DOX in the ADR cell line as compared to its parental HL 60. The IC 50's for DNR in the studies above (i.e. 110 nM and 50 nM) are similar to the IC 50 achieved in the present study of 67 nM (35 ng/ml) in the HL 60 cell line. In the ADR cell line an IC 50 of 1850 nM (975 ng/ml) was achieved, thus the ADR cell line was 28 fold more resistant than the HL 60 cell line. The relative resistances seen by Hindenburg *et al.* (1989) and Bhalla *et al.* (1985) are also higher than that seen here; however, they used the drug resistant cell line HL 60/AR (Bhalla

et al 1985) compared to the ADR cell line used in this study (Marsh *et al.*, 1986). Even though there may be differences between the methods used to determine IC 50's, the HL 60 cell line clearly is drug sensitive whereas the ADR cell line is drug resistant.

Cy A and Tri have been shown to reverse drug resistance. However, it remains uncertain at what concentration these agents can achieve a significant and effective reversal in a clinical setting. Ganapathi & Grabowski (1988) showed that the addition of 5 μM (2.4 μg/ml) Tri decreased the concentration of DOX required to kill drug resistant mouse leukaemia cell lines. The same effect was shown by several authors in a variety of cell lines (Tsuruo *et al.*, 1982; Akiyama *et al.*, 1986; Ganapathi *et al.*, 1984); all these drug resistant cell lines were Pgp positive. The concentrations of the MDR reversing agents (Tri used at 2 - 3 μg/ml) used in the above studies, however, are not possible *in vivo*. In the present study the effect of Tri at a clinically achievable concentration (150 ng/ml, Kaye, 1990), was shown to have no effect on the cytotoxicity of DNR in the drug resistant Pgp positive VLB cells. However, it was able to increase cytotoxicity in the drug resistant Pgp negative ADR cell line, decreasing the relative resistance of ADR from 28 fold to 13 fold. This suggests that clinically achievable concentrations of Tri would be unable to reverse drug resistance in Pgp positive cells but could reverse drug resistance in Pgp negative cells.

Cy A has been shown by Slater *et al.* (1986) to decrease the IC 50 of DNR in a drug resistant T cell lymphatic leukaemia from 8.4 μg/ml to 2.3 μg/ml, the equivalent IC 50 of the parent drug sensitive cell line. Nooter *et al.* (1989) also observed this effect in a drug resistant P388 cell line in which the IC 50 was lowered from 36 μM (19 μg/ml) DNR to 3.8 μM (2 μg/ml) in the presence of Cy A. The concentrations of Cy A used by Slater *et al.* (1986) and Nooter *et al.* (1989) were 13.2 μg/ml and 3 μM (3.75 μg/ml) respectively. These concentrations of Cy A can be toxic to patients and therefore are not clinically achievable. In this study a clinically achievable concentration of 1.5μg/ml Cy A was used. Cy A halved the IC 50 in the drug resistant Pgp positive VLB cell line from 571 ng/ml to 245 ng/ml. In the drug resistant Pgp negative ADR cell line the IC 50 was reduced from 975 ng/ml to 25 ng/ml in the presence of Cy A. This was equivalent to the IC 50 of the parent HL 60 cell line. Therefore Cy A appears to be a more effective agent in reversing drug resistance in non-Pgp resistant cell lines than in Pgp resistant cell lines.

The addition of two or more MDR reversing agents in combination has been described in one study only (Hu *et al.*, 1990). In that study the combination of Cy A and verapamil was used and it was concluded that the actions of the two drugs may be synergistic. In the present study the combination of Cy A and Tri decreased the cytotoxicity by almost half in the VLB cell line and decreased the relative resistance of the drug resistant

Pgp positive VLB cell line from 5 fold in the presence of Cy A alone to 3 fold in the presence of both Cy A and Tri. The combination had no additional effect in the drug resistant Pgp negative ADR cell line, over that of Cy A alone. Indicating that the combination may have some advantages over the individual use of resistance modifiers in the drug resistant Pgp positive VLB cell line but not in the drug resistant Pgp negative ADR cell line.

DNR is extensively metabolised to DOL *in vivo*. Therefore if DOL had similar cytotoxic activity as DNR it would play a major role in the clinical actions of DNR. This study has found that DOL had only 20% of the cytotoxicity of DNR in the CEM cell line and because of this reduced activity, it is not as effective as the parent DNR. The CEM cells accumulated only 18% of DOL, in comparison to the amount of DNR accumulated by the CEM cells. The CEM cells were also over 5 fold resistant to DOL compared to DNR, indicating that the decreased accumulation of DOL in the CEM cell line is most likely responsible for the decreased cytotoxicity of DOL.

As early as 1973, it had been shown that there was a decrease in both drug accumulation and retention in a variety of drug resistant cell lines compared to the parental drug sensitive cell line (Dano, 1973; Ling & Thompson, 1974; Skovsgaard, 1978a, b; Inaba *et al.*, 1979; Kartner *et al.*, 1983; Fojo *et al.*, 1985). This was also evident in the four cell lines studied here. The drug resistant cell lines VLB (Pgp positive) and ADR (Pgp negative) accumulated only 9% and 46% of DNR respectively, of the parent drug sensitive cell lines; and retained only 6% and 26%, respectively. These results are consistent with those seen by Haber *et al.* (1989) for the CEM and VLB cell lines and by Hindenburg *et al.* (1989) and Marsh *et al.* (1986) in HL 60 cells and the drug resistant ADR cell line. Thus the drug resistant cell lines accumulate and retain lower drug concentrations than the parental drug sensitive cell lines. Although the drug resistant Pgp positive VLB cell line was only 12 fold resistant it accumulated significantly lower concentrations of DNR than the drug resistant Pgp negative ADR cell line that was 28 fold resistant. This suggests that two different mechanisms of resistance are present in the drug resistant Pgp positive VLB cell line and the drug resistant Pgp negative ADR cell line, since the accumulation of DNR and the cytotoxicity of DNR do not appear to be related.

Cy A and Tri have been shown to increase accumulation and retention in drug resistant cell lines (Tsuruo *et al.*, 1982; Akiyama *et al.*, 1986; Ganapathi *et al.*, 1984; Coley *et al.*, 1989). All the above studies have used high concentrations of both Cy A (5 μg/ml) and Tri (1-3 μg/ml) to reverse drug resistance. The present study has shown that DNR accumulation and retention can also be increased in drug resistant cell lines by the addition of Cy A at a clinically achievable concentration (1.5 μg/ml). Tri, on the other hand, did not

increase accumulation or retention at a concentration (150 ng/ml) achievable *in vivo*. The combination of Cy A and Tri had no effect in increasing the accumulation of DNR in the drug resistant Pgp negative ADR cell line above that of Cy A alone. For the drug resistant Pgp positive VLB cell line the accumulation of DNR was not enhanced by the combination of Cy A and Tri as compared to Cy A alone. The ability of Cy A to increase DNR accumulation in the drug resistant cell lines VLB (Pgp positive) and ADR (Pgp negative) does not appear to correlate with Cy A ability to increase DNR cytotoxicity. Cy A was able to increase cytotoxicity in the drug resistant Pgp positive VLB cell line by a factor of 2 whereas the accumulation of DNR in the VLB cell line was increased by a factor of 5. In the drug resistant Pgp negative ADR cell line, cytotoxicity was increased by a factor of 39 and DNR accumulation was only increased by a factor of 2. These results indicate that the reversal of drug resistance by Cy A is not related solely to an increase in DNR accumulation and suggests that the actions of Cy A in the Pgp positive VLB cell line may be different from the actions of Cy A in the Pgp negative ADR cell line. Thus Cy A is capable of reversing drug resistance in both Pgp mediated and non Pgp mediated resistant cell lines.

CHAPTER 4

Pharmacokinetics of DNR in patients and the role of P glycoprotein

4.1. Introduction

In vitro, MDR can be associated with the presence of Pgp, which is believed to act as an efflux pump (Chapter 1.2.1). It is hypothesised that intracellular cytotoxic agents are removed from the cell by Pgp, decreasing the intracellular concentration and thereby reducing the effectiveness of these drugs. Although the relationship between cellular drug concentrations and Pgp has been well established in cell lines (Kartner *et al.*, 1983; Fojo *et al.*, 1985), this relationship has not been fully documented in patients undergoing chemotherapy. Previous studies have examined the pharmacokinetics of DNR (Chapter 1.4.2) in patients, but few have investigated the cellular levels of DNR and its major cytotoxic metabolite DOL. A major issue is whether the resistance to DNR is due simply to altered plasma kinetics, resulting in ineffective cellular concentrations, or a mechanism involving Pgp. Ma *et al.* (1987) and Campos *et al.* (1992) have shown previously that the Pgp phenotype is present in patients with leukaemia. In this chapter the plasma and cellular pharmacokinetics of DNR and DOL in patients with acute leukaemia have been examined to test the hypotheses that Pgp is responsible for a decrease in intracellular DNR concentrations

in patients with acute leukaemia, and whether this decrease in intracellular DNR concentrations is responsible for the patients' poor response to chemotherapy.

4.2. Patients

Twenty-seven patients with acute leukaemia (acute myeloid leukaemia, AML, or acute lymphoblastic leukaemia, ALL) were studied (14 females and 13 males). Age ranged from 28 years to 78 years for females and from 16 years to 79 years for males and an overall median of 49 years. The patients were diagnosed according to the FAB classification (Chapter 1.3) and their clinical characteristics at presentation are reported in Table 4.1. Patients received DNR infused over a fifteen minute period (Table 4.1) as part of their induction chemotherapy. For 14 AML patients the chemotherapy protocol consisted of Ara C 100 mg/m^2/day with or without etoposide 75 mg/m^2/day for 7 days and DNR 50 mg/m^2 for 3 concurrent days, 3 patients received reduced doses of DNR due to concern of accumulated cardiotoxicity, 2 AML patients received only DNR 50 mg/m^2 for 3 days. For 9 ALL patients the Hoelzer protocol (Hoelzer *et al.*, 1984) was used, which consisted of daily prednisolone with weekly injections of DNR 25 mg/m^2 and vincristine over the first 4 weeks of induction. One ALL patient was given only weekly DNR (25 mg/m^2) and one relapsed ALL patient received a 50 mg/m^2 dose of DNR. Blood samples were collected (Chapter 2.2.6) at the beginning of chemotherapy for both AML and ALL patients.

Response was determined according to standard criteria as described in Chapter 1.3.1. Briefly, a complete remission (CR) was defined as a reduction of blast cells below 5%, and a return to normal haemopoiesis within 4 weeks after the commencement of chemotherapy; a partial response (PR) was defined as a reduction of blasts in the original population but without adequate normal haemopoetic recovery and no response when there was no alteration or an increase in the blasts. For analysis, patients with a partial response were grouped with those patients that had no response and are termed non responders (NR).

4.3. Patients Response to Treatment

This study included 27 patients (Table 4.1) of which 12 achieved complete remission, 5 had a partial response and 6 did not respond to chemotherapy. Four patients could not be evaluated for response to therapy because they died before a haematological response could be determined. Of the 27 patients, 16 were AML (2 relapsed) and 11 were

Table 4.1. Patient Characteristics

Patient	SEX	AGE	DIAGNOSIS	WCC	BLASTS (%)	DOSE (mg)	DNR DOSE (mg/m2)	Other Drugs at Induction	RESPONSE
1	F	55	AML	10.2	78	85	50	Ara C, VP-16	P
2	M	31	ALL	8.7	67	45	25	Vcr, Pred, Asp	C
3	F	28	ALL	8.1	64	40	25	Vcr, Pred, Asp, Mtx	C
4	F	32	AML	10.9	29	95	50	Ara C	C
5	M	68	AML	15.8	3	90	50	Ara C, VP-16	P
6	F	56	AML	75.6	100	80	50	Ara C, VP-16	NE
7	F	66	ALL	3.8	34	40	25	Vcr, Pred	C
8	F	62	ALL	94.6	92	40	25	Vcr, Pred, Mtx	N
9	M	47	AML	17.1	95	90	50	Ara C, VP-16	C
10	M	65	ALL	3.6	0	40	25	Vcr, Pred, Asp	N
11	F	36	ALL	100	88	35	25		C
12	M	28	ALL	13.1	47	45	25	Vcr, Pred	C
13	M	79	AML	198.9	83	85	50		P
14	M	56	AML	13.5	68	100	50	Ara C, VP-16	P
15	F	46	AML	3.2	10	85	50	Ara C, VP-16	C
16	M	16	ALL	6	50	40	25	Vcr, Pred, Asp	C
17	M	46	R ALL	3.4	31	105	50	Vcr, Pred	N
18	F	48	AML	26	45	80	50		C
19	F	43	AML	4.3	90	75	45	Ara C	NE
20	F	42	ALL	2.9	41	50	25	Vcr, Pred, Asp	C
21	M	19	AML	67.5	70	90	50	Ara C	NE
22	F	64	R AML	2	50	55	35	Ara C, VP-16	N
23	F	78	AML	39.3	40	80	50	Ara C	N
24	M	71	AML	168.4	72	65	30	Ara C	N
25	M	41	R AML	1.4	47	100	50	Ara C, VP-16	NE
26	F	67	AML	49.8	30	80	50	Ara C	C
27	M	36	R ALL	2.4	23	90	50		P

AML-Acute Myeloid Leukaemia
R AML-Relapsed Acute Myeloid Leukaemia
ALL -Acute Lymphoblastic Leukaemia
R ALL -Relapsed Acute Lymphoblastic Leukaemia
wcc - white cell count

C- Complete Remission
P- Partial Response
N- No Response
NE- Not Evaluable

Ara C- Cytosine Arabinoside
VP-16- Etoposide
Pred - Prednisilone
Asp- Asparaginase
Mtx- Methotrexate

ALL (2 relapsed). Five (31% of total, 42% of evaluable) of the AML patients achieved CR; 4 had a PR; 3 did not respond and 4 were not evaluable. Seven (64%) of the ALL patients achieved CR; one had a PR and three had no response. None of the relapsed patients achieved complete remission, one relapsed ALL patient had a PR, one relapsed AML patient was not evaluable and the other two relapsed patients did not respond to treatment.

4.4. Pharmacokinetics

4.4.1. Plasma

The plasma concentration-time curve for each evaluable patient was plotted for both DNR and DOL. The average plasma concentration-time curve for DNR and DOL for patients receiving a 50 mg/m^2 dose of DNR is shown in Figure 4.1 and Figure 4.2 shows those patients receiving a 25 mg/m^2 dose of DNR. As can be seen from the large standard deviations, there is a great deal of inter-individual variation in both the DNR and DOL plasma concentrations. Figures 4.1 and 4.2 demonstrate that plasma DOL concentrations are higher overall than the plasma DNR concentrations during the time period of the study. Generally, DOL plasma concentrations exceed DNR plasma levels from as early as one hour post-administration of DNR.

The pharmacokinetic parameters Cmax, Tmax, AUC (0-24 hr), AUMC (0-24 hr), MRT, CL and Vd were calculated as described in Chapter 2.2.9 and Chapter 1.4.1. The plasma DNR pharmacokinetic parameters are given in Table 4.2 and the plasma DOL pharmacokinetic parameters are given in Table 4.3. Because not all patients received the same dose of DNR, the pharmacokinetic parameters Cmax, AUC (0-24 hr) and AUMC (0-24 hr) of all the patients were corrected for dose (Chapter 2.2.9) and are given in Table 4.4 for DNR and Table 4.5 for DOL.

The mean time to reach maximum drug concentrations was 25 min for DNR and 2.8 hrs for DOL. The average peak plasma DNR concentration was 2.8 ng.ml^{-1}.mg^{-1} DNR for all but one patient, who was excluded because of a peak DNR concentration of 561 ng.ml^{-1}.mg^{-1} DNR (this peak DNR concentration was 130 standard deviations from the mean). The average peak plasma DOL concentration was 1.0 ng.ml^{-1}.mg^{-1} DNR for all but one patient, who was excluded because of a peak DOL concentration of 14 ng.ml^{-1}.mg^{-1} DNR (this peak DOL concentration was 20 standard deviations from the mean). In summary, plasma DNR levels peaked at 2.8 ± 4.3 (n = 25) ng.ml^{-1}.mg^{-1} DNR 25 min after

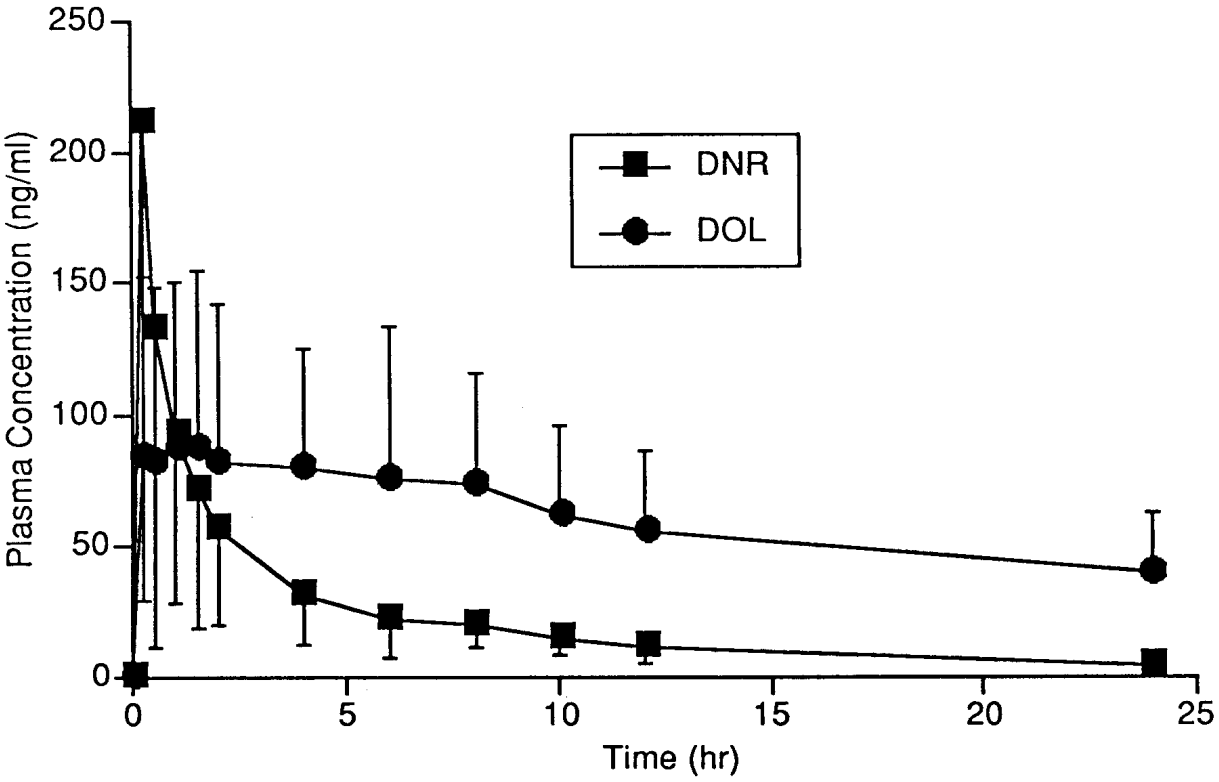


Figure 4.1. Plasma concentration-time curve for DNR and its metabolite DOL for all patients receiving a 50 mg/m² dose of DNR. Each point represents the mean ± SD.

