2001

P-GLYCOPROTEIN-ASSOCIATED ANTHRACYCLINE RESISTANCE IN B-CLL: P0TENTIAL FOR CYTOKINE MODULATION

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http://hdl.handle.net/10026.1/2809

http://dx.doi.org/10.24382/1437
University of Plymouth

P-GLYCOPROTEIN-ASSOCIATED ANTHRACYCLINE RESISTANCE IN B-CLL: POTENTIAL FOR CYTOKINE MODULATION.

by

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A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

Department and Faculty
of
Plymouth Postgraduate Medical School

September 2001

To my family

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P-GLYCOPROTEIN-ASSOCIATED ANTHRACYCLINE RESISTANCE IN B-CLL:

POTENTIAL FOR CYTOKINE MODULATION

VARINIA GRACIELA MUNOZ-RITCHIE

ABSTRACT

The phenomenon of multidrug resistance (MDR) in cancer cells is generally associated with P-glycoprotein (P-gp) expression and presents an obstacle to successful chemotherapy. Attempts to overcome P-gp-associated MDR using P-gp modulators, such as verapamil, have been hindered by their intrinsic *in vivo* toxicity. In 1991, however, Scala *et al.* demonstrated the alteration of P-gp function by interferon-alpha (IFN-α) *in vitro* at non-toxic *in vivo* concentrations, suggesting a basis for the use of IFN-α clinically in patients exhibiting P-gp-associated MDR.

Drug resistance in B-CLL has been linked to the phenomenon of MDR, however, publications regarding this have been conflicting. The contrasting results prompted further investigation of the role of P-gp-associated anthracycline resistance and, using isolated B-lymphocytes from B-CLL patients, this investigation examined P-gp expression, function and IFN-α modulation *in vitro*.

Optimum conditions for *in vitro* analysis of P-gp-associated anthracycline resistance were determined by examining the stability of the anthracycline, daunorubicin, in varying cell culture conditions. The resulting system balanced conditions affecting drug stability with those affecting cell survival. While other investigations have neglected the issue of drug stability, this study demonstrates that the instability of daunorubicin may be a critical variable determining the outcome of drug sensitivity studies. In RPMI + 2mM L-glutamine and 10% (v/v) FBS, loss of drug concentration is due to both adsorption and degradation and these experiments show that the presumed availability of drug may be over-estimated in *in vitro* studies. Furthermore, the degradation products might interfere with P-gp function and modulation.

MDR1 gene mRNA was detected in the B-cells of forty-three out of fifty B-CLL patients analysed, whereas P-gp expression, as measured by flow cytometry, resulted in only sixteen patients out of fifty-five being classed as positive (>10% increase in staining as compared to the control). P-gp functionality and modulation studies on the B-cells of eleven patients confirmed the existence of an efflux mechanism with identical characteristics to P-gp using verapamil, the dye rhodamine 123 (rho123) and daunorubicin. Four patients were classed as functional low expressors (functional P-gp with low P-gp expression (7-10% increase in staining)), six were classed as functional high expressors (functional P-gp with high P-gp expression (20-57% increase in staining)) and one as a non-functional high expressor (non-functional P-gp with high P-gp expression (13.4% increase in staining)). Verapamil modulated rho123 efflux in all ten patients classed as P-gp functional expressors, and daunorubicin efflux in eight of these patients. However, IFN-α modulated rho123 and daunorubicin efflux in only two and one patients, respectively, even at concentrations higher than 500I.U./ml. In contrast to Scala *et al.* (1991), this finding suggests that at a well tolerated concentration IFN-α may not be suitable for use as a P-gp modulating agent *in vivo* in B-CLL, although conclusive evidence would require a larger study.

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LIST OF ABBREVIATIONS

'ABC' -	ATP binding cassette
ADP -	adenosine diphosphate
AIDS -	acquired immune deficiency syndrome
ANOVA -	analysis of variance
ATP -	adenosine triphosphate
B-CLL -	B-cell chronic lymphocytic leukaemia
BMTs -	bone marrow transplants
bp -	base pair
cAMP -	cyclic adenosine monophosphate
CAP -	cyclophosphamide, doxorubicin & prednisone
CD -	cluster of differentiation
cDNA -	complementary deoxyribonucleic acid
CE -	capillary electrophoresis
CHOP -	cyclophosphamide, doxorubicin, vincristine & prednisone
CNS -	central nervous system
COP -	cyclophosphamide, vincristine & prednisone
CSFs -	colony stimulating growth factors
CV -	coefficient of variation
CZE -	capillary zone electrophoresis
DMEM -	dulbecco's modified eagles medium
DMSO -	dimethyl sulfoxide
DNA -	deoxyribonucleic acid
ECACC -	european collection of animal cell culture
EDTA -	ethylenediaminetetraacetic acid
FBS -	foetal bovine serum
FITC -	fluorescein isothiocyanate
GM-CSF -	granulocyte/macrophage colony stimulating factor
GST -	glutathione-S-transferase
GVHD -	graft versus host disease
HGFs -	haematopoietic growth factors
HGPRT -	hypoxanthinecyaninephosphoribosyl transferase
HIV -	human immunodeficiency virus

HPLC	-	high-performance liquid chromatography
I.U.	-	international units
IC_{50}	-	inhibitory concentration resulting in 50% cell death
IFN	-	interferon
Ig	-	immunoglobulin
IL	-	interleukin
LOQ	-	limit of quantification
MAb	-	monoclonal antibody
MDR	-	multidrug resistance
MECC	-	micellar electrokinetic capillary chromatography
mRNA	-	messenger ribonucleic acid
MRP	-	multidrug resistance-associated protein
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
PAGE	-	polyacrylamide gel electrophoresis
PBMCs	-	peripheral blood mononuclear cells
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
PE	-	phycoerythrin
PETG	-	2-phenylethyl-β-D-thiogalactoside
P-gp	-	P-glycoprotein
PI	-	propidium iodide
PK1	-	protein kinase 1
PKC	-	protein kinase C
PMA	-	phorbol 12-myristate 13-acetate
PMT	-	photomultiplier tube
PS	-	phosphatidylserine
rHuIFN-α-2a	-	recombinant human interferon-alpha-2a
RNA	-	ribonucleic acid
RNases	-	ribonucleases
RT-PCR	-	reverse transcriptase-polymerase chain reaction
SDS	-	sodium dodecyl sulphate
TBE	-	Tris-borate in EDTA
TEMED	-	N,N,N',N'-Tetramethyl-ethylenediamine
Topo	-	topoisomerase
Tris	-	2-amino-2-(hydroxymethyl)-1,3-propanediol

ACKNOWLEDGEMENTS

AUTHOR'S DECLARATION

This thesis is the result of my own investigations, except where otherwise stated.

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award. This work has not been previously accepted in substance for any degree.

This study was financed with the aid of a studentship from the University of Plymouth. A programme of study was undertaken, which included a course in the analytical technique of flow cytometry.

Relevant scientific seminars and conferences were attended at which work was often presented; and abstracts prepared for publication.

Publications:

1] Munoz V, *et al*. (1998) Loss of Daunorubicin from *in vitro* systems used in drug resistance studies. *EORTC (European Organisation for Research and Treatment of Cancer) Abstracts.* Winter Meeting, January 21-24th, Nancy, France.

2] Munoz V, *et al*. (1999) Implications of Anthracycline Stability in *in vitro* Systems when Investigating Drug Resistance. *Blood.* **94(10)** Suppl 1, p 194b.

3] Munoz V, *et al*. Drug Resistance Studies - Implications of Daunorubicin Stability in *in vitro* Systems. Science Proceedings, 136th British Pharmaceutical Conference, Cardiff, September 13th-16th. *Journal of Pharmacy and Pharmacology.* (1999) **51, Supplement**.

Presentation and Conferences Attended:

1] Oral presentation at the National Leukaemia Research Fund Meeting in Plymouth, 1997

2] Munoz V, *et al*. Loss of Daunorubicin from *in vitro* systems used in drug resistance studies. Oral presentation. *EORTC (European Organisation for Research and Treatment of Cancer)*. Winter Meeting, January 21-24th 1998, Nancy, France.

3] Oral presentation at Drug Resistance Autumn Workshop, Gatwick, 1998

4] Oral presentation at South West Haematology Society Meeting, Plymouth, 1998.

5] Oral presentation at South West Clinical Pharmacology Meeting, Cardiff, 1999.

6] Munoz V, *et al*. Drug Resistance Studies - Implications of Daunorubicin Stability in *in vitro* Systems. Oral presentation. Science Proceedings, 136th British Pharmaceutical Conference. Cardiff, September 13th-16th, 1999

Signed..

Date..

"If we knew what it was we were doing, it would not be called research, would it?"

.........Albert Einstein.

1. BACKGROUND

1.1   NATURE OF MALIGNANT DISEASE

1.1.1   CELL GROWTH & CELL CYCLE

The term 'Cell Cycle' is used to describe a series of events necessary for the duplication of DNA as well as cell growth (Fig. 1.1).

Fig. 1.1 Four phases of mammalian cell cycle.
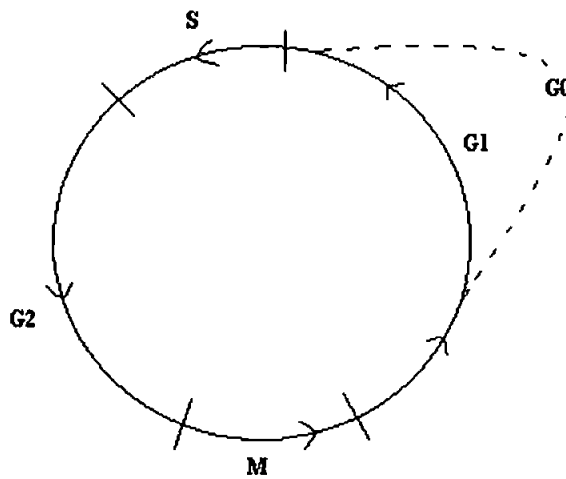
Fig.1.1 G_1-phase - a time gap between M and S-phase; S-phase - the synthetic phase that results in the complete duplication of nuclear DNA; G_2-phase - a time gap between S and M phase; M-phase - the mitotic phase that is characterised by a condensation of chromosomes, spindle attachment and segregation into two; G_0 - sometimes referred to as a separate phase but is actually a quiescent period associated with the G_1-phase.

During the cycle, measures are adopted to counter the threat posed by any cell that may have acquired a flawed genome, the ultimate measure being that of cell death initiation. Failure to initiate cell death is associated with the appearance of cells containing potentially

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damaging mutations and so the strategy adopted to deal with damaged DNA can be split into three components (Rich *et al.* 2000):

a) DNA damage recognition

b) damage assessment enforced by cell cycle checkpoints that monitor the efficacy and the completion of events, culminating in the cessation of cycle progress if conditions are not perfect

c) implementation of the appropriate response (DNA repair or cell death)

1.1.2 APOPTOTIC CELL DEATH

The processes of cell death were being documented as early as the late 19th century and by 1914 enough data was available for a German anatomist, Ludwig Gräper, to publish a paper entitled (in translation) "A point of view regarding the elimination of cells." (Gräper, 1914), where he referred to what is now called apoptotic cell death as a process which,

" ...must exist in all organs in which cells must be eliminated".

The term "apoptosis" was first used by Kerr *et al.* (1972) to describe a highly regulated process of cell death. Since then scientists have distinguished apoptosis as a form of "intentional suicide" based on a genetic mechanism (Ellis *et al.* 1991; Hengartner *et al.* 1992; Hengartner, 2000). However, although extensively described, apoptosis has no absolute, precise definition. Instead, scientists have defined this form of cell death using morphological as well as biochemical criteria.

1.1.2.1 Morphology

Initially, the cell shrinks and the chromatin becomes condensed, being packed into smooth masses normally found against the nuclear membrane, thus giving a "horse-shoe" appearance (Majno and Joris, 1995). The nucleus can break up, a process known as karyorhexis, sometimes being incorporated into cellular processes that tend to bud off the cell, so the detachment procedure is known as budding, the resulting vesicles being termed apoptotic bodies (Fig. 1.2).

Fig. 1.2 Morphological changes associated with apoptotic cell death
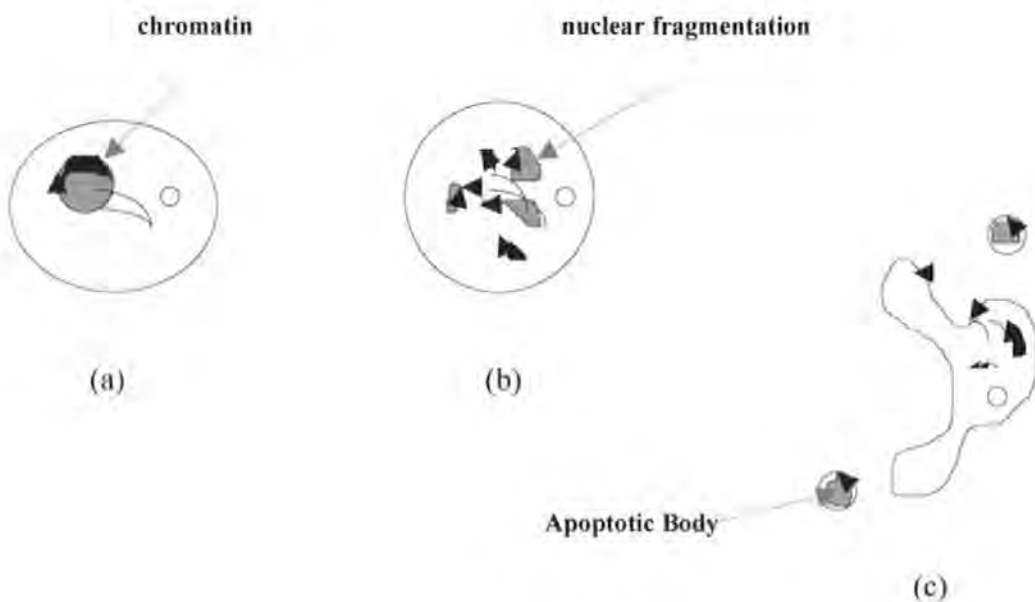
Fig. 1.2 (a) "horse-shoe" chromatin (b) karyorhexis (c) budding.

Although various organelles may become compacted and re-localised, there is little or no swelling of the mitochondria as an intact mitochondrial structure facilitates this genetically controlled, ATP-dependent form of cell death.