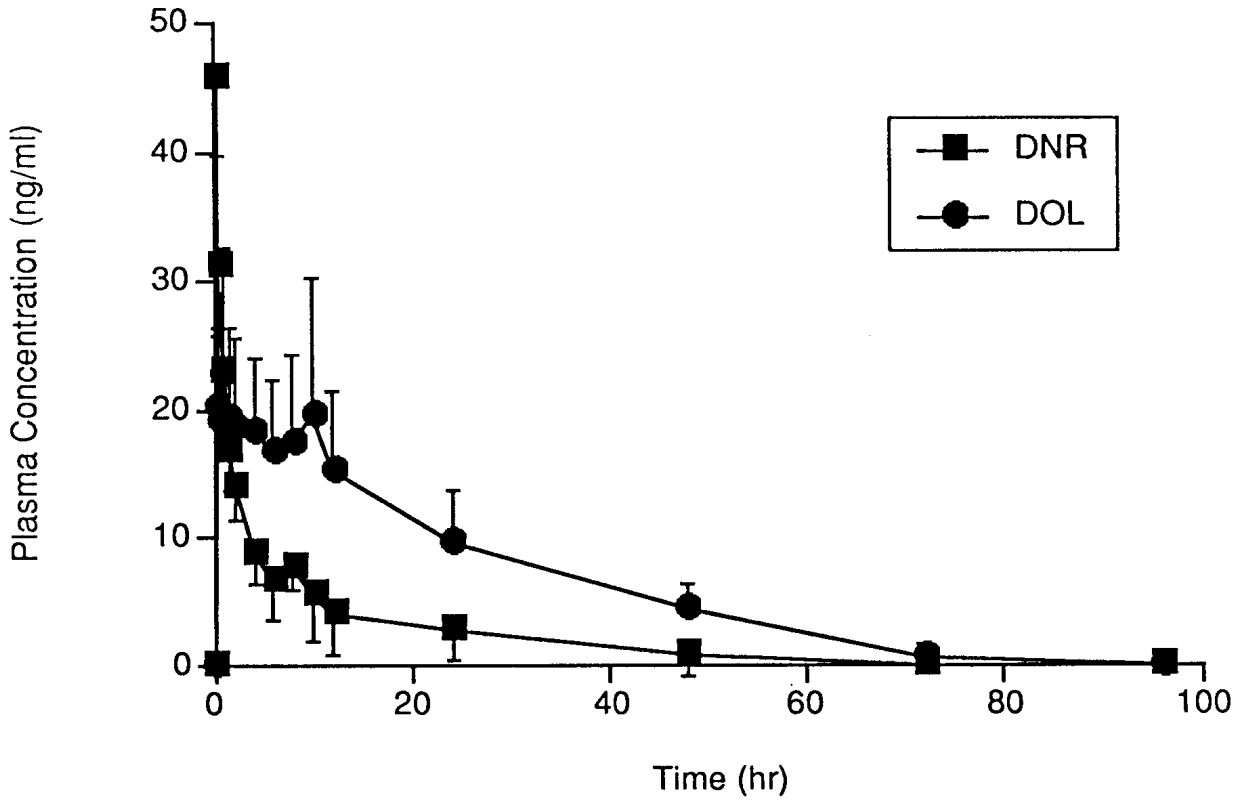


Figure 4.2. Plasma concentration-time curve for DNR and its metabolite DOL for all patients receiving a 25 mg/m^2 dose of DNR. Each point represents the mean ± SD.

Table 4.2. Plasma DNR Pharmacokinetic Parameters

Patient	DOSE (mg)	DNR DOSE (mg/m2)	C max (ng/ml)	T max (min)	AUC (0-24 hr) (ng.hr/ml)	AUMC (0-24 hr) (ng.hr2/ml)	MRT (hr)	CL (L/hr)	Vd (L)
1	85	50	127	18	315	1538	4.9	270	1318
2	45	25	14	40	181	2223	12.3	249	3053
3	40	25	52	19	78	97	1.2	513	638
4	95	50	60	135	267	1521	5.7	356	2027
5	90	50	245	28	713	3184	4.5	126	564
6	80	50	233	17	375	1961	5.2	213	1116
7	40	25	52	16	169	1033	6.1	237	1447
8	40	25	24	60	211	2935	13.9	190	2637
9	90	50	103	18	364	2559	7.0	247	1738
10	40	25	230	16	139	254	1.8	288	526
11	35	25	39	15	232	2184	9.4	151	1420
12	45	25	41	16	200	2260	11.3	225	2543
13	85	50	144	15	591	3790	6.4	144	922
14	100	50	372	17	289	1845	6.4	346	2209
15	85	50	682	17	1216	4607	3.8	70	265
16	40	25	53	21	97	237	2.4	412	1008
17	105	50	163	19	329	1463	4.4	319	1419
18	80	50	126	15	335	2442	7.3	239	1741
19	75	45	1610	15	797	1989	2.5	94	235
20	50	25	49	23	150	860	5.7	333	1911
21	90	50	ND	ND	ND	ND	ND	ND	ND
22	55	35	108	19	268	1476	5.5	205	1130
23	80	50	44855	15	263	1704	6.5	304	1971
24	65	30	64	15	327	2170	6.6	199	1319
25	100	50	121	15	249	1001	4.0	402	1614
26	80	50	120	20	275	4196	15.3	291	4439
27	90	50	255	21	695	4315	6.2	129	804
Average			1921	25	351	2071	6.4	252	1539
SD			8763	24	256	1219	3.5	105	934
N			26	26	26	26	26	26	26

ND = not done

Table 4.3. Plasma DOL Pharmacokinetic Parameters

Patient	DOSE (mg)	DNR DOSE (mg/m2)	C max (ng/ml)	T max (hr)	AUC (0-24 hr) (ng.hr/ml)	AUMC (0-24 hr) (ng.hr2/ml)	MRT (hr)	CL (L/hr)	Vd (L)
1	85	50	134	0.6	1192	11791	10	71	705
2	45	25	17	7.5	266	3267	12	169	2078
3	40	25	31	2.1	342	3300	10	117	1129
4	95	50	85	3.3	1113	9156	8	85	702
5	90	50	154	1.6	2082	19211	9	43	399
6	80	50	115	4.1	1604	15030	9	50	467
7	40	25	24	0.3	305	3305	11	131	1421
8	40	25	43	1.0	915	14204	16	44	679
9	90	50	58	1.0	868	9550	11	104	1141
10	40	25	26	0.3	348	3591	10	115	1186
11	35	25	30	1.5	541	5711	11	65	683
12	45	25	14	6.0	282	3824	14	160	2164
13	85	50	89	7.3	1350	13599	10	63	634
14	100	50	30	8.1	477	5270	11	210	2316
15	85	50	298	1.5	3397	30690	9	25	226
16	40	25	21	2.2	424	7348	17	94	1635
17	105	50	62	0.5	887	8648	10	118	1154
18	80	50	56	4.0	987	11472	12	81	942
19	75	45	57	0.5	593	5538	9	126	1181
20	50	25	27	0.5	332	2544	8	151	1154
21	90	50	ND	ND	ND	ND	ND	ND	ND
22	55	35	63	1.0	1073	11512	11	51	550
23	80	50	1125	0.3	717	7537	11	112	1173
24	65	30	32	12.0	590	6705	11	110	1252
25	100	50	99	1.4	1309	12173	9	76	710
26	80	50	126	1.6	1606	14681	9	50	455
27	90	50	73	2.0	1401	14891	11	64	683
Average			111	2.8	962	9790	11	96	1032
SD			215	3.0	697	6271	2	45	547
N			26	26	26	26	26	26	26

ND = not done

Table 4.4. Plasma DNR Pharmacokinetic Parameters adjusted for administered dose of DNR

Patient	DOSE (mg)	C max (ng/ml/mg DNR)	AUC (0-24 hr) (ng.hr/ml/mg DNR)	AUMC (0-24 hr) (ng.hr2/ml/mg DNR)
1	85	1.5	3.7	18
2	45	0.3	4.0	49
3	40	1.3	2.0	2
4	95	0.6	2.8	16
5	90	2.7	7.9	35
6	80	2.9	4.7	25
7	40	1.3	4.2	26
8	40	0.6	5.3	73
9	90	1.1	4.0	28
10	40	5.7	3.5	6
11	35	1.1	6.6	62
12	45	0.9	4.4	50
13	85	1.7	7.0	45
14	100	3.7	2.9	18
15	85	8.0	14.3	54
16	40	1.3	2.4	6
17	105	1.6	3.1	14
18	80	1.6	4.2	31
19	75	21.5	10.6	27
20	50	1.0	3.0	17
21	90	ND	ND	ND
22	55	2.0	4.9	27
23	80	560.7	3.3	21
24	65	1.0	5.0	33
25	100	1.2	2.5	10
26	80	1.5	3.4	52
27	90	2.8	7.7	48
Average		2.8	4.9	31
SD		4.3	2.8	19
N		26	26	26

ND = not done

Table 4.5. Plasma DOL Pharmacokinetic Parameters adjusted for administered dose of DNR

Patient	DOSE (mg)	C max (ng/ml/mg DNR)	AUC (0-24 hr) (ng.hr/ml/mg DNR)	AUMC (0-24 hr) (ng.hr2/ml/mg DNR)
1	85	1.6	14.0	139
2	45	0.4	5.9	73
3	40	0.8	8.6	83
4	95	0.9	11.7	96
5	90	1.7	23.1	213
6	80	1.4	20.1	188
7	40	0.6	7.6	83
8	40	1.1	22.9	355
9	90	0.6	9.6	106
10	40	0.7	8.7	90
11	35	0.8	15.5	163
12	45	0.3	6.3	85
13	85	1.0	15.9	160
14	100	0.3	4.8	53
15	85	3.5	40.0	361
16	40	0.5	10.6	184
17	105	0.6	8.4	82
18	80	0.7	12.3	143
19	75	0.8	7.9	74
20	50	0.5	6.6	51
21	90	ND	ND	ND
22	55	1.1	19.5	209
23	80	14.1	9.0	94
24	65	0.5	9.1	103
25	100	1.0	13.1	122
26	80	1.6	20.1	184
27	90	0.8	15.6	165
Average		1.5	13.3	141
SD		2.7	7.6	80
N		26	26	26

ND = not done

infusion, whereas the metabolite DOL reached a maximum concentration of 1.0 ± 0.7 (n = 25) $ng.ml^{-1}.mg^{-1}$ DNR 2.8 hrs post-infusion.

The overall availability of a drug is measured by the AUC and AUMC. The plasma AUC (0-24 hr) for DNR was 4.9 ± 2.8 (n = 26) $ng.hr.ml^{-1}.mg^{-1}$ DNR as compared to the plasma AUC (0-24 hr) for DOL of 13.3 ± 7.6 (n = 26) $ng.hr.ml^{-1}.mg^{-1}$ DNR. Thus, there were significantly greater concentrations of the metabolite DOL present in the plasma than there were of the parent drug DNR ($p < 0.0001$, W). Similar findings were seen with the AUMC (0-24 hr) that were 31 ± 19 $ng.hr^2.ml^{-1}.mg^{-1}$ DNR for DNR and 141 ± 80 $ng.hr^2.ml^{-1}.mg^{-1}$ DNR for DOL ($p < 0.0001$, W). Therefore, as seen in Figures 4.1 and 4.2, there were higher amounts of the metabolite DOL present in the plasma than the parent drug DNR (3 to 1 by AUC and 5 to 1 by AUMC).

A measure of the amount of time the drug is in the body, is the mean residence time (MRT). The average plasma MRT for DNR is 6.4 ± 3.5 (n = 26) hr and the average plasma MRT for the metabolite DOL was 10.7 ± 2.1 (n = 26) hr. The MRT for DOL was significantly greater than the MRT for DNR ($p < 0.0001$, W). Therefore not only was there a greater accumulation of the metabolite in plasma, but DOL remained also in the plasma for a longer period of time.

Clearance is a measure of elimination of the drug. The plasma clearance of DNR was 252 ± 105 (n = 26) $L.hr^{-1}$ and DOL was 96 ± 45 (n = 26) $L.hr^{-1}$. Therefore, as would be expected from the MRT, DNR was removed from the plasma at a greater rate than the metabolite DOL ($p < 0.0001$, W).

The apparent volume of distribution (Vd) is a measure of drug lipophilicity. The Vd for the parent drug DNR was 1539 ± 934 (n = 26) L and the Vd of the metabolite DOL was 1031 ± 547 (n = 26) L ($p < 0.01$, W). This indicates both DNR and DOL are distributed into tissues quite readily.

4.4.2. Cellular

The cellular concentration-time curves for the 14 evaluable patients were plotted, and the average cellular concentration-time curve for DNR and DOL for patients receiving a 50 mg/m^2 dose of DNR is shown in Figure 4.3. As was evident with the plasma concentrations, there is a great deal of inter-individual variation in both the DNR and DOL

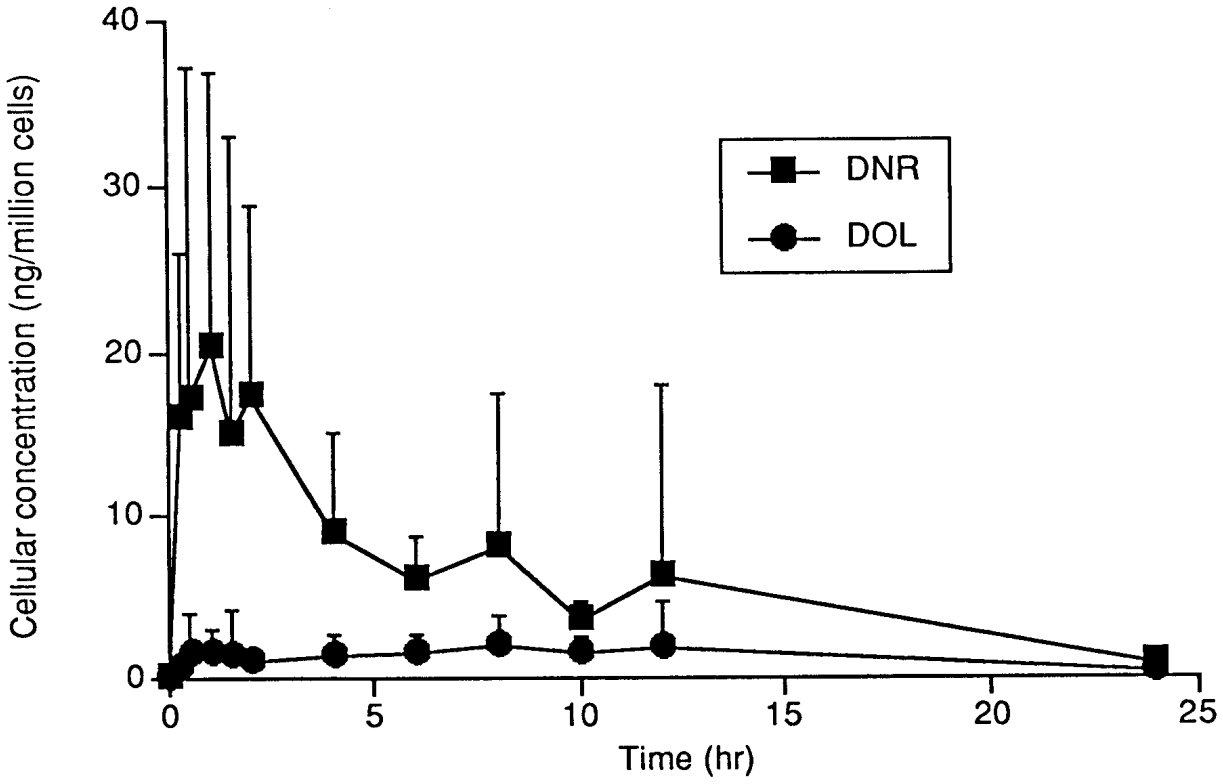


Figure 4.3. Cellular concentration-time curve for DNR and its metabolite DOL for all patients receiving a 50 mg/m^2 dose of DNR. Each point represents the mean ± SD.

cellular concentrations. Figure 4.3 demonstrates that in contrast to plasma drug concentrations of DNR and DOL, cellular DNR concentrations are greater than cellular DOL concentrations.

The cellular DNR pharmacokinetic parameters are given in Table 4.6 and the cellular DOL pharmacokinetic parameters are given in Table 4.7. Because not all patients received the same dose of DNR, the pharmacokinetic parameters Cmax, AUC (0-24 hr) and AUMC (0-24 hr) of all the patients with cellular samples were corrected for dose (Chapter 2.2.9) and are given in Tables 4.8 and 4.9 for DNR and DOL, respectively.

The mean time to reach maximum cellular drug concentrations was 1.1 hr for DNR and 7.7 hr for DOL. The average peak cellular DNR concentration was 0.5 ± 0.9 ($n = 15$) $ng.10^{-6}$ $cells.mg^{-1}$ DNR. The average peak cellular DOL concentration was 0.04 ± 0.05 ($n = 15$) $ng.10^{-6}$ $cells.mg^{-1}$ DNR. Therefore cellular DNR levels peaked earlier and at higher concentrations than the metabolite DOL.

The cellular AUC (0-24 hr) for DNR was 1.4 ± 1.1 ($n = 15$) $ng.hr.10^{-6}$ $cells.mg^{-1}$ DNR and the cellular AUC (0-24 hr) for DOL was 0.4 ± 0.3 ($n = 15$) $ng.hr.10^{-6}$ $cells.mg^{-1}$ DNR. Thus, there were significantly greater concentrations of the parent drug DNR present at the cellular level than there was of the metabolite DOL ($p < 0.001$, W). Similar findings were seen with the AUMC (0-24 hr) that were 11 ± 11 $ng.hr^2.10^{-6}$ $cells.mg^{-1}$ DNR for DNR and 3.9 ± 3.3 $ng.hr^2.10^{-6}$ $cells.mg^{-1}$ DNR for the metabolite DOL ($p < 0.001$, W). Therefore, the ratio of metabolite DOL to parent drug DNR was 1 to 4 by AUC and 1 to 3 by AUMC; this was the inverse of results obtained in plasma.

The average cellular MRT for DNR was 7.9 ± 3.0 ($n = 15$) hr and that for the metabolite DOL was 11.6 ± 3.1 (15) hr. The MRT for DOL was significantly greater than the MRT for DNR ($p < 0.01$, W). Therefore, although there were higher levels of DNR than the metabolite DOL present at the cellular level, the metabolite remained within the cells for a longer period of time.

4.5. Relationships between Pharmacokinetics and Patient Response

The pharmacokinetic parameters for both plasma and cells have been described above. One question to be addressed is: "Is there a relationship between patient

Table 4.6. Cellular DNR Pharmacokinetic Parameters

Patient	DOSE (mg)	DNR DOSE (mg/m2)	C max (ng/million cells)	T max (hr)	AUC (0-24 hr) (ng.hr/million cells)	AUMC (0-24 hr) (ng.hr2/million cells)	MRT (hr)	CL (L/hr)	Vd (L)
2	45	25	2	1.5	17	182	10.7	2647	28339
4	95	50	28	1.5	122	735	6.0	779	4691
6	80	50	22	1.0	72	464	6.4	1111	7160
7	40	25	20	1.0	122	991	8.1	328	2663
8	40	25	3	1.0	30	473	15.8	1333	21022
9	90	50	49	1.0	398	4026	10.1	226	2287
11	35	25	3	0.5	41	409	10.0	854	8516
12	45	25	170	0.3	88	385	4.4	511	2237
13	85	50	7	2.0	65	526	8.1	1308	10582
14	100	50	13	2.1	47	201	4.3	2128	9099
15	85	50	19	1.0	123	667	5.4	691	3747
18	80	50	28	0.3	99	739	7.5	808	6032
24	65	30	11	1.0	64	535	8.4	1016	8490
26	80	50	58	1.6	169	849	5.0	473	2378
27	90	50	7	0.5	64	519	8.1	1406	11404
Average			29	1.1	101	780	7.9	1041	8577
SD			42	0.6	92	925	3.0	661	7357
N			15	15	15	15	15	15	15

Table 4.7. Cellular DNR Pharmacokinetic Parameters adjusted for administered dose of DNR

Patient	DOSE (mg)	C max (ng/million cells/mg DNR)	AUC (0-24 hr) (ng.hr/million cells/mg DNR)	AUMC (0-24 hr) (ng.hr2/million cells/mg DNR)
2	45	0.0	0.4	4.0
4	95	0.3	1.3	7.7
6	80	0.3	0.9	5.8
7	40	0.5	3.1	24.8
8	40	0.1	0.8	11.8
9	90	0.5	4.4	44.7
11	35	0.1	1.2	11.7
12	45	3.8	2.0	8.6
13	85	0.1	0.8	6.2
14	100	0.1	0.5	2.0
15	85	0.2	1.4	7.8
18	80	0.3	1.2	9.2
24	65	0.2	1.0	8.2
26	80	0.7	2.1	10.6
27	90	0.1	0.7	5.8
Average		1.5	13.3	141
SD		2.7	7.6	80
N		26	26	26

Table 4.8. Cellular DOL Pharmacokinetic Parameters

Patient	DOSE (mg)	DNR DOSE (mg/m2)	C max (ng/million cells)	T max (hr)	AUC (0-24 hr) (ng.hr/million cells)	AUMC (0-24 hr) (ng.hr2/million cells)	MRT (hr)	CL (L/hr)	Vd (L)
2	45	25	0.4	7.5	8	119	15	5625	83672
4	95	50	1.4	3.3	11	149	14	8636	116983
6	80	50	2.9	4.1	30	288	10	2667	25600
7	40	25	2.1	6.3	31	267	9	1290	11113
8	40	25	0.3	1.0	7	54	8	5714	44082
9	90	50	15.9	10.3	98	1178	12	918	11039
11	35	25	1.2	10.0	16	226	14	2188	30898
12	45	25	2.9	0.3	11	166	15	4091	61736
13	85	50	0.6	32.1	4	54	14	21250	286875
14	100	50	1.1	6.1	8	47	6	12500	73438
15	85	50	3.9	8.1	32	240	8	2656	19922
18	80	50	1.2	2.0	20	291	15	4000	58200
24	65	30	0.8	12.0	12	155	13	5417	69965
26	80	50	7.6	1.6	61	580	10	1311	12470
27	90	50	1.0	10.3	13	180	14	6923	95858
Average			2.9	7.7	24	266	12	5679	66790
SD			4.1	7.7	25	285	3	5319	69228
N			15	15	15	15	15	15	15

Table 4.9. Cellular DOL Pharmacokinetic Parameters adjusted for administered dose of DNR

Patient	DOSE (mg)	C max (ng/million cells/mg DNR)	AUC (0-24 hr) (ng.hr/million cells/mg DNR)	AUMC (0-24 hr) (ng.hr2/million cells/mg DNR)
2	45	0.01	0.18	2.64
4	95	0.02	0.12	1.57
6	80	0.04	0.38	3.60
7	40	0.05	0.78	6.68
8	40	0.01	0.18	1.35
9	90	0.18	1.09	13.09
11	35	0.03	0.46	6.46
12	45	0.06	0.24	3.69
13	85	0.01	0.05	0.64
14	100	0.01	0.08	0.47
15	85	0.05	0.38	2.82
18	80	0.02	0.25	3.64
24	65	0.01	0.18	2.38
26	80	0.09	0.76	7.25
27	90	0.01	0.14	2.00
Average		0.04	0.35	3.88
SD		0.05	0.30	3.31
N		15	15	15

pharmacokinetics and response to treatment?". In order to answer this question the response of each patient to treatment was recorded as described (Chapter 4.3) and the patients were divided into two groups. The first group included those patients that attained a complete remission (termed the CR group) and the second those patients that only achieved a partial response or no response at all (termed non responders, NR).

4.5.1. Plasma Pharmacokinetics

Table 4.10 and 4.11 summarises the pharmacokinetic parameters in relation to patient responses for DNR and its metabolite DOL, respectively. There were no significant differences between the two patient groups in the following plasma pharmacokinetic parameters for either drug; Cmax, Tmax, MRT, CL, Vd, AUC (0-24 hr) or AUMC (0-24 hr). This suggests that for the patients studied there was no apparent relationship between any of the plasma DNR or DOL pharmacokinetic parameters measured and patient response.

4.5.2. Cellular Pharmacokinetics

The relationship between cellular DNR pharmacokinetic parameters and patient response are summarised in Table 4.12. There were no statistical differences found between the cellular Tmax, MRT or AUMC (0-24 hr) between those patients who achieved complete remission compared to those patients who did not respond to treatment. There was, however, a significantly higher ($p < 0.04$, MW) maximum mean cellular concentration of DNR in those patients achieving complete remission (0.7 ± 1.2 ($n = 9$) $ng.10^{-6}$ cells.mg^{-1} DNR) compared to patients not responding to treatment (0.1 ± 0.05 ($n = 5$) $ng.10^{-6}$ cells.mg^{-1} DNR). With respect to the AUC (0-24 hr) there were also significantly higher values ($p < 0.02$, MW) in complete remission patients (1.9 ± 1.2 ($n = 9$) $ng.hr.10^{-6}$ cells.mg^{-1} DNR) compared to the non responders (0.7 ± 0.2 ($n = 5$) $ng.hr.10^{-6}$ cells.mg^{-1} DNR). There appeared to be higher amounts of DNR as determined by AUMC (0-24 hr) in patients that responded to treatment (14 ± 13 ($n = 9$) $ng.hr^2.10^{-6}$ cells.mg^{-1} DNR) compared to those that did not respond (6.8 ± 3.6 ($n = 5$) $ng.hr^2.10^{-6}$ cells.mg^{-1} DNR), but these were not significantly different. These results indicate that both the maximum cellular DNR concentration and the cellular DNR AUC (0-24 hr) are important in determining whether a patient will respond to DNR combination chemotherapy or not. Clearly, the higher the cellular DNR concentration, the more likely a patient will respond to the DNR treatment.

The relationship between the cellular DOL pharmacokinetic parameters and

Table 4.10. Relationship between the plasma DNR pharmacokinetic parameters and patient response (mean ± SD)

Pharmacokinetic Parameter	Patients achieving Complete Remission (12)	Patients not responding to treatment (11)
T max (min)	30 ± 33	22 ± 19
C max ($ng.ml^{-1}.mg^{-1}$ DNR)	1.7 ± 2.0	2.3 ± 1.5 (10)
MRT (hr)	7.3 ± 4.1	6.1 ± 3.0
CL ($L.hr^{-1}$)	277 ± 117	229 ± 80
Vd (L)	1853 ± 1117	1347 ± 679
AUC (0-24 hr) ($ng.hr.ml^{-1}.mg^{-1}$ DNR)	4.8 ± 2.6	4.6 ± 3.3
AUMC (0-24 hr) ($ng.hr^{2}.ml^{-1}.mg^{-1}$ DNR)	33 ± 20	31 ± 19

Table 4.11. Relationship between the plasma DOL pharmacokinetic parameters and patient response (mean ± SD)

Pharmacokinetic Parameter	Patients achieving Complete Remission (12)	Patients not responding to treatment (11)
T max (hr)	2.6 ± 2.2	3.1 ± 4.0
C max ($ng.ml^{-1}.mg^{-1}$ DNR)	0.94 ± 0.87	0.94 ± 0.46 (10)
MRT (hr)	11 ± 2.6	11 ± 1.7
CL ($L.hr^{-1}$)	103 ± 45	91 ± 49
Vd (L)	1144 ± 603	976 ± 535
AUC (0-24 hr) ($ng.hr.ml^{-1}.mg^{-1}$ DNR)	13 ± 9.5	14 ± 6.3
AUMC (0-24 hr) ($ng.hr^{2}.ml^{-1}.mg^{-1}$ DNR)	134 ± 84	151 ± 86

Table 4.12. Relationship between the cellular DNR pharmacokinetic parameters and patient response (mean ± SD)

Pharmacokinetic Parameter	Patients achieving Complete Remission (9)	Patients not responding to treatment (5)	p =,MW
T max (hr)	0.95 ± 0.52	1.3 ± 0.69	0.30
C max ($ng.10^{-6}$ cells.mg^{-1} DNR)	0.73 ± 1.2	0.10 ± 0.045	0.04
MRT (hr)	7.5 ± 2.4	8.9 ± 4.2	0.74
CL ($L.hr^{-1}$)	813 ± 722	1438 ± 413	0.02
Vd (L)	6766 ± 8359	12119 ± 5110	0.03
AUC (0-24 hr) ($ng.hr.10^{-6}$ cells.mg^{-1} DNR)	1.9 ± 1.2	0.73 ± 0.18	0.02
AUMC (0-24 hr) ($ng.hr^{2}.10^{-6}$ cells.mg^{-1} DNR)	14.4 ± 12.8	6.8 ± 3.6	0.16

patient response are summarised in Table 4.13. There were no statistical differences found between the cellular Tmax or MRT in those patients who achieved complete remission compared to those patients who did not respond to treatment. There was, however, a significantly higher ($p < 0.01$, MW) maximum mean DOL concentration in those patients achieving complete remission (0.06 ± 0.05 (n = 9) $ng.10^{-6}$ $cells.mg^{-1}$ DNR) compared to patients not responding to treatment (0.01 ± 0.00 (n = 5) $ng.10^{-6}$ $cells.mg^{-1}$ DNR). There were also significantly higher ($p < 0.02$, MW) AUC (0-24 hr) in complete remission patients (0.47 ± 0.33 (n = 9) $ng.hr.10^{-6}$ $cells.mg^{-1}$ DNR) compared to the non responders (0.13 ± 0.06 (n = 5) $ng.hr.10^{-6}$ $cells.mg^{-1}$ DNR). The AUMC (0-24 hr) was also significantly higher ($p < 0.01$, MW) in complete remission patients (5.3 ± 3.5 (n = 9) $ng.hr^2.10^{-6}$ $cells.mg^{-1}$ DNR) compared to non responders (1.4 ± 0.83 (n = 5) $ng.hr^2.10^{-6}$ $cells.mg^{-1}$ DNR). These results indicate that the maximum cellular DOL concentration, the cellular DOL AUC (0-24 hr) and the cellular DOL AUMC (0-24 hr) were also important in determining whether a patient will respond to DNR combination chemotherapy or not. As with DNR, the higher the cellular DOL concentrations present the more likely a patient will respond to treatment. Although the high cellular concentrations of DOL may be due to the high levels of DNR present in the cells. Therefore, both the parent drug DNR and its metabolite DOL may be useful in determining whether a patient will respond to treatment.

4.6. P glycoprotein

The overexpression of Pgp was measured by the immunocytochemistry method described in Chapter 2.2.10. Two monoclonal antibodies C 219 and JSB 1 were used to detect Pgp. A blood sample was taken from each available patient at the time of the pre infusion blood sample, and the Pgp expression results are reported in Table 4.14. Of the 27 patients studied, Pgp levels were only measured in 25 (two patients had inadequate samples). Fourteen patients had blast cells that were Pgp negative for C 219 and 11 patients had blast cells that were Pgp positive. Using the JSB 1 antibody 17 patients had blast cells that were Pgp negative and 8 patients had blast cells that were Pgp positive. The correlation between the percentage of blasts stained for the antibodies C 219 and JSB 1, using the Spearman-rank correlation was quite good, with a r value of 0.83, indicating good correlation between the two antibodies. Since there was a good correlation between the two antibodies and since it appears that C 219 is slightly more sensitive than JSB 1, the remaining results expressed in this chapter (unless stated otherwise) will refer to the C 219 antibody only.

Table 4.13. Relationship between the cellular DOL pharmacokinetic parameters and patient response (mean ± SD)

Pharmacokinetic Parameter	Patients achieving Complete Remission (9)	Patients not responding to treatment (5)	p =, MW
T max (hr)	5.5 ± 3.8	12.3 ± 11.9	0.21
C max ($ng.10^{-6}$ cells.mg^{-1} DNR)	0.06 ± 0.05	0.01 ± 0.00	0.005
MRT (hr)	12 ± 2.9	11 ± 3.7	0.26
CL ($L.hr^{-1}$)	3412 ± 2505	10361 ± 6727	0.01
Vd (L)	45115 ± 37615	114044 ± 98346	0.07
AUC (0-24 hr) ($ng.hr.10^{-6}$ cells.mg^{-1} DNR)	0.47 ± 0.33	0.13 ± 0.06	0.01
AUMC (0-24 hr) ($ng.hr^{2}.10^{-6}$ cells.mg^{-1} DNR)	5.3 ± 3.5	1.4 ± 0.83	0.006

Table 4.14. Pgp measurements for patients in the pharmacokinetic study

Patients	% Blasts	C 219		JSB 1		Response
		% Pgp +ve	quantitative	% Pgp +ve	quantitative	
1	90	0	negative	0	negative	N
2	80-90	0	negative	0	negative	C
3	80	0	negative	0	negative	C
4	40-50	100	positive	100	positive	C
5	20	100	positive	100	positive	N
6	>90	0	negative	0	negative	NE
7	50	0	negative	0	negative	C
8	2		N/A		N/A	N
9	90	0	negative	0	negative	C
10	60-70	0	negative	0	negative	N
11			N/A		N/A	C
12	60-70	0	negative	0	negative	C
13	90	20	positive	0	negative	N
14	80	100	positive	10	positive	N
15	45-50	100	positive	25	positive	C
16	63	0	negative	0	negative	C
17	90	100	positive	5	positive	N
18	80	0	negative	0	negative	C
19	50	0	negative	0	negative	NE
20	90	20	positive	5	positive	C
21	80	0	negative	0	negative	NE
22	50	100	positive	10	positive	N
23	80	40	positive	0	negative	N
24	75	100	positive	0	negative	N
25	45-50	0	negative	0	negative	NE
26	50-60	100	positive	100	positive	C
27	50	0	negative	0	negative	N

N/A = not available NE = not evaluable

4.7. Relationship between P glycoprotein and Patient Response

The blast cells from 25 patients were stained with the Pgp antibody C 219. Of the 25 patients, 4 were not evaluable because the patients died, but all 4 had blast cells that were Pgp negative. Of the remaining 21 patients, 10 had blast cells that were Pgp negative and 11 had blast cells that were Pgp positive. Seven of the patients with Pgp negative blast cells achieved complete remission, while three did not respond to treatment. Four of the patients with Pgp positive blast cells attained complete remission and the seven patients with Pgp positive blast cells did not respond to chemotherapy. Overall there was no statistical relationship between the presence of Pgp and the patients' response to treatment ($p = 0.20$, FE).

4.8. Relationship between P glycoprotein and Cellular Pharmacokinetics.

Pgp is found on the cellular membrane of patient blast cells. If Pgp were acting as an efflux pump, there would be decreased intracellular drug concentrations in those patients that are Pgp positive compared to those that are Pgp negative. Therefore the relationship between Pgp and cellular drug accumulation of DNR and its metabolite DOL was examined. Table 4.15 summarises the relationships between cellular AUC and AUMC and Pgp. The DNR AUC (0-24 hr) for the Pgp negative patients was 2 ± 1.5 ($n = 6$) ng.hr.10^{-6} cells.mg^{-1} DNR and for the Pgp positive patients was 1.2 ± 0.6 ($n = 6$) ng.hr.10^{-6} cells.mg^{-1} DNR. Although the Pgp negative patients had a slightly higher AUC than the Pgp positive patients, this did not reach statistical significance ($p = 0.63$, MW). Similar results were seen with the AUMC (0-24 hr): the Pgp negative patients had 16.2 ± 15.8 ($n = 6$) ng.hr^2.10^{-6} cells.mg^{-1} DNR and the Pgp positive patients had 7.1 ± 2.9 ($n = 6$) ng.hr^2.10^{-6} cells.mg^{-1} DNR; again no statistical significance was reached ($p = 0.33$, MW).

The DOL AUC (0-24 hr) for the Pgp negative patients was 0.45 ± 0.39 ($n = 6$) ng.hr.10^{-6} cells.mg^{-1} DNR and for the Pgp positive patients was 0.26 ± 0.27 ($n = 6$) ng.hr.10^{-6} cells.mg^{-1} DNR. Although the Pgp negative patients had a slightly higher AUC than the Pgp positive patients, this result did not reach statistical significance ($p = 0.17$, MW). Similar results were seen with the AUMC (0-24 hr); the Pgp negative patients accumulated 5.3 ± 4.1 ($n = 6$) ng.hr^2.10^{-6} cells.mg^{-1} DNR and the Pgp positive patients 2.5 ± 2.5 ($n = 6$) ng.hr^2.10^{-6} cells.mg^{-1} DNR. Again statistical significance was not achieved ($p = 0.11$, MW).