Changes, especially in early stage apoptosis, are easily distinguished from those associated with necrotic cell death. Membrane disruptants, respiratory poisons and hypoxia cause ATP depletion, metabolic collapse, cellular and organelle swelling and the random spillage of cellular contents into the extracellular surroundings leading to inflammation. These are typical features of necrosis. In contrast, early apoptotic cells show no obvious changes in plasma membrane integrity. In addition, cells undergoing apoptosis display a number of 'eat-me' flags such that this programme of cell deletion *in vivo* is swift, with the final phase being the safe phagocytosis of intact cells. A well characterised example of this would be the early exposure of the 'eat-me' flag, phosphatidylserine, normally restricted to the inner-membrane leaflet (Savill and Fadok, 2000). This phagocyte-dependent clearance allows a large cell turnover without the inflammatory response associated with necrotic material, *i.e.* the mechanisms allowing phagocytes to recognise apoptotic cells as 'unwanted self' are special in that they are uncoupled from inflammatory responses. Indeed, the uptake of apoptotic cells actively suppresses the secretion from activated macrophages of pro-inflammatory mediators such as tumour necrosis factor-α (TNF-α). This safe clearance is beneficial in preventing the secondary necrosis of apoptotic cells, with associated uncontrolled release of damaging contents (Majno and Joris, 1995; Savill and Fadok, 2000).

1.1.2.2 Biochemistry

Regulated destruction of a cell is a complicated process and requires the co-ordinated activation and execution of multiple subprograms. Most of the morphological changes during apoptotic cell death are caused by a set of cysteine proteases activated specifically in apoptotic cells. These proteases belong to a large protein family known as caspases which possess an active-site cysteine and cleave substrates which follow aspartic acid residues. As eliminating caspase activity can prevent apoptosis, caspases are regarded to be the central executioners of the apoptotic pathway.

1.1.2.2.1   Caspase action and activation

In most cases caspase activity results in the inactivation of the target protein but caspases can also activate proteins either directly by cleaving off a negative regulatory domain, or indirectly by inactivating a regulatory subunit (Fig. 1.3).

Fig. 1.3 Caspase mechanism of action
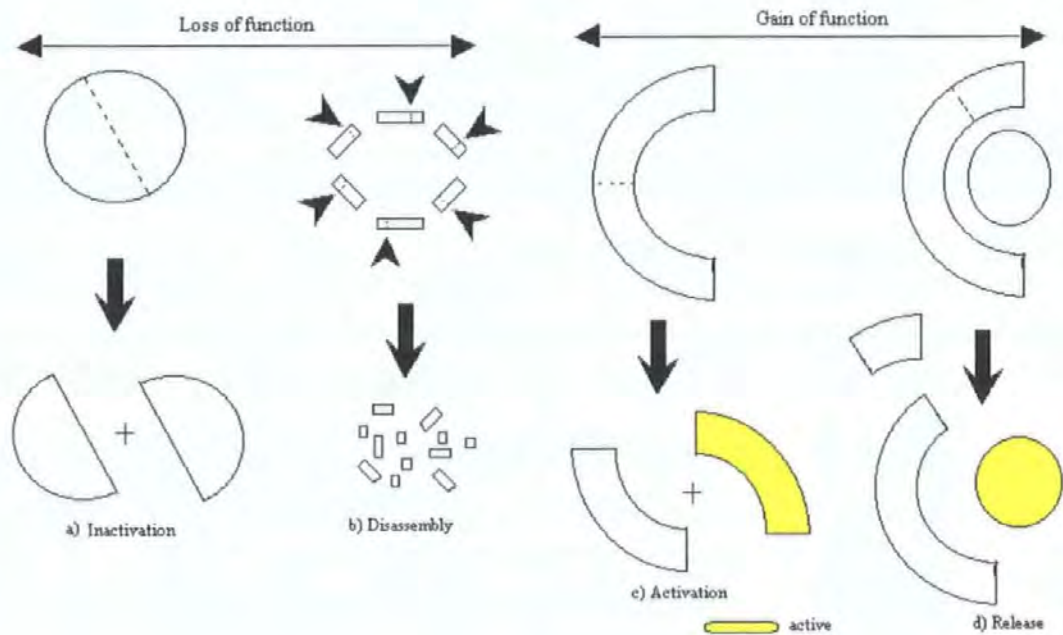
Fig. 1.3 Adapted from Hengartner, 2000.

A good example of this is the generation of the most characteristic biochemical indicator of apoptosis *i.e.* the DNA "ladder" resulting from internucleosomal fragmentation of DNA. This fragmentation is generally considered as evidence of endogenous endonuclease activation (Arends *et al.* 1990). The nuclease, known as caspase-activated DNase (CAD), cuts the genomic DNA between nucleosomes to generate DNA fragments with lengths corresponding to multiple integers of approximately 180 base pairs. CAD pre-exists in living cells as an inactive complex with an inhibitory subunit, dubbed ICAD. Activation of CAD occurs by means of caspase-3-mediated cleavage of the inhibitory subunit, resulting in the release and activation of the catalytic subunit (Enari *et al.* 1998; Hengartner, 2000).

Laddering is widely used in the identification of apoptosis, however, it does not provide definitive criteria for distinguishing between necrosis and apoptosis as "apoptotic-like"

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DNA fragmentation has been observed during hepatic necrosis following treatment of mice with paracetamol (Ray *et al.* 1993). In addition, not all cells show the characteristic laddering pattern during apoptosis (Wiger *et al.* 1997).

As is true of most proteases, caspases are synthesised as enzymatically inert zymogens and three general mechanisms of caspase activation have been described (Hengartner, 2000):-

a)   proteolytic cleavage by an upstream caspase - this is straightforward and effective, and is used mostly for activation of downstream, effector caspases *e.g.* a 'caspase cascade' strategy of activation is used extensively by cells for the activation of three short prodomain caspases, caspase-3, -6 and -7, which are considered the workhorses of the caspase family (Hengartner, 2000).

b)   induced proximity - caspase-8 is the key initiator caspase in the death receptor pathway of apoptosis. Upon ligand binding, "death" receptors such as CD95 (also known as Fas or Apo-1) aggregate and form membrane-bound signalling complexes which then recruit, through adaptor proteins such as FADD (Fas-associated death domain protein), several molecules of procaspase-8, resulting in a high local concentration of zymogen. The model suggests that under these crowded conditions the low intrinsic protease activity of procaspase-8 is sufficient to allow the various proenzyme molecules to mutually cleave and activate each other (Hengartner, 2000; Krammer, 2000; Muzio *et al.* 1998).

c)   Holoenzyme formation - the most complicated mechanism is the one used by caspase-9. Proteolytic processing only has a minor effect on the enzyme's catalytic activity. Instead the key requirement is its association with a dedicated protein cofactor, Apaf-1, forming a caspase-9 holoenzyme. The

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Apaf-1/caspase-9 complex is often referred to as the apoptosome and its large size suggests that it may well contain several additional proteins (Beere *et al.* 2000).

In summary, effector caspases are usually activated proteolytically by an upstream caspase, whereas initiator caspases are activated through regulated protein-protein interactions.

The mechanisms of apoptosis are just beginning to be unravelled and much of the molecular interactions remain unclear, however, protein-protein interactions are one of the underlying themes in apoptosis and can involve many regulatory molecules. One such family of apoptotic regulators is the Bcl-2 family. Named after the first regulator to be described, the Bcl-2 family is comprised of well over a dozen proteins which have been classified into three functional groups (Hengartner, 2000). The members of group I possess anti-apoptotic activity, whereas members of groups II and III promote cell death (Table 1.1).

Group	Bcl-2 family members	Activity
I	Bcl-2	Anti-apoptotic
	Bcl-$_{XL}$	Anti-apoptotic
II	Bax	Pro-apoptotic
	Bak	Pro-apoptotic
III	Bid	Pro-apoptotic

Table 1.1 Example of Bcl-2 family member subclassification

In a normally functioning cell then Bcl-2 family members are presumably in homeostatic balance, but the key function of those in groups II and III seems to be to regulate the release of pro-apoptotic factors, in particular cytochrome c, from the mitochondrial intermembrane compartment into the cytosol. During apoptosis, cytochrome c is released by the mitochondria into the cytoplasm where it associates with the apoptosome complex and facilitates the initiation of a caspase cascade (Hengartner, 2000; Johnson, 2000a).

Other regulatory aspects are emerging and it has been suggested that intracellular pH changes occurring early during apoptosis may play an important role in driving subsequent biochemical changes associated with the death process such as: phosphatidyl serine externalisation (Gottlieb *et al.* 1996; Johnson, 2000a); cytochrome c release; and alterations in mitochondrial membrane potential (Johnson, 2000a; Matsuyama *et al.* 1998).

In addition, there has recently been evidence to suggest that noncaspases, including cathespins, calpains, granzymes and the proteosome complex, also have roles in mediating and promoting apoptosis (Johnson, 2000b), adding to the complexity of this type of cell death.

The complexity of apoptosis means that an understanding of the processes involved is vital especially as this type of cell death is now known to be affected in neuro-degenerative diseases, T-cell depletion in HIV/AIDS, haematopoiesis and cancer (Cohen and Eisenberg, 1992; Groux *et al.* 1992; Hickman, 1992; Orrenius, 1995).

1.2   HAEMATOPOIESIS AND LEUKAEMIA

Progression of a cell through its cycle is dependent on the careful control between growth stimulus and apoptosis. Cancerous growth is primarily due to a disruption of this balance, where abnormal cells escape the normal control parameters monitoring cell status, *e.g.* checkpoints. This deregulation is commonly associated with gene mutations and abnormalities in the suicide program, resulting in the death of vital cells or the survival of dysfunctional cells and gives rise to pathological states such as functional deficiencies or cancer, respectively.

The formation of different types of blood cells, *i.e.* haematopoiesis, is essential for development and survival. New cells from different lineages are produced from stem cells throughout life, however, any abnormalities in this production may result in haematological diseases, *e.g.* haematological malignancies (Sachs, 1996).

The leukaemias, colloquially known as "cancers of the blood", are an example of such malignancies. They are a group of disorders characterised by the accumulation of abnormal white blood cells in the bone marrow leading to bone marrow failure, an increase in the circulating white blood cell count and infiltration of other organs (Hoffbrand and Pettit, 1992).

Historically the leukaemias have been broadly divided into two types: (1) acute, which without treatment is fatal within a few weeks or months, and (2) chronic, which without treatment is fatal in months or years. Further divisions into myeloid and lymphoid leukaemia depend on the haematopoietic cell in which the leukaemic transformation occurs and at which point in differentiation it occurs because this cell and the timing determine the

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phenotype of the leukaemic clone. In addition, extended classification within the myeloid and lymphoid leukaemias can occur, *e.g.* lymphoid leukaemias can be further categorised into T-cell and B-cell dependent on cell type/differentiation (Fig. 1.4).

Fig. 1.4 Lymphocyte differentiation
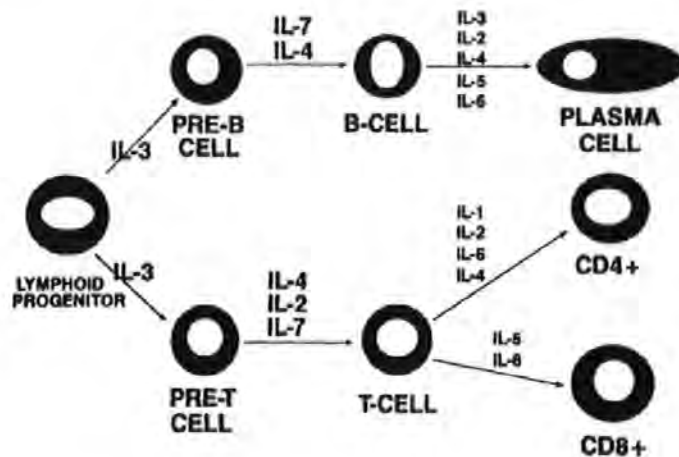
Stated briefly, the leukaemias represent arrested maturation of committed cell lines, where the malignant phenotype and the multiplicity of leukaemic syndromes depends on the stage at which maturation stops.

1.2.1 B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA (B-CLL)

The first ante-mortem diagnosis of patients with chronic leukaemia was made and published in the early 19th century and probably also included patients with chronic myelogenous leukaemia (Fuller, 1846). However, the development of histochemical staining techniques enabled Türk in 1903 to distinguish between myeloid and lymphoid leukaemias and first describe chronic lymphocytic leukaemia as a separate clinical entity (Hamblin, 2000a; Johnson *et al.* 1996).

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B-cell chronic lymphocytic leukaemia (B-CLL) is the most common form of leukaemia in western society, affecting men more than women at a ratio of 2:1. It is a disease of the elderly, the median age of patients being nowadays close to 70 years, however, 10-15% of patients are younger than 50 years (Montserrat, 2000). It comprises 90% of chronic lymphoid leukaemias in the U.S. and Europe (Horning, 1998), and is a neoplastic disease characterised by a clonal expansion of B cells with morphology typical of small, monomorphic lymphocytes in the blood, marrow and lymphatic tissues (Horning, 1998).

B-CLL differs slightly from other leukaemias in that it is the only major adult leukaemia that is not thought to be associated with exposure to ionising radiation, drugs or chemicals, nor is there evidence for viral aetiology. More convincingly, the epidemiology of B-CLL suggests that sex and genetic factors influence the disease susceptibility (Diehl et al. 1999; Steiner et al. 1998).

1.2.1.1 Flow cytometry and immunocytochemistry

Flow cytometry of peripheral blood should follow review of the peripheral smear and immunophenotypically, B-CLL can be characterised by the expression of CD5 (a pan-T-cell marker) as well as CD19, 20, 23 and 43. However, the levels of CD5 & CD20 expression are lower than normal (Almasri et al. 1992; Horning, 1998; Marti et al. 1992). Also expressed on B-CLL cells are CD11a, CD18, and CD54, but due to the low levels of these antigens, they can be reported negative in a certain percentage of cases. Other antigens are not expressed, e.g. CD4, or CD8 (T-cell differentiation antigens) (Beutler et al. 1995). Currently, B-CLL is usually diagnosed based on a combination of morphology and immunophenotype.

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1.2.1.2 Surface immunoglobulin

The leukaemic cells from over 90% of patients express low levels of monoclonal surface immunoglobulin (Ig) with either κ or λ light chains (Beutler *et al*. 1995). Of the heavy chain isotypes, over half of all cases have surface IgM and IgD, a quarter have IgM exclusive of IgD, and approximately 7% have Ig isotypes other than IgM or IgD (usually IgG or IgA).