Table 4.15 Relationship between P-glycoprotein and intracellular DNR or DOL (mean ± SD (n))

	P-glycoprotein		
	positive	negative	p = , MW
DNR AUC (0-24 hr) ($ng.hr.10^{-6}$ cells.mg^{-1} DNR)	1.2 ± 0.58 (6)	2.0 ± 1.5 (6)	0.63
DOL AUC (0-24 hr) ($ng.hr.10^{-6}$ cells.mg^{-1} DNR)	0.26 ± 0.27 (6)	0.45 ± 0.39 (6)	0.17
DNR AUMC (0-24 hr) ($ng.hr^{2}.10^{-6}$ cells.mg^{-1} DNR)	7.1 ± 2.9 (6)	16.2 ± 15.8 (6)	0.34
DOL AUMC (0-24 hr) ($ng.hr^{2}.10^{-6}$ cells.mg^{-1} DNR)	2.5 ± 2.5 (6)	5.3 ± 4.1 (6)	0.11

4.9. Discussion

Complete remission in AML patients treated with DNR combination chemotherapy ranges from 28% (Priesler *et al.*, 1986) to 82% (Gale *et al.*, 1981). The rate of complete remission achieved in AML patients in this study was 31% when all AML patients were included, or 42% complete remission when only the evaluable patients were included. These findings are in agreement with those in the literature. The Toronto leukaemia study group (TLSG, 1986) found that the complete remission rate is very dependent on the way in which the results are analysed. In that study, when no exclusions of AML patients were made the complete remission rate was 43.8%. When only evaluable patients were examined the complete remission rate increased to 60% and when patients over the age of 70 were excluded the complete remission rate increased to 76%. In this study if patients above the age of 70 years were exclude, the complete remission rate increased to 63%. Therefore although the percentage of complete remission is below that of the TLSG study, there is a trend towards their findings. It is clearly important in all patient trials to state the exact exclusion criteria used so researchers are able to compare the results of different studies.

The complete remission response rate in ALL patients ranges from 47% (Gottlieb *et al.*, 1984) to 91% (Linker *et al.*, 1987) with most studies reporting complete remission rates of 70-80%. The complete remission rate achieved in this study for ALL patients was 63% overall, increasing to 77% for de novo ALL patients, and hence in agreement with the expected response rates for ALL patients.

The pharmacokinetic data obtained in this study of leukaemia patients receiving DNR showed, over the time period studied, that the plasma concentrations of the metabolite DOL were significantly higher than the parent drug DNR. However, within the white cells studied the reverse was true i.e. DNR levels were consistently higher than DOL. These results are consistent with previous studies (Speth *et al.*, 1987b; Kokenberg *et al.*, 1988; Paul *et al.*, 1989). DNR is extensively metabolised to DOL and this is predominantly achieved in the liver by an aldo/keto reductase (daunorubicin reductase) (Felsted & Bachur, 1982). The fact that the cellular concentration of the metabolite was relatively low suggests that there is minimal metabolism of DNR at the cellular level and that DOL does not efficiently cross the cell membrane. Huffman & Bachur (1972) have shown that daunorubicin reductase is present in the cells of patients with acute leukaemia. The present results indicate that the presence and the activity of this enzyme in leukaemic cells must be low. Incubating the leukaemic cell line CEM with DNR over 4 hours did not produce any measurable DOL,

confirming the lack of or extremely low level of daunorubicin reductase in these cells. Furthermore, when the metabolite DOL was incubated with CEM cells, only 14% of the metabolite was accumulated compared to the amount of DNR that was accumulated (Chapter 3.8). Therefore, it appears that the differences between plasma and cellular concentrations of DNR are due to the inability of DOL to cross the cell membrane and the lack of daunorubicin reductase in the cells.

There have been few reports on the correlation of plasma and cellular DNR pharmacokinetics and clinical response. In the present study, no correlations between patient response and plasma pharmacokinetics were observed. The average (± S.D.) plasma MRT and plasma clearance of DNR for all patients were 6.4 ± 3.5 hr and 252 ± 105 L/hr, respectively, which is similar to the values obtained by Speth *et al.* (1987b) and Kokenberg *et al.* (1988). Kokenberg *et al.* (1988) found that there were no differences between plasma AUC for DNR or DOL compared to patient response. They also reported no relationship between any other plasma pharmacokinetic parameter and patient response. In this study an inconsistency was noted: patients that received 25 mg/m^2 dose of DNR achieved only approximately one-third of the plasma AUC (0-24 hr) of DNR (170 ± 53) compared to those patients that received 50 mg/m^2 (517 ± 296). One explanation might be that patients receiving a 50 mg/m^2 of DNR, also received Ara C and VP16 in combination, while those receiving a 25 mg/m^2 dose received prednisolone and vincristine in combination. This suggests that either the combination of Ara C and VP16 increases the plasma AUC of DNR, or prednisolone and vincristine decrease the plasma AUC of DNR. Nearly all chemotherapeutic protocols involve the use of more than one agent but there is no good data available on the pharmacokinetic interactions between DNR and any other agent used in chemotherapeutic regimens. However, it would be expected that the concomitant administration of other chemotherapeutic agents would influence the pharmacokinetics of DNR, and this may contribute also to the wide variation observed in measured parameters. The findings reported here would suggest that the combination of DNR, Ara C and VP 16 may be more efficacious.

In this study there was a significant difference in both cellular DNR and DOL levels in those patients that underwent complete remission compared to those that did not respond. This is in contrast to the report of Kokenberg *et al.* (1988) who found that there was no correlation between any pharmacokinetic parameter and response to therapy. One possible explanation for the differences is that Kokenberg *et al.* (1988) compared intracellular concentrations at a single time point (10 minutes after DNR infusion), whereas, the cellular concentration for a 24 hour period and expressed as AUC (0-24 hr) was analysed in this study. A recent study by Marie *et al.* (1993) using a flow cytometric method for detection

of DNR, has shown similar findings to the present study *in vitro*. In the Marie *et al.* (1993) study, 35 evaluable AML patients showed increased cellular DNR concentrations in the leukaemic cells of those patients achieving complete remission (63.6% ± 22.7% DNR uptake) compared to those not responding to treatment (21.6% ± 25.2% DNR uptake). Other drugs used in induction therapy, were given to both responders and non responders, and thus affect both groups equally. In spite of the variables i.e. different drug concentrations, different chemotherapy regimens and different types of leukaemia, a significant difference was observed in the cellular drug concentration between patients responding and those not responding to chemotherapy, implying the correlation was independent of these factors.

This present work appears to be the first investigation of the relationship between Pgp expression and intracellular levels of DNR in patients undergoing chemotherapy. The cellular concentrations of DNR and DOL tended to be lower in those patients with blasts cells that were Pgp positive compared to those with Pgp negative cells (Table 4.15), without reaching a statistically significant difference. Overexpression of Pgp might not be the sole explanation for the lower cellular DNR in patient leukaemic cells. Further studies are required before this can be determined. One possible reason for the lower cellular DNR in patient leukaemic cells could be due to the presence of non-Pgp mechanisms of resistance such as that associated with the HL 60/ADR cell line (Marsh *et al.*, 1986 and Chapter 3.5.4). In that drug resistant cell line there was a decrease in intracellular drug concentration, but no detectable Pgp. Recently, Krishnamachary & Center (1993) have demonstrated the presence of another membrane protein that may be responsible for the decreased cellular drug accumulation present in the HL 60/ADR cell line. This membrane protein has been associated with the overexpression of the *MRP* gene that may play a role in patients with acute leukaemia that do not respond to treatment.

Previous studies looking at the relationship between Pgp and patient response have shown conflicting findings. Chan *et al.* (1991) observed a correlation between Pgp and patient response. Twenty-six out of 31 non-localised neuroblastoma patients who were Pgp negative had a complete remission after treatment, as compared with 6 of 13 patients who were Pgp positive. Campos *et al.* (1992) had similar findings with acute nonlymphoblastic leukaemia in which complete remission rates were significantly lower in Pgp positive patients (23/71, 32%) compared to Pgp negative patients (64/79, 81%). Marie *et al.* (1991) and Pirker *et al.* (1991) also found a correlation between Pgp (mdr 1 gene expression) and patient response. Marie *et al.* (1991) observed a complete remission in 67% of patients with undetectable mdr 1 expression, versus 29% of patients with increased expression. Pirker *et al.* (1991) found the complete remission rate to be 89% in mdr 1 RNA negative patients and 53% in mdr 1 positive patients. In contrast to the above findings, Holmes *et al.* (1989)

established that the amplification of the Pgp gene was not an important mechanism in previously untreated AML. In that study, elevated levels of mdr 1 were seen in two out of eight cases of untreated AML, five out of eight refractory AML and four of five cases of secondary AML. Rothenberg *et al.* (1989) observed that 8/9 patients with ALL at presentation had low levels of mdr 1 mRNA. In 5 patients at primary relapse, none had evidence of mdr 1 overexpression and 3/15 patients with multiple relapses had elevated mdr 1 expression. They concluded that Pgp might play a role in some cases of drug resistance but that other mechanisms of resistance must exist. The present study has found no significant relationship between Pgp and patient response. Of the patients in this study 18/21 were previously untreated. Nine of these patients had blast cells that were Pgp negative for C 219, with seven achieving complete remission (78%), and nine were Pgp positive (4/9 achieving CR, 44%). Of the 3 patients that were previously treated, 2 had blast cells that were Pgp positive and 1 had blast cells that were Pgp negative. None of these patients responded to treatment. The findings of this study are similar to those of Rothenberg *et al.* (1989) in which low levels of Pgp were shown at induction but higher levels of Pgp were shown in multiple relapse patients.

In conclusion, no correlation was found between any of the plasma pharmacokinetic parameters and patient response. However, a correlation between the intracellular DNR and DOL concentrations and patient response was observed in this study. The relationship between Pgp and intracellular drug concentrations was also examined. Although there was no statistical correlation between Pgp and intracellular drug concentrations, there was a tendency for patients that were Pgp positive to have decreased intracellular concentrations of DNR and DOL. A higher proportion of previously treated patients had blast cells that were Pgp positive but the correlation between Pgp and patient response was not statistically significant. These findings suggest that mechanism(s) of drug resistance other than Pgp are also important in clinical resistance to DNR.

CHAPTER 5

Effects of the MDR reversing agents Cy A and Tri on DNR accumulation and retention in patient leukaemic cells

5.1. Introduction

Cy A and Tri have been shown to reverse drug resistance in leukaemic cell lines (Chapter 3.3) but only a few studies have examined the actions of MDR reversing agents such as Cy A or Tri on the blasts cells of patients with leukaemia, and these studies have used high concentrations of either chemotherapeutic agents (1 μg/ml of DNR) or MDR reversing agents (6.25 μg/ml of Cy A) (Ross *et al.*, 1986, 1993; Kessel *et al.*, 1984; Nooter *et al.*, 1990). The concentrations of the MDR agents used in these studies are not clinically achievable and thus the agents may not be as effective *in vivo* as they are *in vitro*. This chapter aims to address these issues by examining the effects of the MDR reversing agents Cy A (1.5 μg/ml) and Tri (150 ng/ml) on patient leukaemic cells at the same clinically achievable drug concentrations used for the leukaemic cell lines in Chapter 3.

5.2. Patients

Peripheral blood or bone marrow samples were obtained from 24 patients. These patients were not the same as those in Chapter 4.2 and their characteristics are described in Table 5.1. The patients included 8 females and 16 males and ranged in age from 19 years to 70 years, with a mean age of 51 years. Of the 24 patients 15 were at initial presentation of either AML or ALL and 9 at relapse. The patients were not on any chemotherapeutic agents when blood samples were taken. Patient leukaemic (blasts) cells were isolated as described in Chapter 2.2.7.1.

5.3. Accumulation of DNR in acute leukaemic patients and the effect of the MDR Reversing agents

5.3.1. Presentation

Patients were divided into two groups: those at initial presentation and those at relapse, and the accumulation of DNR was determined as described in Chapter 2.2.7.2.

5.3.1.1. Initial Presentation

The concentration-time curve for DNR was plotted for the blast cells of each patient and a representative plot from Patient 3 is shown in Figure 5.1. The concentration-time curve for DNR accumulation in patients is similar to the concentration-time curve for DNR accumulation in the leukaemic cell lines (Chapter 3.5). The DNR accumulation is given as the mean AUC (0-4 hr) ± SD (n). The blast cells of patients at initial presentation accumulated 210 ± 205 (15) ng.hr.10^{-6} cells of DNR. It was evident that there was a wide patient to patient variation as was seen with the pharmacokinetics of DNR (Chapter 4). The addition of the MDR reversing agents Cy A or Tri slightly increased the DNR accumulation in the blast cells of patients at initial presentation, but this increase did not reach statistical significance (Table 5.2). The addition of Cy A and Tri in combination however, significantly ($p < 0.01$, Fr) increased the DNR accumulation by 55% in the blast cells of patients at initial presentation (Table 5.2).

Table 5.1. Patient Characteristics

Patient	SEX	AGE	Diagnosis	Stage of Disease	Sample	WCC	% Blasts in PB (BM)
1	M	49	AML	In Pres	PB	198	95
2	M	49	AML	In Pres	PB	198	95
3	M	68	AML	In Pres	PB	58.1	61
4	M	47	AML	In Pres	PB	21	90
5	M	47	AML	In Pres	BM	17	81 (95)
6	M	19	AML	In Pres	PB	31.7	57
7	M	70	AML	In Pres	PB	63.4	25
8	M	63	AML	In Pres	PB	29.9	76
9	M	52	AML	In Pres	PB	56.3	77
10	F	42	AML	In Pres	PB	167.5	96
11	M	70	AML	In Pres	PB	65	18
12	F	38	AML	In Pres	PB	35.9	69
13	M	44	AML	In Pres	PB	52.6	97
14	F	67	AML	In Pres	PB	66.5	65
15	M	68	AML	Rel	PB	158	80
16	M	68	AML	Rel	PB	136	83
17	F	37	AML	Rel	PB	225.9	64
18	M	56	AML	Rel	PB	66.9	27
19	F	48	AML	Rel	PB	98.1	80
20	F	48	AML	Rel	PB	48.5	94
21	F	36	ALL	In Pres	PB	46.1	93
22	M	52	ALL	Rel	PB	4.6	37
23	M	37	ALL	Rel	BM	22.7	43 (90)
24	F	40	ALL	Rel	PB	24.5	78

In Pres = Initial Presentation

Rel = Relapse

PB = Peripheral blood

BM = Bone marrow

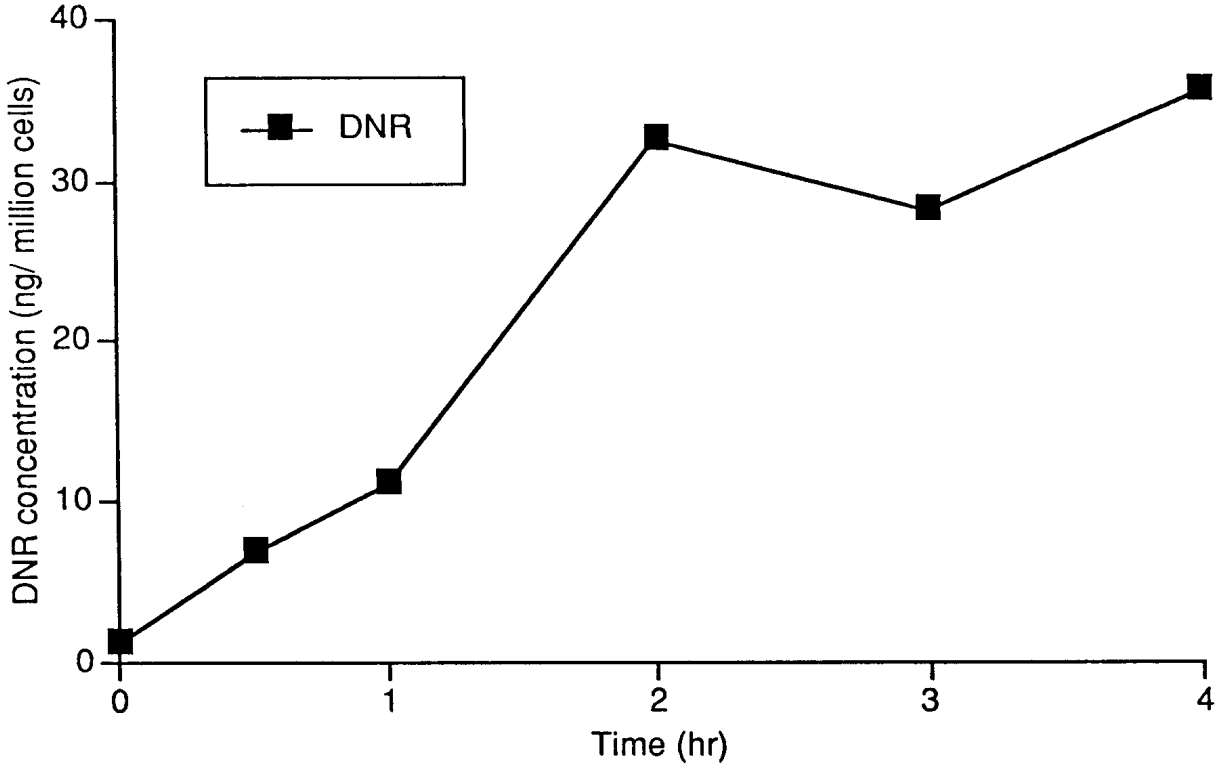


Figure 5.1. Representative concentration-time curve of DNR accumulation in a patient (Patient 3) at initial presentation.

Table 5.2. Accumulation of DNR in the blast cells of patients with acute leukaemia at initial presentation and at relapse (mean ± SD (n)).

	Initial Presentation	Relapse
	(ng.hr.10^{-6} cells)	
DNR	210 ± 205 (15)*	271 ± 408 (9)
DNR + Cy-A	277 ± 232 (15)	279 ± 435 (9)
DNR + Tri	270 ± 221 (15)	303 ± 351 (9)
DNR + Cy-A + Tri	325 ± 256 (15)*	247 ± 329 (9)

* $p < 0.01$ (Fr) DNR vs DNR + Cy A + Tri

5.3.1.2. Relapse

The amount of DNR accumulated in the blast cells of patients at relapse was 271 ± 408 (9) ng.hr.10^{-6} cells, which was slightly higher than the amount of DNR accumulated by the blast cells of patients at initial presentation (210±205 (15) ng.hr.10^{-6} cells). However, there was no significant difference between the amount of DNR accumulated in the blast cells of patients at relapse compared to those at initial presentation. The addition of the MDR reversing agents Cy A, Tri or the combination of Cy A and Tri, had no effect in increasing DNR accumulation in the blast cells of patients at relapse (Table 5.2). This is in contrast to what was seen in the blast cells of patients at initial presentation where the combination of Cy A and Tri significantly increased DNR accumulation.