The immunoglobulins expressed in B-cell CLL often have reactivity for self-antigens, most notably for the constant region of human IgG. These antigens are 'polyreactive' *e.g.* a monoclonal autoantibody produced by a leukaemic B-cell clone may bind to IgG, single-stranded DNA, double-stranded DNA, histones, cardiolipin, actin, thyroglobulin, and/or cytoskeletal components (Beutler *et al*. 1995). Such polyreactivity is a characteristic of some autoantibodies produced during early B-cell development and some CLL B-cells appear to have been selected for their ability to bind self-antigens. Conceivably, normal B cells that express such autoantibodies may be perpetually stimulated, thereby increasing their risk for malignant transformation into CLL. Alternatively, anti-self reactivity may enhance the survival of a B-cell clone subsequent to its malignant transformation. In either case the autoantibody activity of the leukaemic cells may be one factor in the pathogenesis of this disease.

1.2.1.3 Cytogenetic and molecular abnormalities

Today the disease is thought of in terms of molecules and clonal chromosome abnormalities are found in approximately half of all B-CLL, particularly among patients with advanced disease (Montserrat *et al*. 1997). However, molecular cytogenetic

techniques, such as fluorescence *in situ* hybridisation (FISH), have demonstrated abnormalities in more than 80% of patients with CLL (Dohner *et al.* 1998). FISH analysis in a large number of cases has shown strong prognostic associations with these abnormalities and molecular cytogenetics will play an important role in the routine assessment of these patients in the future.

The most common abnormality detected by conventional cytogenetics is trisomy 12 found in around one-third of cases with an abnormal karyotype (Hamblin, 2000b; Juliusson *et al.* 1991). Structural abnormalities of chromosome 12 also occur, most commonly at p11, q13 and q22 and may result in partial trisomy. Trisomy 12 is associated with atypical morphology involving increased numbers of prolymphocytes or lymphoplasmacytic features, atypical immunophenotype, a higher proliferative rate, advanced disease and poor prognosis. The oncogenes that have been identified on chromosome 12 have not yet been shown to be activated or involved in the aetiology of CLL (Juliusson *et al.* 1990).

Structural abnormalities of chromosome 13 are found in around 20% of patients with an abnormal karyotype but long arm deletions of chromosome 13 (usually q14) are the most common abnormality demonstrated using FISH (Dohner *et al.* 1998). 13q14 is the site of the retinoblastoma suppresser gene (RB1) which is not involved in the pathogenesis of CLL. However, a region telomeric to the RB1 gene has been shown to be disrupted in cells from patients with CLL (Brown *et al.* 1993) and may be the site of a recessive tumour suppresser gene known as DBM (deleted in B-cell malignancy). Patients with single del(13q) have a significantly better survival (> 15 years) than patients with del(13q) plus other abnormalities and patients with the other abnormalities studied (Dohner *et al.* 1998) (Table 1.2).

Genetic abnormality	Incidence (%)	Median survival (years)	Clinical features
del(13q) single	36	>15	typical morphology
del(11q23)	17	6.6	bulky disease
+12	15	10.9	atypical morphology
del(17p)	8	3.6	drug resistance
del(6q)	7	11	bulky disease

Table 1.2 Incidence and prognostic significance of genetic abnormalities in CLL

Modified from Dohner *et al.* 1998 and Oscier, 1999.

Inactivation of the p53 tumour suppresser gene as a result of mutation at 17p is found in 8 to 26% of patients with CLL (Dohner *et al.* 1998; Reed, 1998), usually in association with advanced stage. It is also associated with resistance to chemotherapy. Loss of p53 activity can result in both an increase in cell proliferation and also prolonged cell survival

Chromosomes 6 is the next most commonly involved chromosome with long arm deletions or translocations involving either arm. Deletions of the long arm (del(6q)) have been found in 7% of patients (Dohner *et al.* 1998) and these patients had a median survival of 11 years.

Conventional cytogenetics show structural abnormalities of chromosome 14 in 15% of patients with chromosome abnormalities, most commonly q32 (Dohner *et al.* 1998).

Translocations involving the *bcl*-2 gene on chromosome 18q21 with one of the immunoglobulin gene regions on chromosome 2, (κ light chain), 14 (heavy chain), or 22 (or λ light chain), occur in 1 to 4% of patients with CLL (Reed, 1998). These translocations are much more characteristic of follicular lymphomas in 85% of which the t(14;18)

translocation is found. Translocations of the *bcl*-2 gene in CLL more commonly involve light chain loci. The result of these translocations is deregulation of the *bcl*-2 gene and constitutive high expression of the gene product. The *bcl*-2 protein is a potent suppresser of apoptosis.

Although translocations involving the *bcl*-2 gene are uncommon in CLL, high levels of bcl-2 protein, equivalent to those found in follicular lymphoma, are found in approximately 85% of cases (Hanada *et al.* 1993). This appears to be due to hypomethylation of the *bcl*-2 gene promoter region rather than mutation of the gene.

1.2.1.4 Disease staging

The Rai System (five subgroups designated with Roman numerals, I-V [early - advanced]) and the Binet System (three subgroups designated alphabetically, A-C [early - advanced]) are the two clinical staging systems commonly used. Both systems are designed to predict worsening prognosis with advancing stage of disease. It has been suggested that the two systems be amalgamated (IWCLL, 1981, 1989).

A number of other prognostic indicators are used, including lymphocyte doubling time, bone marrow histology and chromosome alterations (Hamblin, 2000). Although there is no distinct correlation between incidence of chromosomal abnormalities and clinical stage, the incidence of abnormalities does increase (from 20-70%) in conjunction with progression from an early to an advanced and more aggressive stage of disease.

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1.2.1.5 Treatment

Although described as indolent in nature, this disease has a highly variable clinical course with death occurring from several months after diagnosis to more than 10 years of life with no major complications, *i.e.* death unrelated to CLL (Juliusson, 1994). In fact, patients with CLL seldom die because of high white cell count, but there are other fatal, clinical outcomes of this disease related to immunodeficiency and autoimmunity.

When making treatment decisions, therefore, the parameters taken into account include the characteristics of the patient (age, associated diseases), the accurate diagnosis of the disease and the prognostic features (systematic staging) with or without additional features, *e.g.* lymphocyte doubling time.

Delaying treatment is considered the best "therapeutic" strategy for patients with indolent presentation and this option is always acceptable for elderly patients who have a high probability of having other debilitating diseases over the following 10 years after diagnosis. It is becoming more apparent, however, that in some patients (usually younger in age) the disease is not indolent. Even though a response to treatment is normally associated with increased survival, patients are still rarely, if ever, cured. The most important aim, therefore, is prolongation of survival (Montserrat, 2000).

1.2.1.5.1   Chemotherapy

1.2.1.5.1.1   Single agent chemotherapy

The alkylating agents such as chlorambucil and cyclophosphamide are important in the treatment of haemopoietic malignancies and have in common the generation of reactive carbonyl groups that attack electron-rich sites on DNA.

Chlorambucil and cyclophosphamide were demonstrated to be active in B-CLL in the mid-1950s (Keating, 1999). Chlorambucil is still first line treatment and is found to decrease the lymphocyte count in approximately two thirds of all patients. In a smaller proportion it will also decrease spleen size and improve platelet and haemoglobin levels. It was initially given at a daily dose of 0.03 to 0.3mg/kg, but now, more frequently, is given 10mg daily for ten days every four weeks for six months. The latter regimen gives similar efficacy but is less toxic. The response rate is 40 to 60% but complete remissions are rare. Side effects include nausea, bone marrow suppression and occasionally skin rashes (Montserrat and Rozman, 1993; Rai, 1993).

Repeated administration of chlorambucil was found to be often ineffective especially in relapsed or refractory disease where the median survival is approximately 15 months (Keating *et al.* 1988). For this reason combination regimens are frequently used.

Cyclophosphamide is occasionally given if chlorambucil is not tolerated. If administered as a single agent the dose is 50-100mg/day orally or 500-750mg/m^2 every 3-4 weeks intravenously. There is, however, no good evidence for its single agent activity and it is more commonly found in combination regimens.

1.2.1.5.1.2   Combination chemotherapy

The most popular combination regimen has been that of chlorambucil and prednisolone, usually given monthly, *e.g.* chlorambucil 10mg daily, prednisone 40mg daily both orally for 10 days, but the responses have ranged from 38-87% (Keating, 1999) primarily due to the wide variation in response criteria and drug dose schedules across all the reported studies.

While the use of this combination of alkylating agent and steroid has proven popular, other drug combinations have included anthracycline drugs in addition to the alkylating agent and steroid, *e.g.* CAP - Cyclophosphamide, Doxorubicin & Prednisone (Beutler *et al.* 1995; Keating *et al.* 1990) and CHOP - Cyclophosphamide, Doxorubicin, Vincristine & Prednisone (Beutler *et al.* 1995; Binet, 1993; Hansen *et al.* 1991).

1.2.1.5.1.3   Emerging chemotherapies in chronic lymphocytic leukaemia: nucleoside analogues

Historically, the malignant process has been associated with an uncontrolled cell proliferation, and therefore, it seemed logical for chemotherapy to target the process of DNA replication. In this respect, most effective cancer drugs either interfere with the synthesis of DNA or produce chemical lesions in DNA that interfere with its replication, *e.g.* alkylating agents, steroids and anthracycline antibiotics.

Although regimens containing such drugs are still often used, the problem with past clinical research has been, in part, that most clinical trials in CLL primarily involved comparisons of various combinations of these agents. However, these agents are more

toxic to cells undergoing proliferation, due to the targeting of the DNA, and as CLL lymphocytes are not proliferating but rather accumulating (most lymphocytes are held in G_0 phase of cell cycle), these agents are not having the optimal desired effect (Cheson, 2000).

In the late 80's purine analogues were developed which had, as one mechanism of action, induction of apoptosis of resting lymphocytes. The purine analogue, fludarabine, has shown significant anti-CLL efficacy and provided a major advance in the treatment of CLL.

Fludarabine is a fluorinated purine analogue that is rapidly dephosphorylated in plasma and enters the cell by a carrier mediated transport mechanism. It is then phosphorylated, by deoxycytidine kinase, to F-ara-adenine triphosphate (F-ara-ATP) which is resistant to deamination by adenosine deaminase and therefore begins to accumulate in the cell. The activity against quiescent cells, which form the majority of malignant cells in CLL, is a result of disruption of nucleoside pools, which inhibits DNA repair and results in activation of poly (ADP-ribose) polymerase, depletion of NAD and ATP, and ultimately cell death by apoptosis (Robertson *et al.* 1993).

Other nucleoside analogues that have shown activity in the treatment of CLL include, pentostatin, GW506U78 and gemcitabine (Cheson, 2000).

Unfortunately, patients are not cured with fludarabine and there are no satisfactory standard options for patients who relapse after initially responding to fludarabine.

1.2.1.5.1.4   Comparative clinical trials

As there has been limited progress in clinical research in chronic lymphocytic leukaemia with failure to improve patient outcome even with the above regimens, many trials have been conducted to investigate the effectiveness of various drug regimens in treating this disease.

Trials by the French Co-operative Group on CLL demonstrated that treatment of patients with early-stage disease with chlorambucil alone or in combination with prednisolone did not result in a survival advantage as compared with a watch and wait approach (Montserrat, 2000).

The North America Intergroup three-arm trial treated patients with advanced, active disease with fludarabine, chlorambucil or fludarabine and chlorambucil. The fludarabine-alone arm resulted in a much higher response rate than the chlorambucil alone and a longer disease-free interval, but, up to the date of the last report no differences in survival have been observed (Keating, 1999; Rai *et al.* 2000).

A European study compared CAP (cyclophosphamide, doxorubicin, prednisolone) with fludarabine in both previously treated and untreated patients. In this study the responses were better with fludarabine as was the progression-free survival and overall survival but the difference did not achieve statistical significance (French Co-operative Group on CLL, 1996; Keating, 1999).

The French Co-operative Group has also compared fludarabine with mini-CHOP (ChOP, with doxorubicin at lower doses than in standard CHOP) and CAP. CAP was found to be

inferior to both the alternatives and, although the results were somewhat better with fludarabine, the difference between CHOP and fludarabine was not statistically different (Keating *et al.* 1998).

In conclusion, the above results demonstrate that in indolent disease the watch and wait approach is preferable until better treatments become available. On the other hand, for early stage patients it may be important to identify those who are likely to progress and, in them, to investigate whether there is a role for fludarabine in changing the natural history of the disease in these patients. In advanced-disease patients, fludarabine has proven to be effective treatment even although survival is not improved (Montserrat, 2000).

In cases where patients relapse after treatment with fludarabine, investigational strategies are appropriate. These include combination regimens with other chemotherapeutic agents (e.g. fludarabine and cyclophosphamide (Flinn, 1998)), emerging therapies involving monoclonal antibodies, vaccines or gene therapy, or combinations of these.

1.2.1.5.2 Emerging therapies

1.2.1.5.2.1 Immunotherapy with antibodies

While idiotype vaccine development has been developed in lymphoma, these have not yet become available for CLL (Keating, 1999).

Two monoclonal antibodies have become available for clinical trial and are being explored in CLL. Rituximab, which is directed against CD20, is a potent treatment for patients with follicular lymphoma, and a clinical trial in CLL using rituximab has shown that the overall

response rate is between 35-40%. There appears to be a dose-response relationship (O'Brien *et al.* 1998).

Treatment with Campath-1H antibody directed against CD52, which is present on almost all patients with CLL, has been explored for a number of years and has demonstrated activity in early stage and late stage patients with CLL (Keating, 1999). A recent trial in 93 patients with fludarabine-refractory CLL has demonstrated that 33% of patients can obtain a remission and occasional patients can obtain a complete remission (Keating *et al.* 1999).

1.2.1.5.2.2   Immunotherapy with genetic modification

Along with differentiation antigens and class I and II molecules of the major histocompatibility complex (MHC), the slowly dividing monoclonal B-cells in B-CLL also express surface immunoglobulin (Ig). The Ig expressed in this disease has features that distinguish it from the Ig expressed by normal, non-malignant cells (Kipps, 1999) and this, coupled to the expression of MHC class I and II molecules, means that the leukaemic B cells should be amenable to host immune recognition and rejection.

Despite expressing MHC class II molecules, CLL cells are ineffective antigen-presenting cells (APCs) and it is therefore necessary to change the genotype, and therefore the phenotype, of the leukaemia cell to stimulate a host anti-leukaemia immune response.

In this respect, the poor APC activity of CLL cells can be corrected (Kipps, 1999). A critical T-cell surface molecule, CD154, which is the ligand for CD40, allows activated T cells to stimulate CD40-bearing normal B cells, monocytes, and/or dendritic cells. Such activation can induce cells to express important accessory surface molecules which are

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required for co-stimulatory cognate interactions with T-cells. This can trigger a cascade of events that can ultimately result in a leukaemic B-cell which expresses a variety of non-expressed, or marginally expressed, stimulatory surface accessory molecules. These changes allow the leukaemic cell to stimulate T cells to respond productively to presented cells.

These findings could develop into a strategy for immune therapy of CLL. The CLL cells can be stimulated either by cells expressing the ligand for CD40 (CD154) or by agents that cross-link CD40 on the leukaemia cell surface. Alternatively, this can be achieved by transferring a gene into the leukaemia cells that encodes a stable and active form of CD154, which can trigger activation of leukaemia cells via its interaction with CD40 (Kato *et al.* 1998). The latter approach has the potential for not only activating the leukaemia cells that have taken up the CD154 gene but also bystander leukemia B cells that have not (Kipps, 1999).

A phase-I study of CLL cells transfected with adenovirus-CD154 (Ad-CD154) has shown encouraging results. In summary, the infusions were well tolerated, and dose-limiting toxicity was not observed. *In vivo* changes in bystander leukaemia cells and significant increases in the numbers of circulating T cells were observed. Also, patients experienced significant falls in leukaemic blood counts and lymph node size. These results have stimulated the design and implementation of phase II trials that examine the effects of administering multiple doses of Ad-CD154-infected autologous leukaemic cells (Kipps, 1999).

<u>1.2.1.5.2.3   Transplantation Therapy</u>

Due to its experimental nature, it is suggested that stem cell transplant (SCT) for CLL should not be performed outside clinical trials. While age alone does not justify the use of experimental treatment approaches, young patients, where B-CLL exhibits poor prognostic features, should be offered experimental therapies in the setting of large and well conducted trials (Montserrat, 2000).

Autologous transplantation usually has a less than 10% transplant-related mortality where the status of the disease at the time of transplantation is the most important factor for survival. However, the constant pattern of relapses (about 50% at 4 years post-transplantation) suggests that autotransplants do not cure CLL. Nevertheless, absence of minimal residual disease after transplantation correlates with a longer disease-free survival.

Allogeneic transplants result in a transplant-related mortality that can be as high as 50%. In contrast to autologous transplants, however, in most series there is a disease-free survival plateau of about 40%. As in the case of autologous transplants, absence of minimal residual disease after transplantation is associated with a longer disease-free interval (Montserrat, 2000).

1.3   ANTHRACYCLINE ANTIBIOTICS

The anthracycline glycosides were first studied in the late 1950's as pigmented antibiotics produced by different strains of *Streptomyces*. The potent antileukaemic activity of the anthracycline daunorubicin, isolated from *S. coeroleorubidus* and *S. peucetius* (Aubel-Sadron and Londos-Gagliardi, 1984; Marco *et al.* 1963), led to the search for other

anthracyclines with anti-tumour activity. In 1969, doxorubicin was isolated from a chemically mutated strain, *S. peucetius var. Caesius* (Arcamone *et al.* 1969). Both daunorubicin and doxorubicin have marked side effects and consequently, various analogues have been developed with the object of retaining antitumour activity whilst reducing toxicity.

1.3.1 STRUCTURE

Anthracyclines are made up of a tetracyclic aglycone chromophore, (imparting the drugs characteristic red, orange or yellow colour) linked to a mono-, di- or tri-saccharide carbohydrate chain (Fig. 1.5).

Fig. 1.5 General structure of the anthracyclines
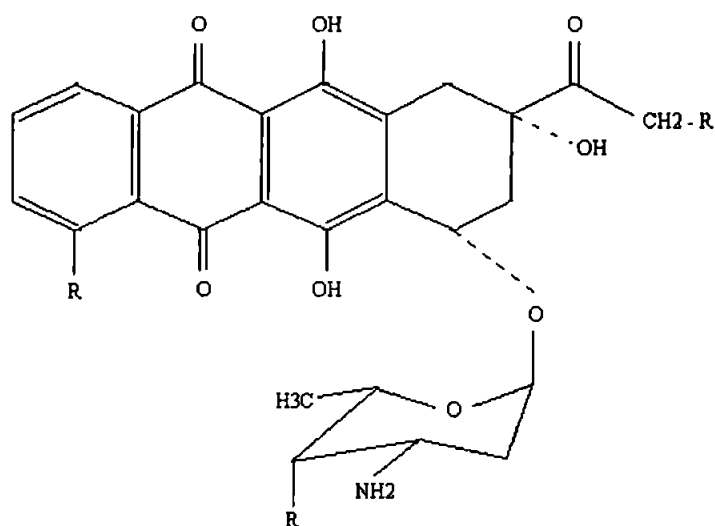
Fig. 1.5 An amino sugar moiety is linked through a glycosidic bond to the C7 of a tetracyclic aglycone.

The structures of some anthracyclines of clinical and experimental importance are shown in Fig. 1.6.

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Fig. 1.6 Chemical structure of various anthracyclines
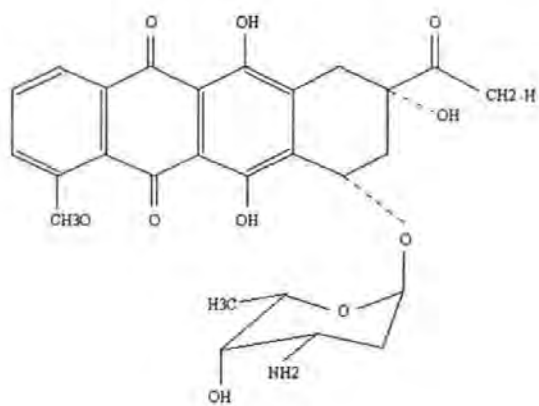
(a)
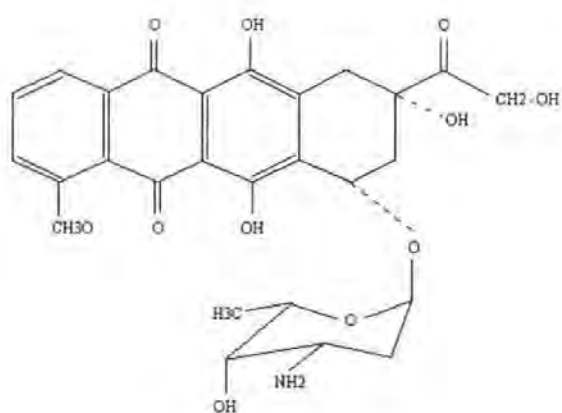
(b)
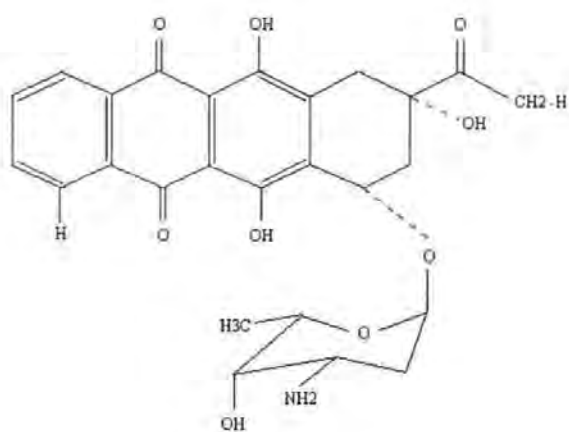
(c)

Fig. 1.6 (a) daunorubicin (b) doxorubicin (c) idarubicin

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1.3.2 MECHANISM OF ACTION

The mode of action of the anthracyclines is still not completely understood but at least ten potentially cytostatic and cytotoxic effects have now been identified. However, it must be considered that while studies involving intact cells utilising extracellular drug concentrations above 1-2μM (the general plasma concentration range after bolus administration versus the nM concentrations found after continuous infusion) may provide information on <u>potential</u> mechanisms of action, studies using such high concentrations are unlikely to reflect the mechanism of action associated with the clinical use of these antineoplastic drugs.

<u>(i) Inhibition of DNA synthesis and interference with macromolecular biosynthesis</u>

The inhibition of DNA biosynthesis may be related to DNA intercalation or inhibition of DNA polymerase activity. Inhibition of DNA synthesis in breast tumour cells and rat hepatoma cells has been observed over the concentration range of 0.1 to 5μM (Fornari *et al.* 1996; Munger *et al.* 1988). However, other studies have shown interference with DNA synthesis over a much lower concentration range, 10^{-8} to 10^{-6}M (Glazer *et al.* 1982).

The contradictory findings have made it difficult to reach a conclusion about inhibition of DNA synthesis in the growth-inhibitory effects of the anthracyclines. Nevertheless, it has been proposed that inhibition of DNA synthesis is an early signalling event that is a cytostatic (and transient) component of drug action related to the growth arrest associated with p53 function rather than the lethality of the anthracyclines themselves (Gewirtz, 1999).

(ii) Role of Free Radicals

The evidence for the involvement of free radical generation in the cytotoxicity of anthracyclines is complex. Under the appropriate conditions, the chemistry of anthracycline drugs lends itself to free radical production.

The quinone structure permits daunorubicin to act as an electron acceptor in reactions mediated by oxoreductive enzymes and the addition of the free electron converts the quinones to semiquinone free radicals. These may induce free-radical injury to DNA themselves or, after interaction with molecular oxygen, form superoxides, hydroxyl radicals and peroxides (Benchekroun *et al.* 1993; Feinstein *et al.*1993). The generation of semiquinone free radicals of doxorubicin has been shown to result in the cleavage or degradation of deoxyribose and/or DNA (Feinstein *et al.* 1993). However, cell-free systems with supraclinical drug concentrations were used for this analysis. It is unclear whether the free radicals are generated at clinically relevant concentrations of the anthracyclines and at normal (*i.e.* hypoxic) oxygen tension in the tumour cell and whether such free radicals could be responsible for anthracycline toxicity to the tumour. Some intact cell studies have been performed but even these used elevated drug concentrations (Benchekroun *et al.* 1993; Ubezio & Civoli, 1994).

One consequence of intracellular generation of reactive free radical species could be DNA damage and some studies have been able to detect this type of damage and block DNA damage using free radical scavengers (Potmesil *et al.* 1984). A few studies have used clinically relevant concentrations of doxorubicin and shown protection from damage using free radical scavengers such as catalase and glutathione peroxidase (Cervantes *et al.* 1988; Doroshow, 1986). However, in contrast to these studies, others have failed to demonstrate protection against doxorubicin toxicity using these free radical scavengers (Gewirtz, 1999).

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In addition, as the free radical scavengers used are large proteins, they should be incapable of crossing the cell membrane. Therefore, protection by these agents may be indicative of free radical generation at the cell surface. Indeed, free radical species could lead to lipid peroxidation, but there is thought to be insufficient evidence to implicate lipid peroxidation (via free radical formation) in the antitumour effects of anthracyclines.

(iii) DNA adduct formation and DNA cross-linking

Various studies have reported the induction of DNA adducts with anthracyclines, but generally they have involved high drug concentrations (Cullinane *et al.* 1994; Cullinane and Phillips, 1990; Gewirtz, 1999; Phillips *et al.* 1989).

The possibility that DNA binding could lead to DNA cross-linking has also been investigated and evidence exists to support the formation of DNA interstrand cross-links but, again, at elevated drug concentrations (Cullinane *et al.* 1994; Skladanowski and Konopa, 1994).

While these findings are consistent with this potential mode of action, the significance of these findings should be verified by studies demonstrating a relationship between DNA cross-linking and cytotoxic effects in intact cells at clinically relevant concentrations of anthracyclines.

(iv) Interference with DNA strand separation and DNA helicase

It has been demonstrated that low concentrations of doxorubicin interfere with DNA unwinding in MCF-7 breast tumour cells (Fornari *et al.* 1994a). Although this finding could be related to DNA interstrand cross-linking, it may be related to drug effects at the level of helicases (Bachur *et al.* 1993; Bachur *et al.* 1998; Tuteja *et al.* 1997).

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(v) Membrane-mediated effects

A number of reports of doxorubicin interaction with real and artificial membranes has led to the hypothesis for a doxorubicin effect at the membrane surface (Rogers *et al.* 1983; Triton and Yee, 1982; Vichi and Triton, 1992).

Vichi and Triton (1992) considered the importance of intracellular and extracellular drug in the cytotoxicity of doxorubicin to L1210 leukaemic cells. The authors' argument proposed that drug must be present at the cell exterior to cause cytotoxicity and they reported that high levels of DNA in the medium prevented drug toxicity, presumably through binding of cell-surface associated drug to the exogenous DNA.

Although this suggests an alternative mechanism of drug action, no insights were provided into the mechanism of action under conventional clinical conditions. In addition, as resistance to the anthracyclines is frequently mediated by the multidrug resistance pump (section 1.5.3) this supports the concept that drug must enter the cell to express its toxicity.

(vi) Induction of DNA damage through interference with Topoisomerase II

Topoisomerase II (topo II) is likely to be one of the primary target sites for the activity of anthracycline antibiotics. The strongest argument in support of this are the data indicating that anthracycline-resistant tumour cells have reduced levels or altered activity of topoisomerase, with a concomitant reduction in the level of drug-associated strand breaks in DNA (Deffie *et al.* 1989; Friche *et al.* 1991; Webb *et al.* 1991).

In conflict with this, however, are reports that have failed to demonstrate a consistent relationship between strand breaks and toxicity of the anthracyclines (Fornari *et al.* 1996; Munger *et al.* 1988; Spadari *et al.* 1986).

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These conflicting data do not clarify that the mechanism of action of anthracyclines is via strand breaks through the inhibition of topo II. However, it has been suggested that gene-specific damage may play a more important role in anthracycline action than bulk damage to DNA (Binaschi et al. 1997; Capranico et al. 1990).

(vii) Growth arrest

Growth arrest may be an alternative to apoptotic cell death with an increase in cell population accumulating in G_2 phase of the cell cycle (Fornari et al. 1996). A study by Ling et al. (1996) demonstrated that G_2 arrest by doxorubicin is related to the disruption of $p34^{cdc2}$/cyclinB activity.

(viii) Metal ion chelation

Anthracyclines have the ability to chelate, or bind, various metals including copper, zinc, and iron. Some of the resulting chelates may be cytotoxic (Hershko et al. 1993; Link et al. 1996; Malatesta et al. 1985).