5.3.2. P glycoprotein

To determine the effect of the MDR reversing agents Cy A and Tri on the accumulation of DNR in the blast cells of patients and their interaction with Pgp, all 24 patients were subdivided as either having Pgp positive blast cells or Pgp negative blast cells. Table 5.3 lists the patients with Pgp positive and Pgp negative blast cells stained with the two antibodies JSB 1 and C 219. The correlation between the two antibodies (using the Spearman-rank correlation) had a r value of 1.00. This indicated that there was no difference between the two antibodies. Therefore, for the remainder of the chapter the Pgp results given will be those for the C 219 antibody, unless stated otherwise. Of the 24 patients the Pgp levels were determined on 23 (Patient 21 had an inadequate sample), 16 of these patients had blast cells that were Pgp negative and 7 had blasts that were Pgp positive.

5.3.2.1. Pgp positive

The patients with Pgp positive blast cells accumulated 126 ± 53 (7) ng.hr.10^{-6} cells of DNR. The addition of Cy A and Tri increased DNR accumulation by 29% and 11%, respectively (Table 5.4), but these increases did not reach statistical significance. The addition of the MDR reversing agents Cy A and Tri in combination, however, increased DNR accumulation by 43% ($p < 0.05$, Fr) (Table 5.4). This is in contrast to the Pgp positive VLB cell line, where Cy A alone was able to increase DNR accumulation and the combination of Cy A and Tri had no additional effect (Chapter 3.5.2).

Table 5.3. Detection of Pgp in patient blast cells

Patient	JSB 1	C 219
1	positive	positive
2	positive	positive
3	negative	negative
4	negative	negative
5	negative	negative
6	negative	negative
7	negative	negative
8	positive	positive
9	negative	negative
10	negative	negative
11	negative	negative
12	positive	positive
13	negative	negative
14	positive	positive
15	negative	negative
16	negative	negative
17	positive	positive
18	positive	positive
19	negative	negative
20	negative	negative
21	N/A	N/A
22	negative	negative
23	negative	negative
24	negative	negative

N/A = not available

Table 5.4. Accumulation of DNR in the blast cells of patients with acute leukaemia according to P glycoprotein (mean ± SD (n)).

	Pgp positive	Pgp negative
	(ng.hr.10^{-6} cells)	
DNR	126 ± 53 (7)^	290 ± 343 (16)
DNR + Cy-A	162 ± 100 (7)	333 ± 371 (16)
DNR + Tri	140 ± 69 (7)	349 ± 308 (16)
DNR + Cy-A + Tri	180 ± 77 (7)^	350 ± 330 (16)

^ $p < 0.05$ (Fr) DNR vs DNR + Cy A + Tri

5.3.2.2. Pgp negative

Patients with Pgp negative blast cells accumulated 290 ± 343 (16) ng.hr.10^{-6} cells of DNR. This appeared to be greater than the amount of DNR accumulated in Pgp positive blasts cells (126 ± 53 (7) ng.hr.10^{-6} cells), but this did not reach statistical significance. The standard deviation in patients with Pgp negative blasts cells was 6 fold higher than patients with Pgp positive blast cells, this was due to two patients having much higher levels of accumulation than the rest of the group. The addition of Cy A, Tri or the combination of both increased DNR accumulation in the Pgp negative blast cells by 15%, 20% and 21%, respectively, but none was a statistically significant increase (Table 5.4). Therefore in contrast to the Pgp positive blast cells, DNR accumulation was not increased in Pgp negative blast cells. There was also no increase in DNR accumulation seen in the Pgp negative CEM and HL 60 cell lines (Chapter 3.5.1, 3.5.3) but Cy A did increase DNR accumulation in the drug resistant Pgp negative ADR cell line (Chapter 3.5.4).

5.4. Retention of DNR in acute leukaemic patients and the effect of the MDR reversing agents

5.4.1. Presentation

Patients were divided into the same two groups as for accumulation, i.e. those at initial presentation and those at relapse, and the retention of DNR was determined as described in Chapter 2.2.7.3.

5.4.1.1. Initial Presentation

The concentration-time curve for DNR was plotted for the blast cells of each patient and a representative plot from Patient 11 is shown in Figure 5.2. The concentration-time curve for DNR accumulation in patients is similar to the concentration-time curve for DNR retention in the leukaemic cell lines (Chapter 3.6). DNR retention is given as the mean AUC (0-4 hr) ± SD (n). The blast cells of patients at initial presentation retained 178 ± 71 (15) ng.hr.10^{-6} cells of DNR. The addition of the MDR reversing agents Cy A or Tri, slightly increased the DNR retention in the blast cells of patients at initial presentation, but this increase did not reach statistical significance (Table 5.5). The addition of Cy A and Tri in

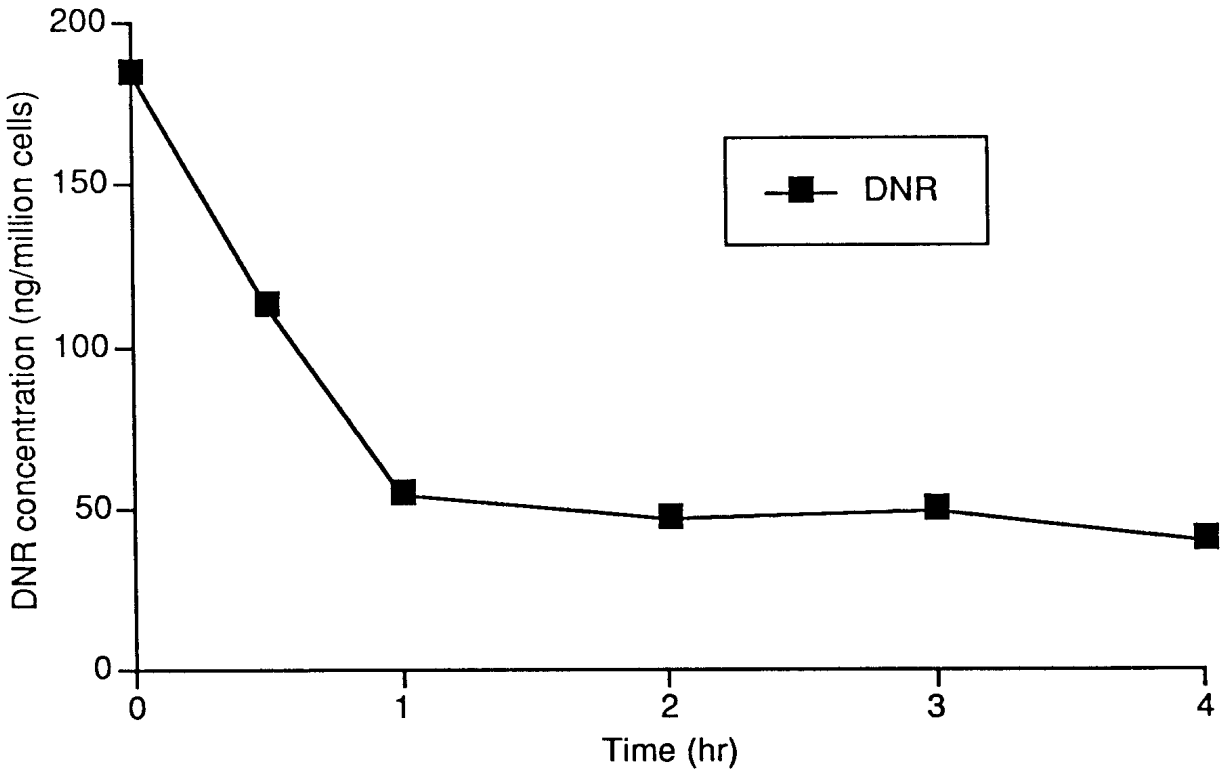


Figure 5.2. Representative concentration-time curve of DNR retention in a patient (Patient 11) at initial presentation.

Table 5.5. Retention of DNR in the blast cells of patients with acute leukaemia at initial presentation and at relapse (mean ± SD (n)).

	Initial Presentation	Relapse
	(ng.hr.10^{-6} cells)	
DNR	178 ± 71 (15)^	584 ± 963 (9)
DNR + Cy-A	201 ± 100 (15)	468 ± 615 (9)
DNR + Tri	190 ± 110 (15)	575 ± 824 (9)
DNR + Cy-A + Tri	226 ± 117 (15)^	542 ± 658 (9)

^ $p < 0.05$ (Fr) DNR vs DNR + Cy A + Tri

combination however, significantly ($p < 0.05$, Fr) increased the DNR retention by 27% in the blast cells of patients at initial presentation (Table 5.5).

5.4.1.2. Relapse

The amount of DNR retained in the blast cells of patients at relapse was 584 ± 963 (9) ng.hr.10^{-6} cells. This appeared higher than the amount of DNR retained in the blast cells of patients at initial presentation (178 ± 71 (15) ng.hr.10^{-6} cells) but was not significantly different due to the large standard deviations. The standard deviation in patients at relapse was 13 fold higher than patients at initial presentation, this was due to two patients having much higher levels of DNR retention than the rest of the group. The addition of the MDR reversing agents Cy A, Tri or the combination of Cy A and Tri, had no effect in increasing DNR retention in the blast cells of patients at relapse (Table 5.5). This is in contrast to what was seen in the blast cells of patients at initial presentation where the combination of Cy A and Tri significantly increased DNR retention.

5.4.2. P glycoprotein

As was the case for accumulation (5.3.2), the patients were reclassified according to Pgp expression (Table 5.3). Sixteen of the patients had Pgp negative blast cells and seven patients had Pgp positive blast cells.

5.4.2.1. Pgp positive

The patients with Pgp positive blast cells retained 149 ± 83 (7) ng.hr.10^{-6} cells of DNR. The addition of Cy A and Tri decreased DNR retention by 18% and 21%, respectively (Table 5.6). The addition of the MDR reversing agents Cy A and Tri in combination, however, increased DNR retention by 24%, but this increase was not statistically significant (Table 5.6). Therefore, the addition of the combination of Cy A and Tri, had no effect on DNR retention in the Pgp positive blast cells of patients. This is in contrast with the effects of Cy A and Tri in combination in the accumulation of DNR in patients with Pgp positive blast cells (5.3) and the Pgp positive VLB cell line, where Cy A alone was able to increase DNR retention (Chapter 3.6.2).

5.4.2.2. Pgp negative

The patients with Pgp negative blast cells retained 416 ± 731 (16) ng.hr.10^{-6}

Table 5.6. Retention of DNR in the blast cells of patients with acute leukaemia according to P glycoprotein (mean ± SD (n)).

	Pgp positive	Pgp negative
	($ng.hr.10^{-6}$ cells)	
DNR	149 ± 83 (7)	416 ± 731 (16)
DNR + Cy-A	123 ± 76 (7)	382 ± 463 (16)
DNR + Tri	119 ± 79 (7)	441 ± 625 (16)
DNR + Cy-A + Tri	185 ± 118 (7)	422 ± 507 (16)

cells of DNR. This appeared to be greater than the amount of DNR retained in those patients with Pgp positive blasts cells (149 ± 83 (7) ng.hr.10^{-6} cells) but did not reach statistical significance. The addition of Cy A, Tri or the combination had minimal effects on the amount of DNR retained (Table 5.6). Therefore, in both the patients with Pgp positive blast cells and those with Pgp negative blast cells, DNR retention was unaffected by the MDR reversing agents Cy A and Tri.

5.5. Relationship between patient presentation and Pgp

Of the 23 patients analysed for Pgp, 16 had blast cells that were Pgp negative and 7 had blast cells that were Pgp positive (Table 5.3). In the nine patients at relapse, two were Pgp positive and seven were Pgp negative and of those patients at initial presentation, five were Pgp positive and nine were Pgp negative. There was no correlation found in this study between the stage of disease and the presence of Pgp ($p > 0.5$, FE).

5.6. Discussion

Few studies have examined the effects of MDR reversing agents in the blast cells of patients with leukaemia. Studies by Kessel *et al.* (1984), Ross *et al.* (1986) and Anderson *et al.* (1987) have shown that verapamil had no effect on increasing cellular drug accumulation in the blast cells of patients with acute leukaemia. In this study, despite the wide variation in the measurement of accumulation and retention from patient samples, it was demonstrated that the MDR reversing agents were more successful in combination than individually. MDR reversing agents were more effective at increasing cellular DNR concentrations in patients at initial presentation than those at relapse. This is an important finding because it suggests that it may be possible to improve patient response if Cy A and Tri were used in conjunction for induction chemotherapy. This may also decrease the number of patients becoming resistant to induction therapy, a major problem associated with chemotherapy.

Recently, Ross *et al.* (1993) and Marie *et al.* (1993) showed that accumulation and retention of DNR could be increased in the blast cells of previously untreated AML patients by the use of Cy A. The present study was unable to confirm that Cy A alone significantly increased accumulation or retention of DNR in patients at initial presentation, although there appeared to be a trend towards an increase in accumulation and retention

(Table 5.2, 5.4). This may be due to the higher concentration (6 μg/ml) of Cy A used by Ross *et al.* (1993), which when equated to blood levels, is a clinically toxic level compared to only 1.5 μg/ml used in the present study.

Nooter *et al.* (1990) examined the effects of an MDR reversing agent and its relationship with Pgp in acute leukaemia. They found that Cy A was able to increase DNR accumulation in leukaemic cells from patients overexpressing the mdr1 gene. By contrast, Marie *et al.* (1993) found the effect of Cy A on DNR uptake (by flow cytometry) did not differ when mdr1 positive and mdr1 negative patients were compared. The present study observed no differences in accumulation or retention of DNR in either Pgp positive or Pgp negative patient cells treated with individual MDR reversing agents, in agreement with the results of Marie *et al.* (1993). However, the combination of Cy A and Tri was able to increase the accumulation of DNR in Pgp positive cells. The different results between the present study and that of Nooter *et al.* (1990) could be due again to the higher concentration of Cy A used by Nooter *et al.* (1990); 3.75 μg/ml compared to 1.5 μg/ml used in the present study. Marie *et al.* (1993) used an even lower concentration of 1μg/ml of Cy A.

The above findings raise a number of issues: (1) is Pgp important in determining whether a patient will respond to treatment or not; (2) does Cy A play a role in reversing the actions of Pgp and/or some other mediator; (3) is the stage of disease more important than the presence of Pgp in terms of the effectiveness of the MDR reversing agents Cy A and Tri; (4) do short term blast cells represent a true indication of *in vivo* behaviour?

In vitro studies undertaken so far using blast cells from patients with acute leukaemia do not consider the achievable concentration of the MDR reversing agents in the clinical situation. This issue needs to be studied carefully since at different concentrations of Cy A different outcomes are possible.

The combination of Cy A and Tri significantly increased accumulation and retention of DNR in the blast cells of patients at initial presentation and this effect was not related to the presence of Pgp. This suggests that the actions of Cy A and Tri are not directly on Pgp and thus some other mechanism(s) must be involved. One possible mechanism for Cy A action is an alteration of cell membranes, resulting in increased membrane fluidity (Slater *et al.*, 1986) thereby allowing more DNR to cross the cell membrane leading to the increased accumulation of DNR. In addition both Cy A and Tri have been shown to bind to calmodulin (Roufogalis *et al.*, 1983; Colombani *et al.*, 1985) and the inhibition of calmodulin may play a role in increased DNR accumulation and retention. Simon *et al.* (1994) have recently shown that intracellular accumulation of DNR can be affected by

changes in pH. Drug resistant cells have a higher pH than drug sensitive cells. Cy A and Tri may decrease intracellular pH and thus lead to increased DNR accumulation. The actions of Cy A and Tri on calmodulin and pH may also be related. Intracellular pH has been shown to be associated with the increased activity of the Na^+/H^+ antiporter (Boscoboinik *et al.*, 1990). This antiporter may be activated by calmodulin or an intermediary of calmodulin. The binding of Cy A and Tri to calmodulin may inhibit this controlling mechanism thus leading to a decrease in intracellular pH and an increase in DNR accumulation. However, no experimental evidence is available to confirm or deny this hypothesis. Therefore, the exact mechanism by which MDR reversing agents influence drug transport remains unclear and further study is required if MDR reversing agents are to have a major part in chemotherapy.

Two clinical trials examining the effects of the MDR reversing agents verapamil (Dalton *et al.*, 1989) and Cy A (Sonneveld *et al.*, 1992) in multiple myeloma have shown favourable results. However, these trials only examined patients that were resistant to standard treatment. A randomised trial is required to examine the effects of MDR reversing agents not only in resistant patients but also patients at initial presentation of disease to determine the role of these MDR reversing agents in acute leukaemia. It may be that the MDR reversing agents are more effective in patients at initial presentation, lengthening the time of remission, as well as increasing the number of patients achieving complete remission.

CHAPTER 6

Actions of Immobilised-DNR

6.1. Introduction

DNR has been shown to have a variety of cytotoxic actions as described in Chapter 1.1. One of these cytotoxic actions is believed to be due to the actions of DNR on the cell membrane. Two reports have previously shown that DOX can exert its cytotoxic action on the murine leukaemia L1210 cell line solely by interaction at the cell surface. (Tritton & Yee, 1982; Tokes *et al.*, 1982). In this chapter, the effects of immobilised-DNR will be examined in human leukaemic cell lines, to determine whether DNR can affect human leukaemic cells by acting solely on the cell membrane.

Pgp is known to cause drug resistance in some cell lines, and has been demonstrated to act as an efflux pump, removing cytotoxic drugs from the cell cytoplasm. If the sole mechanism of MDR results from the ability of Pgp to expel drugs from the cell, then immobilised-DNR should have equivalent cytotoxic actions on both drug sensitive cell lines and Pgp positive drug resistant cell lines. If this is not the case, Pgp must act other than by removal of intracellular drugs that cause drug resistance, otherwise alternative mechanism(s) unrelated to Pgp in the cells would be operating.

The aims of this chapter are (1) to determine whether immobilised-DNR is

cytotoxic, (2) to determine whether MDR cells are more resistant to the membrane associated cytotoxic effect of DNR than non–MDR cells (DNR bound to a solid matrix should not have a differential effect on MDR cells as compared with non–MDR cells if an efflux pump with decreased intracellular drug is the sole explanation of the MDR phenomenon) and (3) to determine whether the MDR reversing agents, Cy A and Tri, are able to potentiate the cytotoxic effects of immobilised-DNR.