(ix) Induction of Apoptosis

There is clear evidence that one consequence of treatment with doxorubicin and daunorubicin is the induction of apoptosis (Bose et al. 1995; Gewirtz, 1999; Jaffrezou et al. 1996; Skladanowski and Konopa, 1993).

Skladanowski and Konopa reported on the induction of DNA fragmentation and cell shrinkage associated with apoptosis at concentrations ranging between 0.7 and 10μM in Hela cells, while Jaffrezou et al. demonstrated that treatment of either HL-60 or U-937 human leukaemic cells with daunorubicin triggered apoptosis at concentrations of 0.5 and 1μM.

(x) Alternative Mechanisms of drug action

One alternative mechanism may be seen during the exposure of cells to nanomolar concentrations of the drug, sustained by continuous infusion. At these low concentrations doxorubicin can induce differentiation of leukaemic cells and in breast tumour cells (Dinnen *et al.* 1993; Fornari *et al.* 1994b), an observation that may have implications for combination drug therapies.

There is also evidence that doxorubicin can interfere with microtubular polymerisation and with the cellular cytoskeleton (Colombo *et al.* 1988; Molinari *et al.* 1990).

1.4 CYTOKINES

The beginnings of cytokine research can be traced back to the demonstration that migration of normal macrophages is inhibited by material released from sensitised lymphocytes upon exposure to antigen (Bloom & Bennett, 1966; David, 1966). The factor responsible for this action was termed macrophage migration inhibitory factor (MIF). Demonstration of MIF activity was followed by the discovery of 'lymphotoxin' activity (Ruddle & Waksman, 1968) which in turn lead to the term 'lymphokine', coined by Dumonde *et al.* in 1969, to designate 'cell-free soluble factors (responsible for cell-mediated immunologic reactions), which are generated during interaction of sensitised lymphocytes with specific antigen'.

The term 'lymphokine' has often been used less discriminately for secreted proteins from a variety of cell sources, affecting the growth or functions of many types of cells. To emphasise that such proteins could be produced by cells other than lymphocytes, Cohen *et al.* (1974) proposed the term 'cytokines'. 'Cytokine' has now become the generally accepted name for this group of proteins along with the interferons (IFNs), haematopoietic

growth factors or colony stimulating factors (CSFs) and some growth factors acting on non-haematopoietic cells *e.g.* transforming growth factor-β (TGF-β).

To designate individual cytokines, a group of participants at the Second International Lymphokine Workshop held in 1979 proposed the term 'interleukin' (IL) in order to develop "a system of nomenclature ...... based on the proteins' ability to act as communication signals between different populations of leukocytes" (Aarden *et al.* 1979). The first two designations were IL-1 and IL-2 and since then the interleukin series has increased greatly (Table. 1.3).

Although the name 'interleukin' implies that these agents function as communication signals among leukocytes, Aarden *et al.* (1979) suggested that the term should not be reserved for factors that can act only on leukocytes. Indeed, a number of proteins that have been labelled as interleukins not only are produced by a variety of non-haematopoietic cells but also affect the functions of many diverse somatic cells *e.g.* IL-1 or IL-6.

Whereas many cytokines are now termed interleukins, others remain known by their older names *e.g.* IFN-α/β, IFN-γ.

CYTOKINE	SOURCE	EFFECTOR FUNCTION
		Interleukins
Il-1	Macrophage, fibroblasts	Proliferation activated B- & T-cells
		Induction PGE₂ & cytokines by macrophages
		Induction neutrophil & T-adhesion molecules on endothelial cells
		Induction IL-6, IFN-ß1 & GM-CSF
		Induction fever, acute phase proteins, bone resorption by osteoclasts
IL-2	T cell	Growth activated T- and B-cells: activation NK cells
IL-3	T cell, Mast Cell	Growth & differentiation haematopoietic precursors
		Mast cell growth
IL-4	CD4 T cell, Mast cell,	Proliferation activated B-, T-, mast & haematopoietic precursor
		Induction MHC Class II
		Isotype switch to IgG1 & IgE
IL-5	CD4 T cell, Mast cell	Proliferation activated B-cells: production IgM & IgA
		Proliferation eosinophils
IL-6	CD4 T cell, Macrophage,	Growth and differentiation B- and T-cell effectors
		Induction acute phase proteins
IL-7	Bone marrow stromal	Proliferation pre-B, CD4- CD8- T-cells & activated mature T-cells
IL-8	Monocytes	Chemotaxis and activation neutrophils
		Chemotaxis T-cells
IL-9	T cell	Growth and proliferation T-cells
IL-10	CD4 T cell, B cell,	Inhibits IFN-γ secretion
		Inhibits mononuclear cell inflammation
IL-11	Bone marrow stromal	Induction acute phase proteins
IL-12	T cell	Activates NK-cells
IL-13	T cell	Inhibits mononuclear phagocyte inflammation
IL-15	Bone marrow stromal	Induce NK cells, T cells & neutrophils, anti-apoptotic, tumourigenic
IL-16	T cells	CD4⁺ T-cell chemotaxis IL-2 receptor expression
IL-17	T cells	Induce secretion of IL-6, IL-8, G-CSF & PGE₂
IL-18	macrophages, adrenal ctx	IFN-γ induction, Fas expression
		Colony stimulating factors
GM-CSF	T cells, macrophages,	Growth granulocyte & macrophage colonies
	Mast cell, endothelium	Activates macrophage, neutrophils, eosinophils
G-CSF	Fibroblasts, endothelium	Growth mature granulocytes
M-CSF	Fibroblasts, endothelium,	Growth macrophage colonies
Steel factor	Bone marrow stromal	Stem cell division
		Tumour necrosis factors
		Tumour cytotoxicity: cachexia
		Induction acute phase proteins
TNF-α	macrophages, T cell	Anti-viral & anti-parasitic activity
TNF-ß	T cell	Activation phagocytic cells
		Induction IFN-γ, TNF-α, IL-1, GM-CSF & IL-6
		Endotoxic shock
		Interferons
IFN-α	Leukocytes	Anti-viral: expression MHC I
IFN-ß	Fibroblasts	" " " " "
IFN-γ	T cell	Anti-viral: macrophage activation
		Expression MHC class I & II on macrophage & other cells
		Differentiation of cytotoxic T cells
		Synthesis IgG2a by activated B cell
		Antagonism several IL-4 actions
		Others
TGF-ß	T cell, B cell	Inhibition IL-2R upregulation and IL-2 dependent T- and B-cell prolif.
		Inhibition (by TGF-ß1) of IL-3 + CSF induced haematopoiesis
		Isotype switch to IgA
		Wound repair and angiogenesis
		Neoplastic transformation certain normal cells
LIF	T cell	Proliferation embryonic stem cells without affecting differentiation
		Chemoattraction and activation of eosinophils

Table 1.3 Some cytokines, their origin and function Adapted from, Essential Immunology. Ivan

Roitt. Eighth Edition. Blackwell Science (1996).

1.4.1   MECHANISM OF ACTION

Cytokines are regulatory polypeptides or glycoproteins, secreted by white blood cells and a variety of other cells in the body, that produce their actions by binding to specific high-affinity cell surface receptors (Thomson, 1998).

The majority of cytokine actions can be attributed to an altered pattern of gene expression in the target cells where, phenotypically, cytokine actions lead to an increase (or decrease) in the rate of cell proliferation, a change in cell differentiation state or a change in the expression of some differentiated functions. The pleiotropic actions of cytokines include numerous effects on cells of the immune system, modulation of inflammatory responses as well as effects on haematopoietic cells. In fact, cytokines share, with many other tissue specific growth factors, the capacity to prevent apoptosis. As apoptosis is a vital cell disposal mechanism, inhibition of apoptosis by cytokines may predispose to oncogenesis (Neshat et al. 2000; Panayiotidis et al. 1994; Schurmann et al. 2000).

Proliferation of haematopoietic cells in marrow, as well as other cytokine actions, are governed by a co-ordinated hierarchy of growth factors. These growth factors and allied cytokines form complex intracellular signal transduction pathways that can act to positively or negatively regulate cell proliferation and differentiation. Consequently, the discovery of the Janus kinase (Jak)/Signal transducers and activators of transcription (STAT) pathway in the early 1990s was important in deciphering cytokine mediated signalling (Touw et al. 2000).

The Jak/STAT pathway is one of the most important signalling pathways downstream of cytokine receptors. Following binding of a ligand to its cognate receptor, receptor-

associated Jaks are activated. STAT proteins are then in turn activated by tyrosine phosphorylation by Jak kinases, allowing their dimerisation and subsequent translocation into the nucleus, where they modulate expression of target genes (Imada and Leonard, 2000).

1.4.2 INTERFERONS

In 1957 Isaacs and Lindenman described a factor that conferred the property of viral interference, leading to the term interferon (IFN).

Three main subspecies of human IFN have now been recognised as alpha (α), beta (β), gamma (γ), produced by leukocytes, fibroblasts and T lymphocytes, respectively (Pestka, 1997). The IFNs have also been divided into two major subgroups by virtue of their ability to bind to common receptor types (Aguet *et al*. 1984; Haque and Williams, 1998; Merlin *et al*. 1985). Type I IFNs all bind to type I IFN receptor and include IFN-α, IFN-β, IFN-ω and IFN-τ. IFN-γ is the sole type II IFN, and binds to a distinct type II receptor (Table 1.4).

Currently at least 23 different IFN-α genes have been identified, coding for 15 functional proteins. There are few chemical differences between the IFN-α subtypes and their similar chemical activity make it unclear as to why there are so many species of IFN-α.

IFN Type	IFN categories	Receptor type	Prototypic cell of origin	Direct Anti-proliferative effects	Stimulates MHC class I expression	Stimulates MHC class II expression	Stimulates NK cell activation
Type I	Alpha (α)	I	Leukocyte	Yes	Yes	No	Yes
	Beta (β)	I	Fibroblast	Yes	Yes	Slightly	Yes
	Omega (ω)	I	Leukocyte	Yes	Yes	No	Yes
	Tao (τ)	I	Ovine Trophoblast				
Type II	Gamma (γ)	II	T-cells NK cells	Yes	Yes	Yes	Less than type I IFNs, delayed

Table 1.4 Interferon classification and properties From Jonasch and Haluska. (2001)

1.4.2.1 Mechanism of action of the interferons

The IFNs possess a broad spectrum of activity and are involved in complex interactions. They display antiviral activity, affect cellular metabolism and differentiation, and possess antitumour activity. The antitumour effects appear to be due to a combination of direct antiproliferative, as well as indirect immune-mediated, effects. Figure 1.7 summarises the intracellular signalling, while figures 1.8 and 1.9 summarise the major effects of IFN-α and -γ on NK cells, antigen-presenting cells, macrophages, T cells and tumour cells.

Fig. 1.7 Interferon intracellular signalling
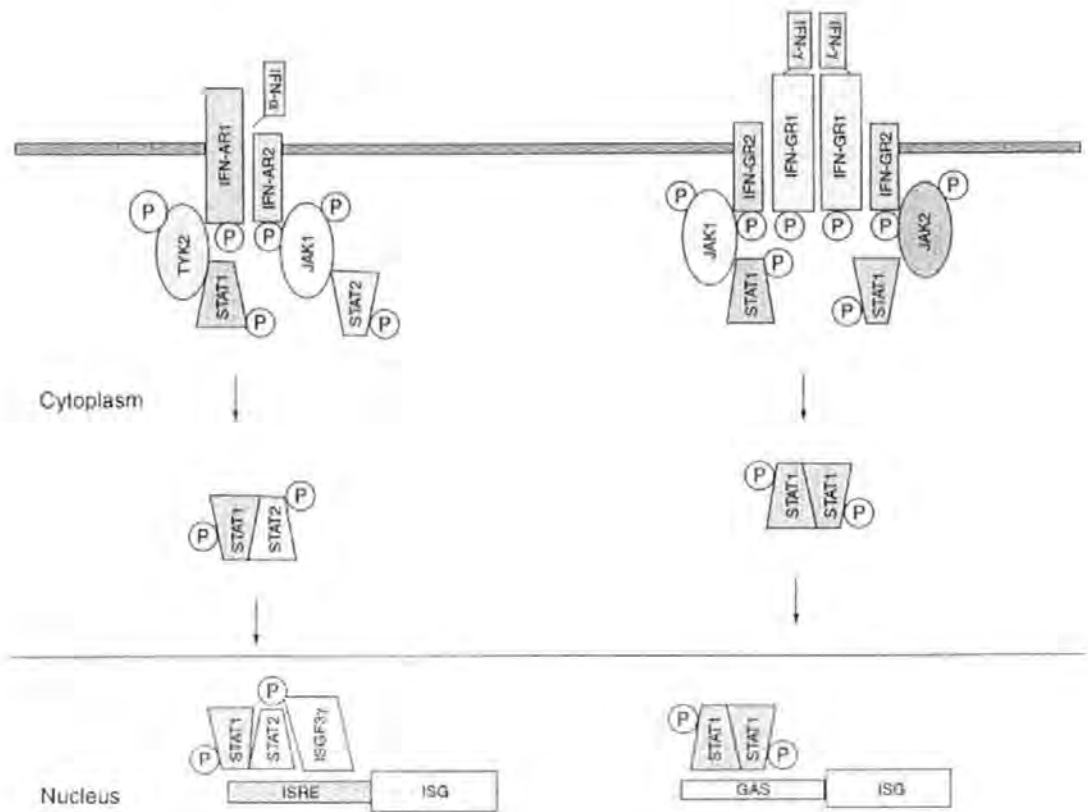
Fig. 1.7 Upon binding of IFN to its receptor, the receptor undergoes oligomerisation, with transphosphorylation of Jaks followed by phosphorylation of the cytoplasmic tails of the receptor molecules. This provides a docking site for the STATS which are then phosphorylated by the Jaks. The phosphorylated STAT dimers are released from the receptor molecules, and translocate to the nucleus, where they activate transcription of IFN-stimulated genes (ISGs). For type I IFNs, ISGs can be identified by the presence of an IFN-stimulated response element (ISRE) in their promoter regions. Enhancers of IFN-γ-inducible genes contain a unique element called the IFN-γ activation site (GAS). From Jonasch and Haluska. (2001).

Fig. 1.8 IFN-α effects on NK cells, antigen-presenting cells, macrophages, T cells and tumour cells
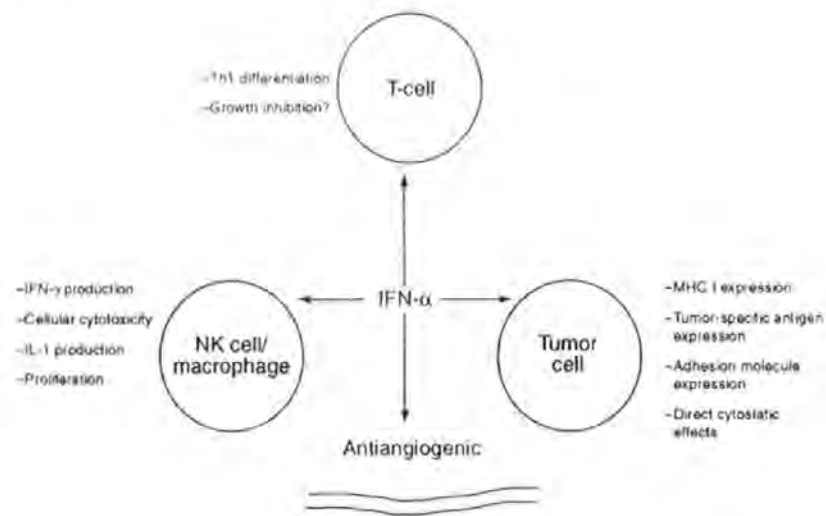
Fig. 1.8 From Jonasch and Haluska. (2001)

Fig. 1.9 IFN-γ effects on NK cells, antigen-presenting cells, macrophages, T cells and tumour cells
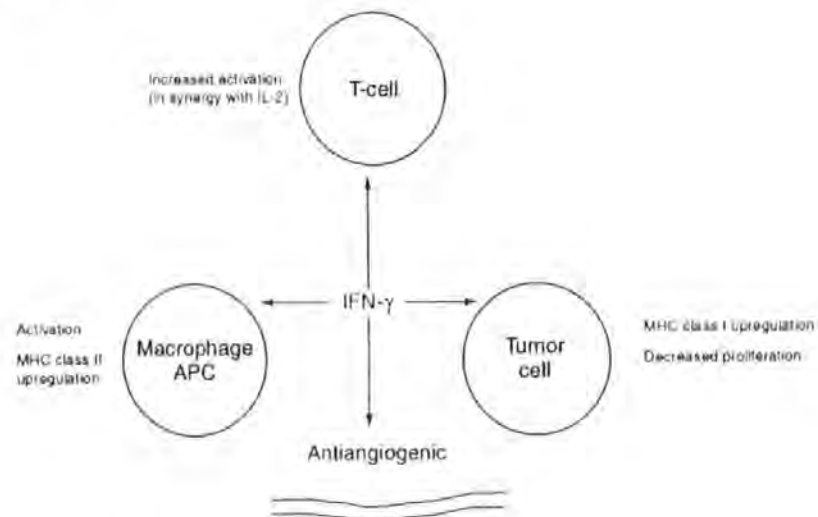
Fig. 1.9 From Jonasch and Haluska. (2001)

1.4.2.2 Clinical application

Their antitumour effect means that the IFNs (predominantly IFN-α) have been tested clinically in the treatment of lymphoproliferative disorders such as Follicular lymphoma, Hairy cell leukaemia, Myeloma and B-cell chronic lymphocytic leukaemia (B-CLL) (Jonasch and Haluska, 2001; McSweeney *et al.* 1993; Montserrat *et al.* 1991).

The use of IFN-α as maintenance of remission following chemotherapy has been an attractive concept for B-CLL. Various post-remission strategies have been tried to improve the complete remission rate and survival of CLL patients. However, even though this agent has activity in patients with early stage disease and can be given on a continuous basis, the use of interferon in CLL showed that only one patient out of nine who was in partial remission at the time of starting treatment was able to achieve a complete remission on interferon (Keating, 1999).

1.4.2.3 Control of apoptosis by interferons

In addition to the IFN effects mentioned above, reports of the anti-apoptotic roles of IFNs have been published. In 1994 Panayiotis *et al.* published work reporting that IFN-α protects B-CLL cells from apoptotic cell death *in vitro*. B-CLL cells are prone to spontaneous apoptosis when cultured *in vitro*, and this study analysed the effects of IFN-α on DNA fragmentation, bcl-2 protein levels and cell survival in purified B-cells from 16 CLL patients. IFN-α was shown to reduce the degree of spontaneous fragmentation and this inhibition was accompanied by an increased survival in comparison to control cells. These *in vitro* results, coupled with other studies showing similar anti-apoptotic effects of IFN-α (Jewell *et al.* 1994), and IFN-γ (Buschle *et al.* 1993), suggest that the clinical responses of

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some CLL patients to IFN-α cannot be explained by a direct cytotoxic effect of IFN-α on circulating CLL cells.

In addition, IFNs may have another role to play, which could result in their use in chemotherapy schedules for patients with drug resistant tumours. It has been reported that IFN-α in particular may modulate drug resistance (Scala *et al*. 1991), thus restoring chemosensitivity to drug resistant cells.

1.5 DRUG RESISTANCE

The failure of chemotherapy to cure more than a minority of tumours is predominantly due to drug resistance. This resistance may be inherent or acquired, either as a stable change within the cell or induced following drug administration and can be attributed to various mechanisms (Fig. 1.10).

1.5.1 GLUTATHIONE TRANSFERASES (GSTs)

The glutathione-S-transferases (GSTs) are a supergene family of dimeric enzymes involved in drug detoxification (McKenna, 1997; Strange *et al*. 2000) and are found to be distributed in all organs but primarily in the liver and kidney. Two supergene families encode proteins with glutathione-S-transferase activity. The family of soluble enzymes comprises at least 16 genes while the separate family of microsomal enzymes comprises at least 6 genes (Hayes and Strange, 2000).

These two families are believed to exert a critical role in cellular protection against oxidative stress and toxic foreign chemicals. Their mode of action is by conjugation of

electrophilic drugs, toxins and carcinogens to reduced glutathione (GSH), via the sulphur atom of its cystine residue, before elimination from the cell. Their importance is suggested by the finding that GST enzymes are expressed in probably all life forms.

In man, GSTs exist in microsomal and cytosolic forms, and there are three different classes of the cytosolic form – pi(π), alpha(α), mu(μ) (Waxman, 1990).

Fig. 1.10 Schematic diagram of various drug resistance mechanisms identified in mammalian cells.
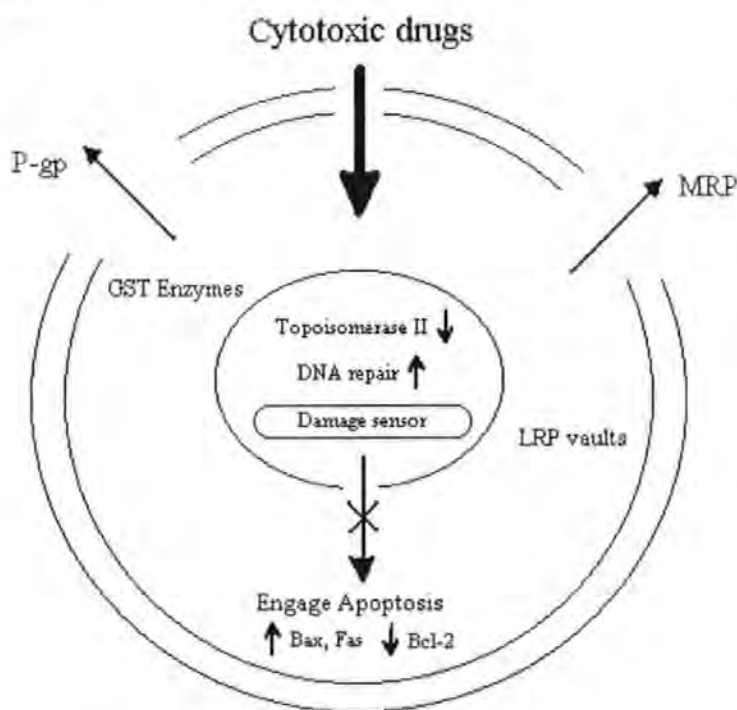
Fig. 1.10 P-gp and MRP proteins can actively efflux cytotoxic drugs from the cell. GST enzymes can detoxify drugs in the cytoplasm, and LRP vaults may be involved in the redistribution of drugs away from the nucleus. Alterations in drug target e.g. topoisomerase II, increased DNA repair, and decreased susceptibility to apoptosis can also confer a drug-resistant phenotype. Adapted from McKenna, 1997.

GSTπ constitutes the predominant isozyme found in human cancers with a 2- to 4-fold increase in its RNA levels in tumours of the colon, bladder, ovary and stomach, relative to

normal tissues. In addition, it has been found to be over-expressed in several multidrug resistant cell lines, particularly in doxorubicin resistant lines (Harris and Hochhauser, 1992). In contrast, however, other studies have shown little difference between GSTs in tumour cells and normal cells. In a study of patients with CLL, no correlation was found between chlorambucil resistance and GST expression in patient cells, which also did not vary significantly between control cells and CLL lymphocytes (Schisselbauer *et al.* 1990).

It still remains to be shown that the GST activity changes observed, amount to more than associated stress responses, rather than definitive resistance mechanisms. However, an increased protection against doxorubicin, although small, has consistently been found to be correlated with an elevation of GSTπ levels (Nakagawa *et al.* 1990).

1.5.2 TOPOISOMERASE II

Topoisomerase II (topo II) is the eukaryotic homologue of bacterial DNA gyrase, and is a 170kD homodimeric protein which plays a role in DNA replication, chromosome scaffold formation, chromosomal segregation, and possibly recombination and gene transcription (Fortune and Osheroff, 2000; Liu, 1989; McKenna, 1997; Wang, 1985).

The enzyme acts by producing DNA single and double strand breaks and then attaches covalently to the 5' ends of the break. Subsequent strand passage occurs allowing both supercoiling and DNA relaxation, such that, by its DNA passage reaction, topo II is able to regulate DNA over- and under-winding.

The importance of this enzyme in cell growth and survival is demonstrated by the many anti-topo II drugs which exist. Some of these topo II 'poisons' transform the enzyme into a

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potent cellular toxin (Fortune and Osheroff, 2000) by increasing the concentration of covalent enzyme-cleaved DNA complexes that are normally fleeting intermediates in the catalytic cycle of topo II. As a result of their action these drugs generate high levels of enzyme-mediated breaks in the genetic material of treated cells and ultimately trigger apoptosis. Alternatively, a second group, the catalytic inhibitors, prevent topo II from carrying out its physiological functions (Fortune and Osheroff, 2000). Drugs of both categories vary widely in their mechanisms of action, yet, characteristically, there is cross resistance to the full range of anti-topo II drugs.

Different forms of topos exist with various clinical implications. A good example of this is the ability to reduce topo II resistance by molecular design, *e.g.* one mechanism of resistance to the topo II inhibitor amsacrine (mAMSA) was due to altered transport. By altering substituents on the anilino acridine nucleus of mAMSA this resistance has been overcome (Findlay *et al.* 1990).

Although the resistance demonstrated by topos to anti-topo agents is important in itself, with respect to cross-resistance, there may be a link between MDR and topo II regulation, with a possible inverse correlation of these. A study of MDR induction has shown simultaneous topo II reduction although this may simply be due to a general inhibitory effect of the drugs on cellular metabolism rather than a specific action on topo II regulation (Chin *et al.* 1990a).

1.5.3   MULTIDRUG RESISTANCE

Among the different mechanisms of resistance, the appearance of the multidrug resistance (MDR) phenotype is the most frequently observed in many cancer cell lines exposed to a variety of cytotoxic agents.

MDR, or pleiotropic drug resistance, means that exposure to one drug induces cross-resistance to a variety of other agents to which the cell has not been exposed. These agents are usually structurally unrelated cytotoxic compounds of natural origin such as anthracyclines (doxorubicin, daunorubicin, idarubicin), vinca alkaloids, epipodophyllotoxins, taxanes and amsacrine.

The most common phenotypic marker associated with MDR is the overexpression of a 170kDa protein known as P-glycoprotein (P-gp) which is located in the plasma membrane of cells and is encoded by the MDR1 gene located on chromosome 7. In addition to P-gp, several other proteins have been described that can also lead to the development of pleiotropic resistance to cytotoxic agents *e.g.* MRP and LRP (Sonneveld, 2000).

1.5.3.1 Resistance associated with MDR1

There are two different genes encoding for P-gp in humans: MDR1 and MDR3, both located on chromosome 7 (Figs 1.11 and 1.12).

Fig. 1.11 Map of human MDR1 gene

Fig. 1.11 The MDR1 gene includes 28 introns, 26 of which interrupt the protein-coding sequence. Exons are indicated by vertical lines. Adapted from Chen *et al.* 1990

Fig. 1.12 Map of human MDR3 gene
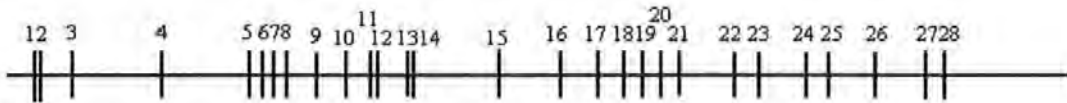
Fig. 1.12 The MDR3 gene contains 28 exons and 27 of these contain coding sequences for the two homologous halves of the protein that correlate with functional domains. Exons are indicated by numbered vertical bars. Adapted from Lincke *et al.* 1991

Even though the structure of MDR3 is virtually identical to that of the human MDR1 gene and MDR3 has been shown to be functional in cells (Arai *et al.* 1997), only the MDR1 gene is associated with MDR.

The product of the MDR1 gene is a transport protein, and as such the function of P-gp means that it is able to extrude drugs from the intracellular matrix preventing therapeutic concentrations from being reached.

P-gp belongs to a super-family of ATP-binding cassette (ABC) transporters, a family of ATP-dependent transport proteins. The protein consists of two structurally homologous

halves, each with six transmembrane domains and one ATP-binding site (Hrycyna, *et al.* 1996; Sonneveld, 2000) (Figure 1.13).