6.2. Preparation of immobilised-DNR

The method of preparing immobilised-DNR is shown in Figure 2.2. The first step involves the acid-catalyzed cross linking of polyvinyl alcohol (PVA) with terephthalaldehyde to form the cross-linked polymer (CL-PVA). CL-PVA is then activated using cyanuric chloride to allow the attachment of DNR through the free amine group on the duanosamine sugar residue.

After synthesis, immobilised-DNR was washed extensively as described in chapter 2.2.11.2 to ensure removal of free DNR. The presence of free DNR would cause cytotoxicity and hence invalidate experiments using immobilised-DNR. After washing with methanol, acetonitrile, phosphate buffer, NaCl, water and then more methanol, the absence of a DNR peak in the wash buffer was confirmed by UV spectrophotometry. To determine if further DNR was released by the final methanol wash, an HPLC method for DNR detection was used. The amount of DNR released in this methanol wash was found to be 400 ng DNR per ml methanol and this concentration of DNR would be cytotoxic to cell lines (Chapter 3). Therefore, further washing of immobilised-DNR was required.

The immobilised-DNR was then washed twice with RPMI at 37°C and twice with RPMI at room temperature. Each of the fractions was collected and the amount of DNR released was measured (Table 6.1). The cytotoxicity of these RPMI washes was analysed using the MTT assay (Chapter 2.2.4) and the results are given in Table 6.1 in which is shown a decrease in the amount of DNR released associated with an increase in cell viability of the CEM cells. The viability of the CEM cells is equivalent to that shown for the cytotoxicity of free DNR in Chapter 3.

In the final RPMI wash 35 ng/ml of DNR was released. Therefore, the immobilised-DNR was washed with more RPMI. The DNR release rate was reduced to 17 ng/ml. To reduce the DNR release rate further, the immobilised-DNR was washed with

Table 6.1. The amount of DNR released and cytotoxicity of the media.

RPMI media	Volume (ml)	DNR released (ng/ml)	% viability
1st wash	20	132	60
2nd wash	20	184	ND
3rd wash	25	112	62
4th wash	25	35	92

ND = not determined

methanol again. The methanol wash caused an increase in the amount of DNR (167 ng/ml) released. This suggests that more DNR is released in the presence of methanol than RPMI. The immobilised-DNR was washed extensively with more methanol, until the release rate of DNR, was ≤ 3 ng of DNR per ml of methanol and then no further washings were performed. Since the amount of DNR released in methanol is greater than the amount of DNR released in RPMI, there should be minimal amounts of free DNR released during the immobilisation experiments.

6.3. Amount of DNR bound to PVA

The amount of DNR bound to PVA was determined as described in section 2.2.11.3. With quantitative hydrolysis of the bound DNR it was found that 1.86 μg (3.3 nmoles) of DNR was bound to each mg of PVA.

6.4. Amount of DNR released in immobilised-DNR experiments

To confirm that there was no free DNR released during the immobilised-DNR experiments as described in Chapter 2.2.11.5, the supernatant and cells were assayed for free DNR by HPLC (Chapter 2.2.8). There was no detectable free DNR found in the RPMI or the cells in any of the immobilised-DNR experiments. This indicates that any findings in these experiments would not be due to the cytotoxic actions of free DNR.

6.5. Cytotoxicity of immobilised-DNR

The cytotoxicity of immobilised-DNR on the four human leukaemic cell lines CEM, VLB, HL 60 and ADR was determined as described in Chapter 2.2.5. This involves a clonogenic assay that relies on the number of colonies of cells formed over a specified time. The drug sensitive HL 60 cells were the most prolific of the cell lines with 762 ± 343 colonies formed over 10 days. This was a plating efficiency of 30%. The drug resistant Pgp negative ADR cell line had 352 ± 170 colonies formed at a plating efficiency of 14% while the drug resistant Pgp positive VLB cell line formed 353 ± 128 at a plating efficiency of 14%. The drug sensitive CEM cells were the poorest growing cells, with a plating efficiency of only 4.5%. The CEM cell line formed 223 ± 151 colonies over the 10 day growth period.

The cytotoxicity of immobilised-DNR was studied at two concentrations of DNR: 2 μg/ml and 20 μg/ml (per ml of assay medium). This was equivalent to 1.08 mg and 10.8 mg of CL-PVA (per ml of assay medium) bound DNR, respectively. In the remainder of this chapter the 2 μg/ml of DNR will be referred to as the low concentration and the 20 μg/ml of DNR will be referred to as the high concentration. As a control the effect of the equivalent amounts of CL-PVA was also examined to determine whether the support to which DNR was bound had any cytotoxic actions. The results of these experiments are summarised in Table 6.2 for CEM and its drug resistant subline VLB, and Table 6.3 for HL 60 and its drug resistant subline ADR.

In the CEM cell line the low (1.08 mg/ml) CL-PVA (control) had no effect on cell viability and the low concentration of immobilised-DNR also had no effect on viability. On the other hand, the higher concentration (10.8 mg/ml) of CL-PVA (control) caused a 70% decrease in the amount of cells grown and the high concentration of immobilised-DNR produced a 93% decrease in colony formation. There was no significant difference (Friedman two-way analysis of variance) between the effect produced by the high concentration of CL-PVA alone and the effect of the high concentration of immobilised-DNR. Thus the cytotoxic actions of the high concentration of immobilised-DNR were due to non specific effects produced by the presence of the CL-PVA. At low concentrations of CL-PVA (control) there was no cytotoxic effect. Therefore, immobilised-DNR is not cytotoxic to the drug sensitive CEM cell line.

Immobilised-DNR showed no effect on colony formation of the drug resistant Pgp positive VLB cell line with either concentration of DNR. The percentage change in colony formation was +5% for the low CL-PVA, -3% for both the low concentration of immobilised-DNR and the high CL-PVA, and -8% for the high concentration of immobilised-DNR. Thus the immobilised-DNR had no effect on the viability of the drug resistant Pgp positive VLB cell line.

The high concentration of immobilised-DNR produced a 37% reduction in colony formation in the drug sensitive HL 60 cell line whereas the low concentration of immobilised-DNR reduced cell growth by 23%. However, both the low and high CL-PVA produced a small reduction in the amount of colonies formed, reducing them by 27% and 24%, respectively. There was no significant reduction in colonies formed by cells incubated with the low and high CL-PVA (control) or the low immobilised-DNR. There was a significant reduction in colonies produced by HL 60 incubated with the high concentration of immobilised-DNR. There was also a significant difference between the high CL-PVA and the high concentration of immobilised-DNR. This suggests that the cytotoxic actions

Table 6.2. The effect of CL-PVA and immobilised-DNR on the drug sensitive CEM cell line and its drug resistant subline VLB. The data is given as the average number of colonies formed ± SD (n).

Treatment	CEM	VLB
Control	223 ± 151 (28)*†	353 ± 128 (16)
l-PVA	265 ± 227 (12)	370 ± 160 (12)
l-Imm-DNR	246 ± 212 (12)	344 ± 98 (12)
h-PVA	66 ± 35 (12)*	343 ± 158 (12)
h-Imm-DNR	15 ± 24 (12)†	328 ± 151 (12)

Control - cells only

l-PVA - 1.08 mg of CL-PVA, l-Imm-DNR - 2 μg/ml of immobilised-DNR

h-PVA - 10.8 mg of CL-PVA, h-Imm-DNR - 20 μg/ml of immobilised-DNR

* $p < 0.01$ (Fr) Control vs h-PVA

† $p < 0.01$ (Fr) Control vs h-Imm-DNR

Table 6.3. The effect of CL-PVA and immobilised-DNR on the drug sensitive HL 60 cell line and its drug resistant subline ADR. The data is given as the average number of colonies formed ± SD (n).

Treatment	**HL 60**	**ADR**
Control	762 ± 343 (20)*	352 ± 170 (20)
l-PVA	559 ± 336 (12)	341 ± 75 (12)
l-Imm-DNR	589 ± 365 (12)	321 ± 99 (12)
h-PVA	583 ± 311 (12)^	278 ± 76 (12)
h-Imm-DNR	483 ± 320 (12)*^	270 ± 100 (12)

Control - cells only

l-PVA - 1.08 mg of CL-PVA, l-Imm-DNR - 2 μg/ml of immobilised-DNR

h-PVA - 10.8 mg of CL-PVA, h-Imm-DNR - 20 μg/ml of immobilised-DNR

* $p < 0.01$ (Fr) Control vs h-Imm-DNR

^ $p < 0.05$ (Fr) h-PVA vs h-Imm-DNR

of the high concentration of immobilised-DNR are due to the presence of DNR bound to the CL-PVA and not due to the CL-PVA itself, contrary to that seen with the CEM cell line. Therefore, immobilised-DNR is cytotoxic to the drug sensitive HL 60 cell line.

The immobilised-DNR had no effect on the drug resistant Pgp negative ADR cell line. The low concentration of immobilised-DNR produced a 9% decrease in colony formation, while the low CL-PVA decreased the colony formation by only 3%. The high concentration of immobilised-DNR caused a 23% decrease in colony formation and the high CL-PVA produced a 21% decrease in the amount of colonies formed. There were no significant differences between the decrease in colony formation produced by the high concentration of immobilised-DNR and the high CL-PVA in the drug resistant ADR cell line. Therefore, in contrast to the drug sensitive HL 60 cell line, the drug resistant Pgp negative ADR cell line was unaffected by the immobilised-DNR.

6.6. Effects of MDR reversing agents

MDR reversing agents have been thought to act by inhibiting the actions of Pgp on the removal of intracellular drug. This begs the question that, if the cytotoxic actions of DNR are extracellular, could the MDR reversing agents also increase cytotoxicity? If this were the case then the actions of Pgp in causing drug resistance would not be solely due to Pgp's ability to transport drugs out of the cells. Therefore, the effect of the two MDR reversing agents Cy A and Tri were examined to see if they had any effect on the cytotoxicity of immobilised-DNR.

The effects of Cy A and Tri on the viability of the CEM cell line and its drug resistant subline VLB are given in Table 6.4. In CEM cells Cy A itself produced a 76% reduction in colonies, suggesting that Cy A itself was cytotoxic to these cells. Tri produced a 30% reduction in the CEM cell line colonies but this was not a statistically significant effect. Cy A was also slightly cytotoxic in the drug resistant cell line, reducing colony formation by 21% and Tri reduced colony formation by 13% in the VLB cell line.

Table 6.5 lists the effect of Cy A and Tri on HL 60 and ADR cell lines. Cy A caused a 34% increase in colony formation of the HL 60 cells and caused an 11% increase in the colony formation of the drug resistant ADR cell line. Tri decreased HL 60 colony formation by 6% and increased ADR colony formation by 12%. There were no statistically

Table 6.4. The effect of the MDR reversing agents Cy A and Tri on the CEM and VLB cell lines. The data is given as the average number of colonies formed ± SD (n).

Treatment	**CEM**	**VLB**
Control	223 ± 151 (28)*	353 ± 128 (16)^
Cy A	53 ± 13 (16)*	279 ± 61 (8)^
Tri	157 ± 35 (16)	308 ± 45 (8)

Control - cells only

* $p < 0.01$ (Fr) Control vs Cy A

^ $p < 0.05$ (Fr) Control vs Cy A

Table 6.5. The effect of the MDR reversing agents Cy A and Tri on the HL 60 and ADR cell lines. The data is given as the average number of colonies formed ± SD (n).

Treatment	**HL 60**	**ADR**
Control	762 ± 343 (20)	352 ± 170 (20)
Cy A	1019 ± 486 (14)	392 ± 198 (16)
Tri	719 ± 151 (16)	395 ± 254 (16)

Control - cells only

significant effects in the HL 60 and ADR cell lines. Therefore, the effects of Cy A on HL 60 and ADR cell lines tended to contrast with its effects on CEM and VLB cell lines.

The low concentration of immobilised-DNR had no effect on the CEM cell line (Table 6.6). On the addition of the MDR reversing agent Cy A, the colony formation decreased by 78%. This effect was totally due to the actions of Cy A alone, since Cy A on its own caused a 76% reduction in colony formation of the CEM cells. On the other hand, the addition of Tri also led to a significant reduction in the colony formation of the immobilised-DNR treated drug sensitive CEM cell line, reducing the colony formation by 86%. Tri on its own only produced a 30% reduction in colony formation; therefore, the addition of Tri to the immobilised-DNR caused a significant further reduction in colony formation suggesting that Tri can increase the cytotoxicity of the immobilised-DNR in the drug sensitive CEM cell line. The cytotoxic actions of the high concentration of immobilised-DNR on the CEM cell line were mainly due to the high CL-PVA (Table 6.6). The addition of Cy A or Tri had no effect above that of the immobilised-DNR or CL-PVA.

At both the low and high concentrations of immobilised-DNR, the MDR reversing agents had only a slight effect on increasing the cytotoxicity of the immobilised-DNR in the drug resistant Pgp positive VLB cell line (Table 6.7). Cy A produced a 21% and 20% reduction in VLB colony formation with both the low and high concentrations of immobilised-DNR, respectively, while Tri produced a 14% and 15% reduction, respectively, in colony formation. These reductions in colony formation are probably due to the actions of the MDR reversing agents themselves, since Cy A alone produced a 21% reduction in colony formation while Tri alone produced a 13% reduction. Thus, the MDR reversing agents were unable to increase the cytotoxicity of immobilised-DNR in the drug resistant Pgp positive VLB cell line. This is in contrast to the drug sensitive cell line CEM where Tri was able to increase cytotoxicity, indicating that Tri can reverse drug resistance in non-Pgp positive cell lines.

The low concentration of immobilised-DNR produced a 23% reduction in colony formation in the HL 60 cell line (Table 6.3) but this reduction was not statistically significant. The MDR reversing agents Cy A and Tri increased (not significant) this cytotoxicity to 37% and 31%, respectively (Table 6.8). Thus, the MDR reversing agents slightly enhanced the cytotoxicity of the low concentration of immobilised-DNR. The high concentration of immobilised-DNR caused a 37% reduction in cytotoxicity and this was significantly ($p < 0.05$) increased to 51% and 48% cytotoxicity with the addition of Cy A and Tri, respectively (Table 6.8). Thus the MDR reversing agents can increase the cytotoxicity of immobilised-DNR in the drug sensitive HL 60 cell line, as did Tri in the drug

Table 6.6 Effect of the MDR reversing agents on the cytotoxicity of immobilised-DNR in the drug sensitive CEM cell line. The data is given as the average number of colonies formed ± SD (n).

Treatment	**Low**	**High**
Control	265 ± 227 (12)*†	66 ± 35 (12)*†°
Imm-DNR	246 ± 212 (12)	15 ± 24 (12)°
Imm-DNR + Cy A	58 ± 31 (12)*	14 ± 24 (12)*
Imm-DNR + Tri	38 ± 26 (12)†	14 ± 21 (12)†

Control - equivalent amount of PVA

Imm-DNR - immobilised-DNR

Low - 2 μg/ml of immobilised-DNR

High - 20 μg/ml of immobilised-DNR

* $p < 0.01$ (Fr) Control vs Imm-DNR + Cy A

† $p < 0.01$ (Fr) Control vs Imm-DNR + Tri

° $p < 0.01$ (Fr)Control vs Imm-DNR

Table 6.7 Effect of the MDR reversing agents on the cytotoxicity of immobilised-DNR in the drug resistant VLB cell line. The data is given as the average number of colonies formed ± SD (n).

Treatment	**Low**	**High**
Control	370 ± 160 (12)	343 ± 158 (12)
Imm-DNR	344 ± 98 (12)	328 ± 151 (12)
Imm-DNR + Cy A	291 ± 70 (12)	276 ± 131 (12)
Imm-DNR + Tri	318 ± 129 (12)	292 ± 157 (12)

Control - equivalent amount of PVA

Imm-DNR - immobilised-DNR

Low - 2 μg/ml of immobilised-DNR

High - 20 μg/ml of immobilised-DNR

Table 6.8 Effect of the MDR reversing agents on the cytotoxicity of immobilised-DNR in the drug sensitive HL 60 cell line. The data is given as the average number of colonies formed ± SD (n).

Treatment	Low	High
Control	559 ± 336 (12)	583 ± 311 (12)*†
Imm-DNR	589 ± 365 (12)	483 ± 320 (12)
Imm-DNR + Cy A	479 ± 278 (12)	372 ± 326 (12)*
Imm-DNR + Tri	524 ± 353 (12)	399 ± 252 (12)†

Control - equivalent amount of PVA

Imm-DNR - immobilised-DNR

Low - 2 μg/ml of immobilised-DNR

High - 20 μg/ml of immobilised-DNR

* $p < 0.01$ (Fr) Control vs Imm-DNR + Cy A

† $p < 0.01$ (Fr) Control vs Imm-DNR + Tri

sensitive CEM cell line. This indicates that MDR reversing agents may have additional effects in drug sensitive tumours.

At the low concentration of immobilised-DNR, there was only a small non significant reduction (9%) in the colony formation of the drug resistant ADR cell line (Table 6.9). This was increased by the addition of the MDR reversing agent Cy A to a 34% reduction in colony formation. The addition of Tri had no effect on the colony formation at the low concentration of immobilised-DNR. At the high concentration of immobilised-DNR there was a 23% reduction in colony formation. The addition of Cy A doubled this reduction in colony formation to 46%. As with the low concentration of immobilised-DNR, Tri had no effect on increasing cytotoxicity with the high concentration of immobilised-DNR. Thus, Cy A is effective in increasing the cytotoxicity of the immobilised-DNR in both the drug sensitive HL 60 cell line as well as its drug resistant subline ADR. Tri, however was only able to increase the cytotoxicity of the immobilised-DNR in the drug sensitive cell line and not in the drug resistant subline.

6.7. Discussion

A number of studies have shown that the anthracyclines interact with the cell membrane (Chapter 1.1.3). Alterations in the transition temperature (Tm) are expected to be wrought by molecules that bind to the bilayer and perturb the packing of the phospholipid molecules. There is an inverse relationship between Tm and membrane fluidity. Tritton *et al.* (1978) found that the Tm of 34.6°C for liposomes prepared from dipalmitoylphosphatidyl choline (DPPC) decreased to 33.3°C in the presence of DOX, indicating an increase in membrane fluidity. Using other positively and negatively charged phospholipids to prepare liposomes, an increase in membrane fluidity was also observed. However, on incorporation of cardiolipin (a negatively charged phospholipid) in membranes treated with DOX, the Tm increased, indicating a decrease in membrane fluidity and the opposite effect to all other phospholipids. Thus DOX has a unique effect on membranes that contain cardiolipin.

Ferrer-Montiel *et al.* (1988) found that in experiments with relatively low drug/lipid ratios, the anthracycline appeared to be predominantly located in domains near the hydrophilic surface, although a second more deeply embedded drug location arises at higher drug/lipid ratios. Circular dichroism studies by Henry *et al.* (1985) and Henry-Toulme *et al.* (1988) provide evidence for two distinct types of membrane binding sites for the drug. They suggest that in site I the amino sugar of DOX is bound to a charged phosphate with

Table 6.9 Effect of the MDR reversing agents on the cytotoxicity of immobilised-DNR in the drug resistant ADR cell line. The data is given as the average number of colonies formed ± SD (n).

Treatment	**Low**	**High**
Control	341 ± 75 (12)	278 ± 76 (12)
Imm-DNR	321 ± 99 (12)	270 ± 100 (12)
Imm-DNR + Cy A	233 ± 51 (12)	191 ± 34 (12)
Imm-DNR + Tri	386 ± 120 (12)	331 ± 145 (12)

Control - equivalent amount of PVA

Imm-DNR - immobilised-DNR

Low - 2 μg/ml of immobilised-DNR

High - 20 μg/ml of immobilised-DNR

the chromophore lying outside the bilayer. Whilst in site II, the sugar phosphate interaction persists, but the chromophore is embedded in the bilayer. Adler & Tritton (1988) developed a technique for determining the angular rotation of molecules in membranes. Their results showed that most membrane components take on an angle that is either parallel with or perpendicular to the bilayer. DOX, on the other hand, adopted an angle of about 55°, which could be very disruptive to orderly bilayer packing. Therefore, there is clear evidence that the anthracyclines interact with the cell membrane.

Previous studies by Tritton & Yee (1982) and Tokes *et al.* (1982) have shown that DOX bound to an immobilised support had cytotoxic actions on a variety of cell lines. In this chapter the effects of immobilised-DNR were examined to determine whether DNR could have cytotoxic actions solely via cell membrane interaction. Immobilised-DNR was not cytotoxic at 2 μg DNR bound per ml on the drug sensitive Pgp negative CEM cell line. The cytotoxicity of the immobilised-DNR increased to 93% in the presence of 20 μg DNR bound per ml. However the drug support, CL-PVA, on its own produced a 70% decrease in viability, indicating that the support itself was capable of causing cytotoxicity in the CEM cell line. Therefore at 10.8 mg CL-PVA/ml, the CL-PVA itself was cytotoxic to the CEM cell line but at 1.08 mg CL-PVA/ml, it was not. Wingard *et al.* (1985) found that 4 mg CL-PVA/ml had no effect in the S 180 cell line. In the present work, 1.08 mg CL-PVA/ml exhibited no cytotoxicity (Table 6.2). Although the immobilised-DNR caused an increase in the cytotoxicity of the CEM cell line, from 70% to 93%, this difference was not significant (Friedman two-way analysis of variance). It must then be assumed that the immobilised-DNR at both 2 and 20 μg/ml had no effect on the viability of the drug sensitive CEM cell line or what effect there was could not be delineated due to the significant effect of the CL-PVA. In contrast, Tokes *et al.* (1982) showed that 12 nM (6.9 ng/ml) of DOX, covalently bound to polyglutaraldehyde microspheres (PGLs), was able to cause a 50% reduction in the viability of the CEM cell line. This suggests that immobilised-DOX and immobilised-DNR may have different actions, which may explain why DOX has a wider spectrum of activity than DNR.

The immobilised-DNR had no cytotoxic actions in the drug resistant Pgp positive VLB cell line. CL-PVA alone also had no cytotoxicity, in contrast to that seen in the parent cell line CEM. Therefore, the changes that have occurred in the drug resistant VLB cell line allow this line to be unaffected by the CL-PVA. One possible explanation for this difference is that CL-PVA interacts with the cell membrane of the CEM cells causing cytotoxicity. In the drug resistant VLB cell line the presence of Pgp may help to stabilise the cell membrane and circumvent the cytotoxicity seen in the CEM cell line.

In the drug sensitive HL 60 cell line the CL-PVA on its own produced a 27% and 24% decrease in viability at the low and high concentrations respectively, these differences were not statistically significant. The low concentration of immobilised-DNR caused a similar effect to that of the CL-PVA alone, and thus had no real cytotoxic action. The high concentration of immobilised-DNR, on the other hand, significantly reduced the HL 60 cell viability by 37% and this was also significantly different from the high CL-PVA alone. The HL 60 cell line was not significantly affected by the CL-PVA itself, in contrast to the CEM cell line in which the CL-PVA caused 70% cytotoxicity. Thus, the immobilised-DNR was able to cause cytotoxicity in the drug sensitive HL 60 cell line. This indicates that there are differences in the way different cell lines are affected by immobilised-DNR. Work by Panneerselvam *et al.* (1987) showed that exposure to 30 μg/ml immobilised-DOX caused 70% cytotoxicity in the murine L1210 leukaemia cell line; whereas only 10-15% cytotoxicity was observed in the human melanoma cell lines SK-MEL-170 and SK-MEL-93-2. When the concentration of the immobilised DOX was reduced to 10 μg/ml, the L1210 cell line still showed 50% cytotoxicity while the viability of the melanoma cells was no longer affected. These results indicate that, as with immobilised-DNR, different cell lines react differently to immobilised-DOX. This was demonstrated also in the present work: different cell lines (CEM and HL 60), responded differently to the actions of immobilised-DNR and this was affected also by the concentration of immobilised-DNR. An increase in the amount of immobilised-DNR may lead to an increase in cytotoxicity. However, this will also result in a concomitant increase in the amount of CL-PVA present, which itself may cause cytotoxicity, as seen in CEM. Ideally, immobilised-DNR with a greater amount of DNR bound to CL-PVA should be used to reduce the actions of the CL-PVA itself.

The immobilised-DNR showed no cytotoxicity in the drug resistant Pgp negative ADR cell line. There was a small decrease in viability with the high dose immobilised-DNR but this was due to the CL-PVA alone which produced the same decrease in cell viability. Therefore, in contrast to the drug sensitive HL 60 cell line the drug resistant ADR showed no decrease in cell viability. Thus the ADR cell line appears to be resistant to the actions of the immobilised-DNR. At the low concentration of immobilised-DNR, no cytotoxic actions were seen in any of the four cell lines. At the high concentration of immobilised-DNR, the drug sensitive cell lines had reduced viability, although the reduced cell viability of the CEM cell line was probably due to the CL-PVA. Both the drug resistant Pgp positive VLB cell line and the drug resistant Pgp negative ADR cell line were unaffected by the actions of immobilised-DNR.

The MDR reversing agents, Cy A and Tri have previously been shown to be effective in reversing drug resistance (Chapter 1.2.1; Chapter 3.3). The mechanism by

which these drugs work is generally thought to be by the inhibition of the membrane pump Pgp. This would prevent the removal of intracellular drug from the cells and thereby reverse drug resistance. If the cytotoxic actions of DNR were on the cell membrane, then the mechanism by which the MDR reversing agents increase cytotoxicity cannot be due to intracellular accumulation since there is no free intracellular drug present.

Cy A caused cytotoxicity on its own in the drug sensitive CEM cells and, to a lesser extent, in the drug resistant Pgp positive VLB cell line. Cy A is known to have a very wide range of effects, and one of these effects is interaction with the cell membrane (Colombani *et al.*, 1985). In the CEM and VLB cell line Cy A may interact with the cell membrane leading to destabilisation, thereby causing cytotoxicity to the cells.

Cy A was, however, able to lead to an increase in the DNR cytotoxicity of the drug sensitive HL 60 cell line and the drug resistant Pgp negative ADR cell line. In both cell lines Cy A, in addition to the immobilised-DNR at 20μg/ml of DNR, was able to produce approximately a 50% reduction in colony formation. Cy A and the addition of the immobilised-DNR at 2μg/ml of DNR was unable to significantly increase the cytotoxicity, but there did appear to be some decrease in cell viability. In contrast to what was found in the CEM and VLB cell lines, Cy A itself had no effect on the viability of the HL 60 or ADR cell line. Cy A appeared to increase the colony formation of the HL 60 cell line. The difference seen in the actions of Cy A in the two cell lines is probably due to the origins of the lines. CEM is a lymphocytic T cell line whereas the HL 60 cell line is a promyelocytic cell line. Cy A has previously been shown to act on T cell lines by restricting a number of functions vital to the growth of T cells (Colombani *et al.*, 1985).

Tri is another agent shown to reverse multidrug resistance, Tri had no increased effect to that of immobilised-DNR on either of the drug resistant cell lines, ADR or VLB. It was, however, able to increase the cytotoxicity of the immobilised-DNR in the drug sensitive CEM and HL 60 cell lines. The cytotoxicity of the immobilised-DNR at low concentrations was increased by over 80% with the addition of Tri in the CEM cell line. At the high concentration this effect was not seen because the CL-PVA itself was cytotoxic. Tri also increased the cytotoxicity in the HL 60 cell line at the high concentration of immobilised-DNR by an additional 15%. However, it had no effect at the low concentration of immobilised-DNR. These results suggest that Tri may interact with the cell membrane in such a way to allow the immobilised-DNR to act on the cell membrane, thus enhancing its cytotoxic actions.

Although the MDR reversing agents, Cy A and Tri have been shown to reverse

drug resistance by increasing the intracellular concentration of cytotoxic agents, these agents also appear to be capable of increasing the cytotoxicity of immobilised-DNR when there is no free DNR present. Adler & Tritton (1988) showed that DOX adopted an angle of about 55° in the cell membrane, which could be very disruptive to orderly bilayer packing. The immobilised-DNR could insert itself similarly in the cell membrane, thus disrupting the cell membrane and leading to cell death.

CHAPTER 7

General Discussion

7.1. Overview of the studies conducted

The pharmacokinetics of DNR and its metabolite DOL was studied in 27 patients. Twelve achieved a complete remission (CR), 5 had a partial response, 6 had no response to treatment (the 5 partial response and 6 no response patients were grouped together and termed non responders (NR)) and 4 were not evaluable (Chapter 4.3). The plasma and cellular pharmacokinetic parameters of DNR were determined and are summarised in Tables 4.2 - 4.9. There were no relationships found between any of the plasma pharmacokinetic parameters for either DNR or DOL and the patients response to treatment (Chapter 4.5.1). There were, however, significant differences between the cellular DNR pharmacokinetic parameters and patient response (Chapter 4.5.2). Patients who responded to DNR treatment had higher maximum cellular concentrations (C max) of both DNR and DOL, as well as higher cellular AUC (0-24 hr) and higher cellular AUMC (0-24 hr) for both DNR and DOL. These three cellular pharmacokinetic parameters indicate that higher concentrations of both the parent drug DNR and its metabolite DOL were achieved in those patients who responded to treatment compared to those patients who did not. Patients who responded to DNR chemotherapy also had a significantly lower cellular clearance and volume of distribution compared to those who did not. This indicates that those patients who cleared DNR at a faster rate, thereby reducing the amount of DNR

present in the cells, were not able to respond to DNR chemotherapy as well as those patients who did not clear the drug as quickly. This suggests that cellular concentrations of DNR are important in determining whether a patient will respond to treatment or not. The effects of the metabolite DOL are a little more difficult to interpret. Although the *in vivo* findings are almost identical to those of DNR, the relationships between DOL and patient response may be due simply to the presence of DNR and not the metabolite itself.

The overexpression of Pgp has been shown to correlate with drug resistance in some leukaemia cell lines. The clinical significance of this is uncertain. In this study the overexpression of Pgp was determined by immunocytochemistry, and of the 21 evaluable patients, 10 were Pgp positive (7 CR, 3 NR) and 11 Pgp negative (4 CR, 7 NR). There was no statistical relationship between Pgp and patient response (p=0.20, FE). A recent study (Wattel *et al.*, 1995) has also found no correlation between Pgp and patient response to treatment, in fact there was a slightly better response to treatment in the Pgp positive patients (29 CR, 3 NR) than Pgp negative patients (14 CR, 4 NR). There was also no significant relationship between the amount of DNR accumulated by the cells and Pgp (Chapter 4.8). These results indicate that the presence of Pgp is not a major reason for treatment failure in patients receiving DNR chemotherapy. One possible reason for the lower cellular DNR in patient leukaemic cells could be due to the presence of non-Pgp mechanisms of resistance such as that associated with the HL 60/ADR cell line (Marsh *et al.*, 1986 and Chapter 3.5.4). In that drug resistant cell line there was a decrease in intracellular drug concentration, but no detectable Pgp. Krishnamachary & Center (1993) have demonstrated the presence of another membrane protein that may be responsible for the decreased cellular drug accumulation present in the HL 60/ADR cell line. This membrane protein has been associated with the overexpression of the *MRP* gene and may play a role in patients with acute leukaemia that do not respond to treatment. Therefore the amount of DNR accumulated by the cells appears to be a more important factor in the ability of a patient to respond to treatment than the presence of Pgp.

Previous *in vitro* studies examining the actions of cytotoxic agents and drug resistance have used high concentrations of either DNR or MDR reversing agents. This study examined the effects of clinically achievable concentrations of DNR, Cy A and Tri, firstly in leukaemic cell lines (Chapter 3) and then in the blast cells of patients with acute leukaemia (Chapter 5). The amount of DNR required to kill 50% of the cells (IC 50) for the 4 cell lines used in this study (CEM, VLB, HL 60 and ADR) are summarised in Table 7.1. The CEM cell line is a drug sensitive cell line and the VLB cell line is a drug resistant subline of CEM. The VLB cell line is also Pgp positive. The other series of cell lines used were the drug sensitive HL 60 cell line and its drug resistant ADR subline. The ADR cell line

Table 7.1. Cytotoxicity and accumulation of DNR in drug sensitive (CEM, HL 60) and drug resistant (VLB, ADR) cell lines with and without MDR reversing agents.

Cell Line	DNR Cytotoxicity (ng/ml)	DNR Accumulation (ng.hr/million cells)	DNR Retention (ng.hr/million cells)
CEM	49	378	388
CEM + Cy-A	34	428	355
CEM + Tri	110	384	322
CEM + Cy A + Tri	44	452	276
VLB	571	33	25
VLB + Cy-A	245	176	99
VLB + Tri	945	41	28
VLB + Cy A + Tri	130	176	93
HL 60	35	397	257
HL 60 + Cy-A	20	471	267
HL 60 + Tri	44	395	252
HL 60 + Cy A + Tri	29	544	267
ADR	975	185	66
ADR + Cy-A	25	341	122
ADR + Tri	446	226	71
ADR + Cy A + Tri	134	403	129

Data summarised from Tables in Chapter 3. Cy A = 1.5μg/ml, Tri = 150ng/ml

although being drug resistant is Pgp negative. The MDR reversing agent Cy A was able to decrease the IC 50 of DNR in both the VLB and ADR resistant cell lines, whereas Tri was only able to decrease the IC 50 in the ADR cell line. The combination of Cy A and Tri had no additional effect on the IC 50 above that of each of the agents individually (Table 7.1). Cy A and Tri, either alone or in combination had no effect on the drug sensitive CEM or HL 60 cell lines.

The amount of DNR accumulated and retained by each of the cell lines is also summarised in Table 7.1. The drug sensitive CEM cell line accumulates almost 12 times more DNR than the drug resistant Pgp positive VLB cell line. The amount of DNR accumulated in the CEM and VLB cell lines is consistent with the findings of Haber *et al.* (1989). The addition of Cy A to VLB was able to increase DNR accumulation by over 5 times, however this was still only half of the DNR accumulated by the CEM cell line. Slater *et al.* (1986) and Hu *et al.* (1990) have both shown that increasing the concentration of Cy A leads to an increase in the ability to reverse drug resistance. A higher concentration of Cy A would probably lead to DNR accumulation similar to that of the CEM cell line, however concentrations greater than 1.5μg/ml of Cy A are not readily achieved in *in vivo* situations, thus could not be used clinically. Tri had no effect on DNR accumulation in the VLB cell line. All previous studies (Tsuruo *et al.*, 1982; Akiyama *et al.*, 1986; Ganapathi & Grabowski, 1988) have used Tri at concentrations of 1-5 μg/ml to show increased accumulation and retention in drug resistant cell lines, this is at least ten times the concentration used throughout this work and indicates that at a clinically achievable concentration of 150 ng/ml, Tri is not capable of increasing accumulation or retention of DNR in drug resistant cell lines. The combination of Cy A and Tri had no additional effect on the VLB cell line above that of Cy A alone. The MDR reversing agents had no effect on the accumulation of DNR in the drug sensitive CEM cell line, which is consistent with the findings of other studies in which the MDR reversing agents had no effect on the drug sensitive cell lines (Tsuruo *et al.*, 1982; Akiyama *et al.*, 1986; Ganapathi & Grabowski, 1988; Haber *et al.*, 1989). The amount of DNR accumulated by the drug resistant Pgp negative ADR cell line was almost half of that accumulated by the drug sensitive HL 60 cell line in agreement with the findings of Marsh *et al.* (1986). The addition of Cy A increased DNR accumulation in the ADR cell line to that of the drug sensitive parent HL 60 cell line. As seen in the drug resistant VLB cell line, Tri had no effect on the accumulation of DNR in the drug resistant ADR cell line. The combination of Cy A and Tri, did not significantly increase DNR accumulation over the action of Cy A alone. Similar to results in the drug sensitive CEM cell line, Cy A and Tri had no effect on DNR accumulation in the drug sensitive HL 60 cell line. The effects of the MDR reversing agents on DNR retention reflected those on DNR accumulation.

The above results indicate that when Cy A is used at clinically relevant concentrations, it is able to reverse drug resistance in both the Pgp positive VLB cell line and the Pgp negative ADR cell line. Cy A was able to only partially reverse drug resistance in the Pgp positive VLB cell line but was able to completely reverse drug resistance in the Pgp negative ADR cell line. This leads to the conclusion that Cy A has the ability to reverse not only Pgp mediated drug resistance but other forms of transport mediated drug resistance which must be acting in the Pgp negative ADR cell line. A recent study (Cole *et al.*, 1994) confirms the finding that Cy A is capable of reversing non Pgp mechanisms of drug resistance. At non-toxic clinical concentrations of the other MDR reversing agent, Tri, drug resistance was not reversed in the leukaemia cell lines studied. The combination of Cy A and Tri together did not appear to have any additional effects over that of Cy A alone.