Fig. 1.13 Schematic diagram of the MDR1 product, P-glycoprotein

Glycosylation sites
Photoaffinity labelled regions
Phosphorylation sites
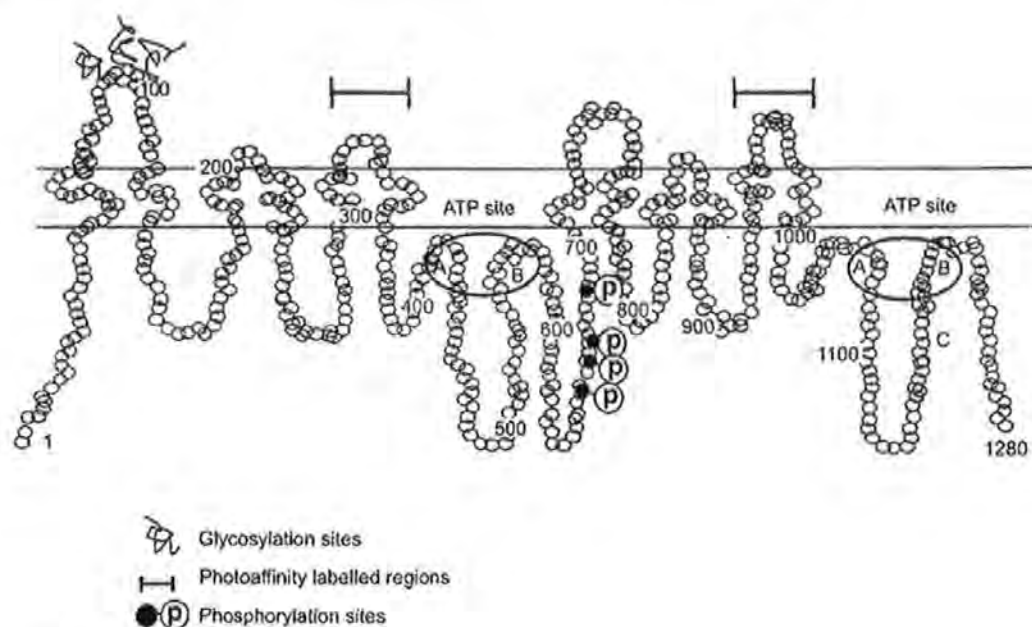
Fig. 1.13 From Sonneveld, 2000.

Although it has been suggested that phosphorylation of P-gp might be essential for drug transport (Germann, 1996a), two different groups have shown that a mutation of the major phosphorylation sites within P-gp did not affect its transportation function (Germann *et al.* 1996b; Goodfellow *et al.* 1996). The glycosylated sites at the cellular outside are probably involved in routing and stability of the protein, but may also act as antigens for monoclonal antibodies recognising P-gp (Schinkel *et al.* 1993a).

1.5.3.2 P-gp Mechanism of Action

Drugs affected by MDR are hydrophobic and positively charged and therefore bind readily to the negatively charged phospholipid head groups of the membrane. It has been suggested that in cells exposed to drugs *in vitro*, the low drug concentrations in the medium are in equilibrium with a pool of high drug concentrations present in the outer leaflet of the plasma membrane (Eytan and Kuchel, 1999) (see Fig. 1.14).

Fig. 1.14 Movement of MDR-susceptible drugs in P-gp overexpressing cells.
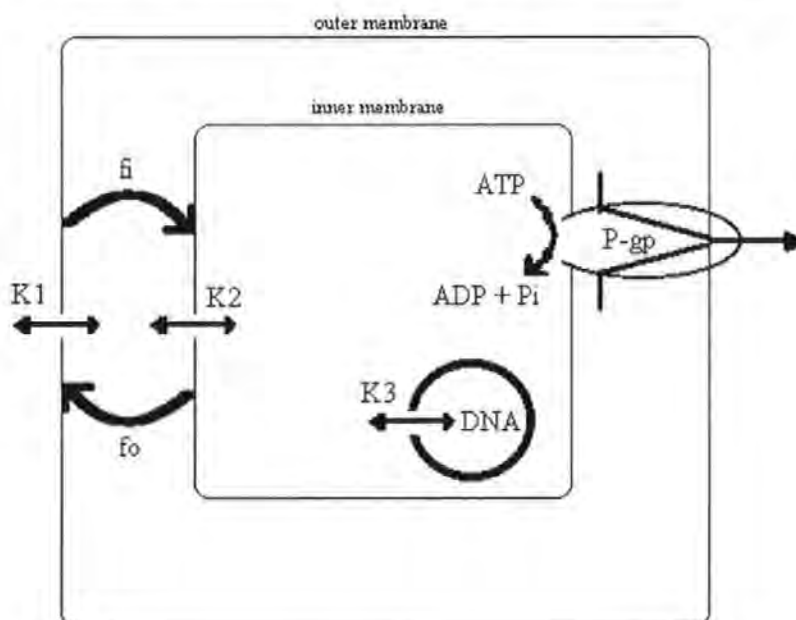
Fig. 1.14 MDR susceptible drugs added to the extracellular medium bind to and are in practical equilibrium (K1) with the drug pool in the outer leaflet of the plasma membrane. The drugs flip-flop across the plasma membrane (fi and fo). The drug pool in the inner leaflet of the membrane is in "effective" equilibrium with the drug pool in the cytoplasm (K2). Drugs in the cytoplasm are largely bound and in equilibrium (K3) with the intracellular molecular sinks represented here, for simplicity, as DNA. P-gp appears to extract its substrates from the inner leaflet of the plasma membrane and flip them outwards. Adapted from Eytan and Kuchel, 1999.

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The drug pool adsorbed to the cell surface is the direct source of drug influx into the cells. Drug transported from this pool into the cells is immediately replenished by drug from the medium. In this instance, the transmembrane movement of MDR-susceptible drugs (such as daunorubicin) across the plasma membrane, inward and outward, occurs within about 1 min. The drug reaching the inner leaflet of the plasma membrane is equilibrated rapidly with the drug pool in the cytoplasm. Due to the high affinity of the MDR-susceptible drugs for membranes, low concentrations of drug in the cytoplasm is in equilibrium with relatively high drug concentrations in the inner leaflet of the plasma membrane and drug reaching the cytoplasm is adsorbed by high-capacity molecular sinks such as DNA (capable of binding anthracyclines) or tubulin (capable of binding vinca alkaloids) (Eytan and Kuchel, 1999).

In relation to the above mechanism, it is no longer believed that P-gp is a 'classical' pump which binds substrates and transports these over the membrane down a gradient present in the lipid core (Eytan and Kuchel, 1999). Instead, interaction of substrate with P-gp has been shown to take place within the membrane (Raviv et al. 1990). In this case P-gp would extract its substrates directly from the inner leaflet of the plasma membrane, functioning as a 'flippase' within the membrane (Eytan and Kuchel, 1999; Higgins and Gottesman, 1992). A long residence lifetime of a drug in the membrane leaflet therefore increases the probability that P-gp will remove it from the cell.

This is one proposal for the mechanism of action of P-gp, but there is controversy over whether the protein protects cells by translocating drugs directly (as some type of pump) or indirectly (through modulating biophysical parameters of the cell). P-gp overexpression can change the plasma membrane electrical potential and intracellular pH and these changes will greatly affect the cellular flux of a large number of compounds to which P-gp

overexpression confers resistance. It may be these biophysical alterations that are responsible for many MDR mediated phenomena that have often been hypothesised to be due to direct drug transport by P-gp (Wadkins and Roepe, 1997).

1.5.3.3 Mechanisms of regulation

While antineoplastic agents are important substrates of P-gp, a variety of other clinically relevant drugs are also transported by P-gp. Therefore, in addition to examining P-gp overexpression in tumour cells, understanding the physiological mechanisms of P-gp regulation should help to explain patient variability with regards to drug disposition. In this respect, delineating regulatory pathways could enable prediction and manipulation of the expression of the MDR1 genes in order to improve the clinical effectiveness of P-gp substrates.

One of the functions of P-gp is to protect cells from harmful chemicals and metabolites, therefore, it is possible that these transporters play an important role in the cellular response against stress. In fact, numerous "stress-evoking" stimuli have been reported to alter MDR1 expression *e.g.* an 8-fold increase in MDR1 mRNA levels was seen in the renal carcinoma cell line HDP 46 in response to heat shock, ethanol, and cadmium (Chin *et al.* 1990b). This is consistent with a role for P-gp as a stress inducible gene product following environmental insults (Sukhai and Piquette-Miller, 2000) (Fig. 1.15).

Fig. 1.15 MDR1 promoter region
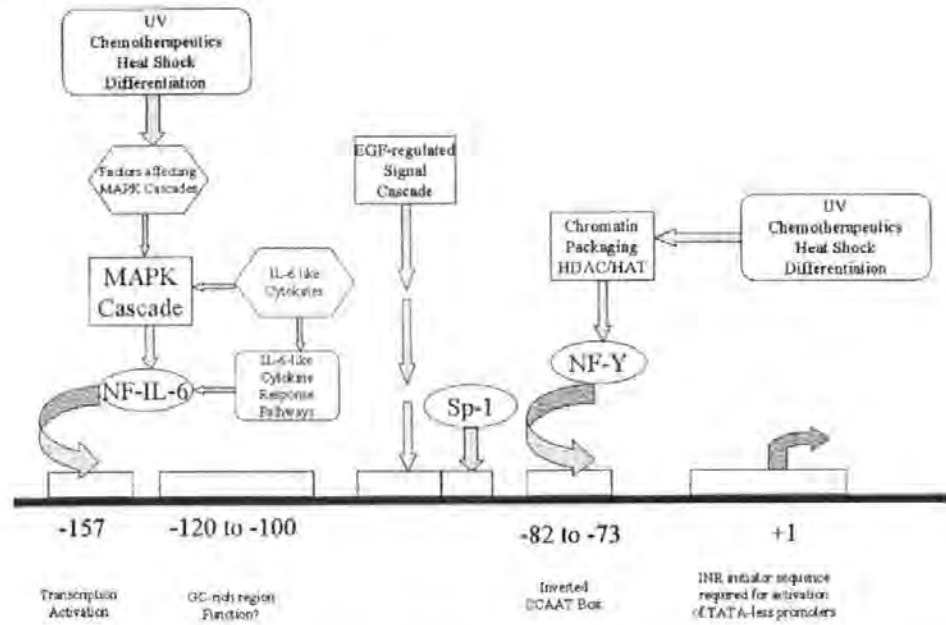
Fig. 1.15 A schematic diagram showing the relative locations of transcription factor binding sites, as well as interacting transcription factors and signal transduction pathways. The putative binding sites for various "stress" transcription factors are shown including, Sp-1, NF-Y and NF-IL-6 (also known as C/EBPβ). From Sukhai and Piquette-Miller, 2000.

A variety of physical and chemical agents affect expression and activity of P-gp either pre- or post-transcriptionally. For instance, protein kinase C activators which increase P-gp activity and drug resistance have been found to enhance MDR1 gene expression via both transcriptional and translational pathways (Chaudhary and Roninson, 1992). In addition, modulations in protein stability, plasma membrane incorporation, mRNA stability and processing, gene transcription and gene amplification have each been reported for P-gp (Sukhai and Piquette-Miller, 2000). Of these, alterations in P-gp expression that occur at the level of mRNA are perhaps the most frequently observed (Germann, 1996a).

1.5.3.3.1   Heat shock

Heat shock proteins are proteins that are synthesised in response to stressful stimuli. These often include proteins that are thought to help in stabilising and repairing cell damage. Identification of two strong heat shock consensus elements within the human MDR1 gene promoter, as well as an observed *in vitro* increase in MDR1 mRNA following cellular exposure to high temperature and toxic heavy metals (stress stimuli), suggest that MDR1 could function as a heat shock gene. It has been shown that basal activity of the MDR1 promoter requires heat shock factor (HSF) - mediated transactivation (Kim *et al.* 1997). Indeed, inhibition of the DNA-protein complex formation between HSF and its response element has been found to block MDR1 basal transcription, sensitising drug resistant cells to anticancer drugs (Kim *et al.* 1998).

Further, inhibition of protein kinase A (PKA) suppresses HSF DNA-binding activity as well as reducing expression of the heat shock proteins hsp90 and hsp70 (Kim *et al.* 1997). In addition, cells treated with antisense oligonucleotides to both hsp90 and MDR1 have been demonstrated to display vastly decreased P-gp half-lives and increased doxorubicin sensitivity (Bertram *et al.* 1996). Consequently, hsp90 has been implicated as a possible "chaperone protein" for P-gp and is thought to aid in the maintenance of P-gp functional activity and protein half-life. Suppression of hsp90 expression would, therefore, likely result in decreased P-gp half life and activity (Bertram *et al.* 1996).

Similarly, experiments by Kim *et al* (1996) have shown that the heat shock element may be involved in alterations of MDR1 transcription rates through pathways that are dependent upon PKA and the Raf oncogene, *i.e.* Raf activation by heat shock resulted in an induction of P-gp activity whereas an inhibition of PKA blocked the heat shock potentiation of P-gp

activity. These data indicate multiple pathways of control of MDR1 expression by cellular pathways that define the heat shock response.

1.5.3.3.2   Irradiation

In addition to initiating genetic mutations, ionising radiation may initiate cellular responses that can affect MDR1 gene expression. It has been demonstrated that induction of MDR1 expression incurred by ultraviolet irradiation results from increased MDR1 gene transcription rates (Ohga *et al.* 1998).

Evidence also suggests that the superstructure of chromatin plays a role in transcriptional regulation during UV irradiation. Specifically, the histone acetyltransferases and deacetylases that modulate DNA packaging into histones are believed to be involved. Incubation of a human carcinoma cell line (SW620) with an inhibitor of histone deacetylase has been reported to induce a 20-fold increase in MDR1 mRNA levels (Jin and Scotto, 1998). This induction may be due to an increased transcription, requiring the sequence from -82 to -73, which contains an inverted CCAAT box element, as point mutations in that sequence abolished promoter response to the histone deacetylase inhibitor. Gel mobility shift assays establishing binding of NF-Y (Fig. 1.17) to the inverted CCAAT box and the involvement of NF-Y in intrinsic histone acetyltransferase activity also appear to indicate regulatory mechanisms of human MDR1 gene expression via chromatin acetylation/deacetylation pathways. The CCAAT sequence has also been implicated in induction imposed by various stimuli including differentiation (Morrow *et al.* 1994), heat shock (Mickley *et al.* 1989) and cytotoxic drugs (Ohga *et al.* 1996) as this sequence is thought to play a role in maintaining basal MDR1 promoter activity (Miyazaki

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et al. 1992). This implies that MDR1 may be induced by radiation through a general non-specific cellular response to environmental stress.