After establishing the actions of the MDR reversing agents on DNR accumulation and retention in leukaemia cell lines (Chapter 3), the actions of Cy A and Tri on DNR accumulation and retention were examined in the blast cells of patients with acute leukaemia (Chapter 5). The accumulation and retention of DNR was studied in patients at initial presentation of leukaemia and at relapse. In patients at initial presentation both Cy A and Tri alone produced small (but statistically non-significant) increases in both accumulation and retention. However, a significant increase in both DNR accumulation and retention was achieved when the combination of Cy A and Tri was used. For the patients at relapse the MDR reversing agents had no effect on increasing either accumulation or retention, suggesting that MDR reversing agents may be more appropriate for patients at initial presentation than those at relapse. Most studies have examined the use of MDR reversing agents in patients that are inclined to be resistant to treatment (Kessel *et al.*, 1984; Ross *et al.*, 1986; Andersson *et al.*, 1987) and minimal work has been done on patients at initial presentation. Studies from resistant patients have showed minimal effects of MDR reversing agents. It may be possible to use the MDR reversing agents at initial presentation in order to increase DNR accumulation and thereby minimising the chances of a patient going into relapse, thus increasing patients chances of achieving a complete remission.

The patients involved in the accumulation/retention study were also classified according to the presence of Pgp. Of the 24 patients in the *in vitro* study, 16 had blast cells that were Pgp negative and 7 had blast cells that were Pgp positive. There was inadequate sample for one of the patients. There appeared to be no real difference between the AML patients at initial presentation (5 Pgp positive and 9 Pgp negative) or at relapse (2 Pgp positive and 4 Pgp negative) in terms of Pgp status. All of the ALL (3) patients were at relapse and Pgp negative. These results are similar to the findings of Kuwazuru *et al.* (1990b) who found only 8 out of 20 patients at initial presentation were Pgp positive and

Ito *et al.* (1989) who found no Pgp positive patients at either initial presentation (0/14) or relapse (0/18). The MDR reversing agents had no effect on either the accumulation or the retention of DNR in the blast cells from Pgp negative patients. This is in agreement with the drug sensitive CEM and HL 60 cell lines in which there was no increased DNR accumulation or retention. The combination of Cy A and Tri caused a significant increase in DNR accumulation in the Pgp positive blast cells of patients. Only one previous study has examined the possibilities of using two MDR reversing agents together (Hu *et al.*, 1990), in that study it was suggested that there was a synergistic mechanism present when Cy A and verapamil were used in combination at concentrations lower than what could be used individually. Therefore, the possibility of using two or more MDR reversing agents together at more clinically appropriate concentrations may be an avenue for further investigation. An increase in DNR retention was seen, but was not statistically significant. Therefore, the MDR reversing agents individually were unable to significantly increase DNR accumulation or retention in Pgp positive patient blast cells, in contrast to Cy A's ability to increase both accumulation and retention in the drug resistant Pgp positive VLB cell line (Chapter 3). The ability of Cy A, however, to increase accumulation and retention in the drug resistant Pgp negative ADR cell line indicated an alternate mechanism for Cy A. The ADR cell line has the membrane transport protein (MRP) present, which is believed to be responsible for the cell lines' drug resistance (Krishnamachary & Center, 1993). MRP is a membrane transport protein similar to Pgp and thus Cy A may also inhibit the actions of MRP, leading to increased DNR accumulation. MRP has recently being transfected into two cell lines, NIH/ 3T3 (Breuninger *et al.*, 1995) and HeLa cells (Cole *et al.*, 1994), these MRP transfectants displayed increased resistance to a number of drugs including DNR, DOX, epirubicin, etoposide, vincristine and vinblastine. Decreased DNR (Breuninger *et al.*, 1995) and vincristine (Cole *et al.*, 1994) accumulation and increased efflux was seen in these MRP transfectants, and both Cy A and verapamil were able to increase vincristine toxicity (Cole *et al.*, 1994). Breuninger *et al.* (1995) concluded that MRP functions to extrude drugs from cells, while Cole *et al.* (1994) demonstrated that the multidrug resistance phenotype conferred by MRP is similar but not identical to that conferred by Pgp. This indicates that Cy A is able to reverse drug resistance in both Pgp (VLB) and non Pgp (ADR) mediated resistance mechanisms in cell lines. However, the MDR reversing agents may not be able to play a significant role in the reversal of MDR resistance in Pgp positive patients blast cells.

The finding in Chapter 3 that Cy A was able to reverse drug resistance in a Pgp negative resistant cell line, ADR, indicates that Cy A does not work solely by acting on Pgp. Tritton *et al.* (1978) showed that membrane fluidity was increased in the presence of DOX, and Henry *et al.* (1985) and Henry-Toulme *et al.* (1988) provided evidence for two distinct

membrane binding sites for DOX. They suggested that in site I the amino sugar of DOX is bound to a charged phosphate with the chromophore lying outside the bilayer, whilst in site II the sugar phosphate interaction persists, but the chromophore is embedded in the bilayer. Thus, evidence existed for the interaction of DNR with the cell membrane. Therefore, in an attempt to understand the process of drug resistance further, the effects of DNR on the cell membrane were examined. This was achieved by immobilising DNR, that is attaching DNR to an inert support so it was unable to enter the cell. The method for immobilisation is described in Chapter 2.2.11, and the results obtained from the work are in Chapter 6. It was found that the immobilised-DNR was only able to kill the drug sensitive HL 60 cell line and had no effect on the drug resistant ADR or VLB cell line. The effects on the drug sensitive CEM cell line could not be determined because the PVA support itself was able to kill the CEM cell line. This suggests that immobilised-DNR may be effective against drug sensitive cell lines but not against drug resistant cell lines. There may be changes associated with the cell membrane in these drug resistant cell lines which do not allow immobilised-DNR to exert a cytotoxic action via the cell membrane.

Cy A itself had an effect on both the CEM cell line and its drug resistant VLB cell line, thus any effects seen in the presence of Cy A in these two cell lines were due in large part to Cy A alone. Tri was able to increase the cytotoxic actions of low concentrations of immobilised-DNR in the CEM cell line. Thus, Tri, an MDR reversing agent, is capable of potentiating the actions of immobilised-DNR in a drug sensitive cell line. This suggests that the actions of Tri are not solely due to altering the action of Pgp, and indicates there is an alternative mechanism for the actions of Tri. Tri is known to bind to calmodulin. This binding may have led to changes in the cell membrane which in turn allowed the interaction of further immobilised-DNR with the cell membrane, leading to increased cell death.

The MDR reversing agents Cy A and Tri had no effect in increasing the cytotoxicity of immobilised-DNR in the drug resistant Pgp positive cell line. This, indicates that the actions of Cy A and Tri at the concentrations used here in the VLB cell line had no effect in the presence of extracellular DNR. This is in contrast to the effects seen in Chapter 3 where DNR cytotoxicity and accumulation were increased in the presence of Cy A. This suggests that there are two separate actions for DNR: an extracellular activity which is not affected by Cy A and an intracellular activity which is affected by Cy A. The MDR reversing agents also had no effect in the HL 60 cell line or its drug resistant Pgp negative subline ADR, indicating two different actions for DNR: an extracellular action which may involve the insertion of DNR in the lipid bilayer and thus disrupting the cell membrane, and an intracellular action where DNR intercalates with DNA.

Acute leukaemia patients who responded to chemotherapy had higher cellular DNR and DOL compared to non responders. Overexpression of Pgp was not the sole explanation for the lower cellular DNR levels as was expected from the *in vitro* studies. Similarly the MDR reversing agents Cy A and Tri do not work solely by inhibiting the actions of Pgp. The results also indicate that these MDR reversing agents may be more useful in patients at initial presentation than in patients at relapse.

7.2. Implications and future work

It is not uncommon to find large variations in data obtained from patients (Marie *et al.*, 1993; Ross *et al.*, 1993). It is therefore very important to try to design experiments and clinical trials which minimise some of the biological variations to which these studies are prone. The use of non parametric statistics (which have been used throughout this thesis) help in overcoming some of the difficulties in interpreting results so that some conclusions can be drawn. Caution must be used in all studies when examining results with large variations and comparing them to studies of a similar nature. Although large variations do occur, these studies are very important in attempting to understand the *in vivo* actions of drugs such as DNR and the MDR reversing agents Cy A and Tri. The actions of Cy A and Tri were shown to be different in patient blast cells (Chapter 5) than in cell lines (Chapter 3), indicating that conclusions drawn from experimental models do not always reflect what may be occurring *in vivo*. The differences between the cell lines and patients could be due to the cell lines being generally homogeneous, whereas patient cells are heterogeneous, thus the cell line is possibly only a small proportion of what is seen in the patients' cells. The CEM and VLB cell lines used in this work are T cell leukaemia cell lines, whereas most of the patients used were mainly AML and non T cell ALL. The differences between the cell lines and patient cells could also be due to the method of drug selection, the drug resistant cell lines are selected for resistance by growing them in the presence of a single cytotoxic agent, whereas the patients cells have been exposed to a variety of different cytotoxic agents.

In any statistical procedure there are two types of errors that can be made: Type I or α error, the possibility of concluding that a treatment has an effect when in actual fact it does not (false positive); and Type II or b error, concluding that a treatment has no effect when it does (false negative). To be more confident that a treatment has an effect, that is, make a smaller, you increase your chances of missing a true effect, that is, make β bigger. The chance of detecting a true positive, that is, reporting a statistical significant difference when the treatment produces an effect, is $1-\beta$ and this is called the statistical power of the

test. Traditionally an α value of 0.05 is chosen and a β value of 0.2, this means that there is a 5% chance of a false positive and a 20% chance of a false negative, with a power of 80%.

The most statistical power in any of the studies trying to relate Pgp with patient response was by Campos et al. (1992). In that study 23/71 (32%) Pgp positive patients responded to treatment, whereas 64/79 (81%) Pgp negative patients responded to treatment. Campos et al. (1992) had 80% power to detect a difference of 23%. Two other studies showing a relationship between Pgp and patient response had 80% power to pick up a 48% difference (Marie et al., 1991) but only detected a 38% difference which was significant and 80% power to pick up a 35% difference (Pirker et al., 1991) in which they detected a 36% difference between the two groups. Wattel et al. (1995) on the other hand had 80% power to detect a 29% difference and concluded that there was no relationship between Pgp and patient response, in that study Pgp positive patients (29/32, 91%) had a better response rate than Pgp negative patients (12/18, 67%). Gruber et al., (1992) also showed no difference between Pgp and patient response with 80% power to pick up a 36% difference they only detected a 15% difference between Pgp positive patients (12/18, 67%) and Pgp negative patients (23/28, 82%). The study trying to correlate Pgp and patient response (Chapter 4.7) in which 7/10 (70%) patients that were Pgp negative responded to treatment while only 4/ 11 (36%) Pgp positive patients responded to treatment had 80% power to detect a 57% difference between the two groups. The actual difference seen between the two groups was only 34%, and for the study to detect a 34% difference with 80% power would require another 40 patients, to give group sizes of 30 patients. Therefore even with adequate power to pick up a 34% difference, their may have not been a relationship between Pgp and patient response.

The same situation is seen in the study of cellular accumulation and Pgp (Chapter 4.8). For example for the relationship between DNR AUC and the presence of Pgp in which only 6 patients were obtained in each group the study had 80% confidence in showing a difference of 2 between the groups, the study however only showed a difference of 0.8. To be 80% certain that a difference of 0.8 was significant at the 0.05 level a sample size of 25 per group is required. Therefore it is important when designing any study that some form of power analysis be done to calculate the chances of concluding incorrectly, obviously the larger the sample size the better. However on occasions we have to consider detecting larger differences with confidence when only smaller sample sizes are available. In cancer studies where patient numbers are difficult to obtain there is a fine line between having enough patients and whether the studies are worth doing. For example in the Pgp vs response study only 21 patients were obtained over a 3 year period to recruit a further 40 patients would require the study to run over a period of nine years. Therefore these decisions

need to be considered when a clinical study is being designed. There have been virtually no studies trying to relate cellular accumulation with Pgp in patients, therefore even though there was only a small sample size in this study, clinical information of this nature is still required.

The fact that a relationship was seen between the intracellular accumulation of DNR and patient response (Chapter 4) indicates that this may be a useful variable to help determine the predicted outcome of a patient being treated with DNR. If low intracellular levels of DNR were found then increased doses of DNR could be given to these patients in an attempt to overcome their poor response to treatment. This may be difficult to examine as there is a move in clinical practice away from DNR to the analogue idarubicin (IDA). Several trials (Berman *et al.*, 1991; Vogler *et al.*, 1992; Vogler *et al.*, 1989; Wiernik *et al.*, 1992; Wiernik *et al.*, 1989) have compared the effectiveness of DNR and Ara C against IDA and Ara C and these studies have shown that complete remission rates have increased from 58% with DNR and Ara C to between 67%-80% with the use of IDA and Ara C, indicating that IDA plus Ara C may be a more effective regimen than DNR plus Ara C. The higher intracellular levels of IDA has been postulated to be the major factor for its enhanced effectiveness (Berman & McBride, 1992). Therefore, any further studies along this line will more than likely be conducted with IDA. Because, in contrast to results in cell lines, no relationship was found between the presence of Pgp and patient response, further work examining the relationships of other possible MDR causing mechanisms, such as MRP and topoisomerase II, and patient response should be looked at in patients with acute leukaemia. Recent studies have shown MRP to be present in patients with acute leukaemia (Zhou *et al.*, 1995; Schneider *et al.*, 1995; Hart *et al.*, 1994), therefore the role of MRP needs to be examined in further detail when investigating resistance mechanisms present in acute leukaemia.

Pgp can be detected at the either the DNA level (Southern blot, dot blot), RNA level (Northern blot, slot blot, polymerase chain reaction (PCR), RNAse protection assay, *in situ* hybridization) or at the protein level (immunohistochemistry, Western blots, flow cytometry). All these methods have advantages and disadvantages to their use. The ideal method needs to be both sensitive (detect low levels of Pgp) and specific (distinguish resistant from sensitive cancer cells and cancer cells from reactive host cells). Herzog *et al.* (1992) used eight methods for Pgp detection including Northern blot, slot blot, PCR, *in situ* hybridization, immunofluoresence, immunocytochemistry, fluoresence activated cell sorting and immunoblot analysis in a panel of 9 cell lines with varying amounts of Pgp. They concluded that for detection of mdr1/Pgp there was no clearly superior method, multiple factors needed to be considered prior to deciding on a particular method, and no method is

indicated in all situations, but PCR analysis offered the advantage of being the most sensitive.

Brophy *et al.* (1994) compared four methods of mdr1/Pgp detection, immunocytochemistry with MRK 16, *in situ* hybridisation, slot blot and PCR in 36 cell lines established from children with acute leukaemia. They showed that in 14 cases (39%) all four methods agreed and in another 39% the results differed on a single test result. They considered these 78% (28) of cases as assessable and the consensus results were considered correct. They defined sensitivity as the true positives divided by the addition of the true positives and false negatives, and specificity as the true negatives divided by the sum of the false positives and true negatives. They found that slot blot (100%) was the most sensitive assay, followed by PCR (93%), *in situ* hybridisation (92%) and immunocytochemistry (80%). However, slot blot was the least specific of the methods at 54%, while immunocytochemistry was at 80% and *in situ* hybridization and PCR at 100%. They concluded that these four techniques yield discordant results for mdr1 detection, and recommended the use of at least two methods, PCR for its relative simplicity and specificity and a technique capable of detecting heterogeneity of Pgp among cells such as immunocytochemistry or flow cytometry.

Zhou *et al.* (1992) compared slot blot to immunohistochemistry using the C219 antibody in a group of 42 AML patients and using the same definitions as Brophy *et al.* (1994) for sensitivity and specificity, found the slot blot method more sensitive than immunohistochemistry (78% vs 57%), however immunocytochemistry was more specific than the slot blot method (84% vs 61%). Zhou *et al.* (1992) also used the MRK 16 antibody in 33 of the patients, and showed that C219 agreed with MRK 16 on 28 of 33 occasions. The 5 disagreements were positive for C219 and negative for MRK16, of these results 3 were negative for slot blot and 2 were positive.

It is evident that immunohistochemistry may lack the sensitivity of some of the molecular techniques such as PCR, *in situ* hybridization and slot blots, but it has the advantage of detecting Pgp in an individual cell and the ability to differentiate malignant from non-malignant cells, thus giving the method good specificity. Immunohistochemistry is also well suited to clinical situations where only limited samples are available and methods such as slot blot, Northern blot, RNase protection assay, immunoblot and Western blotting require large samples. There is the need to optimize a number of conditions in immunohistochemistry, including the antibody to be used, the fixation steps, the use of either fresh samples or frozen samples (which may affect recognition of the epitope by antibodies) and the possibility of cross reaction of antibodies which can effect the

specificity. Three antibodies are widely used for Pgp detection these are C219, MRK 16 and JSB 1 and all 3 recognize a different epitope of Pgp, C 219 and JSB 1 recognize an intracelullar epitope where as MRK 16 recognizes an extracellular epitope, it is feasible to use a panel of antibodies to avoid non specific staining which can be associated with these antibodies as was done in this study to help increase the specificity of these antibodies.

There appears to be no standard method for immunohistochemistry of Pgp and this may be one of the reasons why there are a number of conflicting results in studies trying to relate the presence of Pgp and patient response. If some of these problems are to be overcome then standard methods for Pgp need to be determined, probably the use of PCR as well as immunocytochemistry in all studies to obtain a greater understanding of the relationship between Pgp and treatment outcome, this however still leaves one problem associated with methods used to detect Pgp and that is, is the detected Pgp functional. An assay for Pgp function may also be required.

The use of MDR reversing agents was examined in Chapters 3, 5 and 6. Cy A was able to increase DNR accumulation in cell lines that were drug resistant. However, this was not seen in the blast cells of patients with acute leukaemia. The combination of Cy A and Tri was, however, able to increase accumulation in the blast cells of patients at initial presentation but not those in relapse. Therefore, it will be important to examine the role of the MDR reversing agents not only in poor prognosis patients (who are thought to be resistant) but also in patients at initial presentation of disease. Recent clinical studies have shown that Cy A can increase both DOX (Bartlett *et al.*, 1994; Erlichman *et al.*, 1993) and DNR (List *et al.*, 1993) accumulation in patients with poor prognosis. This suggests that Cy A can be used as a clinical modulator to lead to increased DNR accumulation, which has been shown to be important in determining whether patients respond to treatment or not (Chapter 4). It is also reasonable to suggest that MDR reversing agents be used in patients at initial presentation, to attempt to achieve higher intracellular concentrations of DNR, thus increasing patients' chances of responding to treatment and resulting in more efficient therapy.

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