1.5.3.3.3   Genotoxic Stress

Multiple pathways involving alterations of phosphorylation of proteins and transcription factors mediate cellular responses to DNA damage. These alterations occur through several distinct protein kinases, such as ERK and JNK/SAPK, as well as the tumour suppresser protein p53. In particular the cyclic AMP (cAMP) responsive transcription factors such as NF-6B, AP-1 and CREB, transduce signals in response to protein kinase C activation.

Several lines of evidence show a correlation between kinase activity and MDR1 expression which suggests that activation of cAMP-dependent protein kinases may induce the multidrug resistant phenotype of some tumour cells (Rohlff and Glazer, 1995).

The c-Jun NH_2-terminal protein kinase (JNK) is activated in response to many stressful stimuli including heat shock, UV irradiation and inflammatory cytokines (Cobb and Goldsmith, 1995; Hibi *et al.* 1993; Kyriakis *et al.* 1994). It has been reported that JNK is activated in human carcinoma cells by treatment with a number of anticancer drugs and that this activation correlates with increased MDR1 expression (Osborn and Chambers, 1996). Therefore, JNK may play a role in cellular development of the multidrug resistant phenotype.

Additionally, JNK is known to phosphorylate and activate c-Jun, which comprises half of the heterodimeric AP-1 transcription factor (Hibi *et al.* 1993). It is also known that there are AP-1 binding sites on the promoters of MDR1 genes across species (Ikeguchi *et al.*

1991) and a positive correlation between AP-1 activation and MDR1 transcription has been reported (Volm, 1993). It is therefore possible that induction of MDR1 expression correlates with JNK activation and thus could be traced to trans-activation by AP-1. Other agents which affect the JNK or protein kinase cascades may also affect MDR1 expression in this manner.

1.5.3.3.4 Inflammatory response and cytokines

Induction of an acute inflammatory response in experimental models of inflammation in rats (Piquette-Miller *et al.* 1998) and mice (Hartmann *et al.* 2001) has been demonstrated to decrease the hepatic expression and activity of P-gp at the level of mRNA.

As the majority of effects seen during an acute inflammatory response are associated with the release of a few of the pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, it is possible that these mediators are also involved in P-gp regulation and the control of MDR gene expression during an inflammatory response. Indeed, *in vitro* treatment of cultured hepatocytes with recombinant IL-1β and IL-6 elicit dose- and time-dependent reductions in P-gp expression and activity (Sukhai *et al.* 2001). Results demonstrated decreases in MDR1 mRNA expression in IL-6 but not IL-1 treated cells, suggesting that IL-1β mediates effects on P-gp expression via post-translational mechanisms, whereas IL-6 likely influences P-gp expression by either decreasing MDR1 mRNA stability or reduced transcription rates.

Several studies also indicate that TNF-α, which primarily acts through NF-κB, suppresses MDR1b (MDR1 gene equivalent in rodents) gene expression (Stein *et al.* 1996a; Stein *et al.* 1996b; Walther and Stein, 1994). These *in vitro* studies reported down-regulation of

PGP protein and MDR1 gene expression as well as enhanced chemosensitivity in continuous human intestinal cell lines treated with TNF-α. A binding site for NF-κB exists on the MDR1b promoter (Zhou *et al.* 1996) which may implicate the potential involvement of this transcription factor in MDR1b down regulation. On the other hand, others have also reported a TNK-α mediated induction of MDR1b expression in cultured rat hepatocytes that can be suppressed by addition of an anti-inflammatory steroid, dexamethasone (Hirsch-Ernst *et al.* 1998; Kreuser *et al.* 1995).

In terms of species differences, although the inflammatory response mediates a suppression of P-gp in rats and mice, this phenomenon has yet to be examined in humans. Some reports indicate a diminished MDR1 gene expression and/ or potentiation of chemosensitivity in human colon carcinoma cell lines incubated with a number of these cytokines including IFN-γ and TNF-α (Stein *et al.* 1996a; Walther and Stein, 1994). While information in this area is limited, IFN-γ and TNF-α effects are thought to be mediated through an inhibition of MDR1 gene transcription. Studies with IFN-α have also demonstrated an IFN-α mediated downregulation of MDR1 in a human hepatoma cell line (Takeuchi *et al.* 1999) as well as an IFN-α mediated functional reversal of the MDR1 phenotype in LoVo/Dx cells by affecting P-gp function (Scala *et al.* 1991). Although the molecular pathways involved in the cytokine regulation of MDR1 gene expression have not yet been elucidated, it is likely that the down-regulation of MDR1/P-gp in hepatocytes occurs through inhibition of gene transcription. Cytokines mediate their effects through unique signal transduction pathways involving only a handful of nuclear transcription factors *e.g.* NF-6B, C/EBP and APRF (Sukhai and Piquette-Miller, 2000). Although the effects of cytokines on P-gp expression have not been fully characterised, the many overlapping and synergistic effects of the cytokines suggest that their action on MDR1 gene expression is likely to occur

through elaborate interaction and unique effects dependent upon cytokine concentrations, cell type and species.

1.5.3.4 Post-translational Modification

Post-translational modification of MDR is highly significant and, in man, a 140kD precursor protein is gradually converted to a 170kD form (P-gp) over 2 to 4 h.

In addition to this conversion, phosphorylation may also be important. P-gp appears to be phosphorylated in its basal state by protein kinase C (PKC), that may in turn affect drug transport. Drug accumulation assays in a multidrug resistant human carcinoma cell line showed that phorbol-ester (PMA) treatment significantly reduced ^{3}H-vinblastine accumulation and that basal phosphorylation of P-gp was increased 6-fold (Aftab *et al.* 1994). Staurosporine and H7 (inhibitors of PKC and cAMP-dependent protein kinase activity), however, did not affect overall P-gp phosphorylation, suggesting perhaps a different mechanism of phosphorylation.

Another group has reported that treatment of multidrug resistant MCF-7/adriamycin resistant cells with heat shock can increase the phosphorylation of P-gp and that this response was not seen in the sensitive MCF-7 cell line (Yang *et al.* 1995). U-73112, an inhibitor of phospholipase C and staurosporine, in turn, an inhibitor of protein kinase C, both decreased the heat-shock-induced phosphorylation of P-gp. This suggests that heat shock induced phosphorylation of P-gp is mediated through the activation of the phospholipase C/protein kinase C pathway.

Studies of vincristine resistant HL-60 cells identified another membrane-associated protein kinase (PK1) which also phosphorylates P-gp on serine and threonine residues and may regulate levels of multidrug resistance (Chambers *et al.* 1990; Staats *et al.* 1990).

It is interesting that although the phosphorylation of P-gp (outlined above) has been appreciated for many years, little is known about the significance of this process. It has been suggested that the phosphorylation of P-gp might be essential for drug transport (Germann, 1996a), however two different groups have shown that a mutation of the major phosphorylation sites within P-gp did not affect its transportation function (Germann *et al.* 1996b; Goodfellow *et al.* 1996).

In contrast, the activity of P-gp as an ion channel regulator has been reported to be affected by phosphorylation. (Vanoye *et al.* 1999). Several proteins belonging to the ATP-binding cassette superfamily can affect ion channel function, including P-gp. Vanoye *et al.*, measured whole cell swelling-activated Cl- currents (ICl, swell) in parental cells and cells expressing wild-type MDR1 or a phosphorylation-defective mutant. While PKC and PKA stimulation reduced the rate of increase in ICl, swell in cells that expressed MDR1, their effects were absent in the phosphorylation-defective mutants.

1.5.3.5 Mutation

Mutations in the MDR1 gene can alter the patterns of cross resistance to certain agents (Szabo *et al.* 2000). A study conducted by Ruth *et al* (2001) reported that the glycine to valine mutation at position 185 (G185V) near transmembrane domain 3 of human P-gp increased the relative ability of P-gp to transport several drugs, including etoposide, but decreased the transport of other substrates. Another substitution (I186N), adjacent to

G185V, increased resistance to all the tested drugs and augmented the effect of G185V on etoposide resistance. By ligand binding analysis, the K(m) value that reflects the apparent affinity of drugs for P-gp, and the Hill number, reflecting the apparent number of drug-binding sites, were monitored. The results showed that an increase or decrease in drug resistance relative to that of the wild type was accompanied by a corresponding increase or decrease in the K(m) and the Hill number, suggesting that these mutations alter the ability of P-gp to transport agents due to a change in the affinity and number of drug-binding sites in P-gp.

Similarly, Vo and Gruol (1999) have reported that a series of mutations located within transmembrane domains 4-6 of P-gp, proximal to the cytoplasmic interface, cause a reduced ability to bind steroids. The presence of hydroxyl groups, associated with specific steroid carbon atoms, regulates the ability of corticosteroids to be transported and this specificity was demonstrated by their experiments measuring the ability of steroids to inhibit drug transport. The results indicated that a keto oxygen, associated with the 3- and 20- carbon atoms, as well as a 17-carbon hydroxyl group, acts to enhance steroidal P-gp inhibitory activity. The reported mutations seem to alter the recognition of the 17 alpha-hydroxyl group and the 20-keto oxygen atom.

1.5.3.6 Methods of P-gp detection

A variety of methods exist to detect P-gp at the RNA or protein level.

1.5.3.6.1 Detection of RNA

Some investigations have used MDR1 probes in conjunction with slot blot analysis which is specific, sensitive and quantitative (Goldstein *et al.* 1989), while others have used reverse transcriptase-polymerase chain reaction (RT-PCR) to amplify the RNA signal (Noonan *et al.* 1990).

Sensitive RNA assays are very useful in determining drug resistance in cell lines but the use of pooled RNA from the tumour sample can be a major disadvantage as it does not reflect the heterogeneity of P-gp expression in individual tumour cells. A given measurement of RNA could reflect a tumour with a large number of cells expressing low levels of RNA or a few tumour cells expressing high levels of MDR1 RNA. In addition there is no cut- off level of measured RNA above which clinically significant multidrug resistance can be measured. *In situ* hybridisation (ISH) is able to measure RNA expression at a cellular level therefore avoiding the pitfalls of measuring total tumour RNA levels (Vergier *et al.* 1993). ISH is more sensitive than dot blot hybridisation but cannot detect very low expression, in addition to which it is technically demanding and therefore not widely used.

1.5.3.6.2 Immunohistochemistry

Immunohistochemistry has been used often but with varying and often contradictory results (Ferry, 1998).

A large variety of antibodies is available (Table 1.5) but, where staining can be reliably achieved in tumours containing high levels of P-gp, the technique is unreliable when P-gp levels are low.

Monoclonal Antibody	Epitope
C219	Intracellular
JSB-1	Intracellular
MRK16	Extracellular
4E3	Extracellular
UIC2	Extracellular

Table 1.5 Some monoclonal antibodies against P-gp

1.5.3.6.3   Flow Cytometry/ functional assays

By employing both anti-mouse and anti-human monoclonal antibodies, flow cytometry has been used to locate P-gp as well as to study its function (Ferry, 1998; Guerci *et al.* 1995).

Functional assays employ many fluorescent agents to monitor their cell flux and hence follow the activity of P-gp. Most use rhodamine 123 and anthracycline agents such as daunorubicin or doxorubicin however, rhodamine 123 (the most widely used fluorescent probe for this application) is also transported by the gene product of another efflux pump gene, multidrug resistance-associated protein (MRP) (Twentyman *et al.* 1994).

1.5.3.7 Clinical significance

Using the techniques mentioned above, the highest P-gp levels in normal tissue have generally been found in the kidney, adrenal cortex, stomach, duodenum, colon and placenta. Expression occurs primarily in specialised epithelial cells on luminal surfaces, but

strong expression has been found in endothelial cells of capillary blood vessels at blood-tissue barrier sites such as CNS and testes (Cordon-Cardo *et al.* 1989; Fojo *et al.* 1987). These observations are significant since the brain and testes constitute sanctuary sites in which relapse following systemic chemotherapy occurs, presumably because of failure of drug penetration.

The most extensive study of P-gp expression in cancerous tissue reported on levels of MDR1 mRNA in over 400 human cancers (Goldstein *et al.* 1989). The overall findings were that there is an inverse correlation between levels of MDR1 expression and chemosensitivity of the tumour type, though numerous exceptions have been found. The exceptions may reflect the sensitivity of the assay and uncertainty as to the level at which MDR1 expression becomes clinically significant within the clinical context. Whilst the Goldie-Coldman hypothesis predicts treatment failure if 1 cell in 10^6 expresses a resistance mechanism (Ferry, 1998), no method of analysis yet described can reliably achieve this degree of detection.

From the point of view of prognosis, however, a few studies have reported a relationship between MDR1 expression and either the absence of remission or the presence of refractory disease especially in AML (Marie *et al.* 1991; Pirker *et al.* 1991; van den Heuvel-Eibrink *et al.* 2000; Zhou *et al.* 1992) (section 1.6.1).

1.5.3.8 Other multidrug resistance proteins

While many effects were thought to be due to just one drug-transport protein, *i.e.* the 170kDa P-gp, several other membrane proteins of 300kD, 180kD, 170kD, 95kD and 85kD have been reported and are associated with MDR. For example, MCF-7 breast carcinoma

cells were made resistant to doxorubicin by exposing them to the drug in the presence of verapamil. P-gp expression was not elevated in these cells but a novel uncharacterised 95kD surface membrane protein was expressed, and the presence of this protein in clinical samples of breast cancer refractory to doxorubicin suggested actions on membrane mechanisms other than P-gp (Chen *et al.* 1990). In fact a specific breast cancer resistance protein (BCRP) has now been identified (van den Heuvel-Eibrink *et al.* 2000).

In addition to P-gp, the multidrug resistance protein (MRP) and lung resistance protein (LRP) share the ability to act as drug transport proteins and as such contribute to MDR (Almquist *et al.* 1995; Sonneveld, 2000; van den Heuvel-Eibrink *et al.* 2000). Furthermore, five new homologues of MRP (MRP2 or MOAT, MRP3, MRP4, MRP5, and MRP6) have now been identified, as have other membrane-associated drug transport proteins such as the transporter associated with antigen processing (TAP), the anthracycline resistance-associated protein (ARA), and sister of P-glycoprotein (sP-gp) (van den Heuvel-Eibrink *et al.* 2000). The clinical significance of these proteins has not yet been reported.

1.5.3.9 Modulation of MDR

Pharmacological modulation of P-gp function to increase drug bioavailability is one approach to enhance therapeutic effectiveness. As such, a large number of compounds have been investigated for their ability to reverse P-gp mediated MDR.

Many agents have been found to reverse MDR and range from the calcium blockers such as verapamil and nifedipine, to tamoxifen, phenothiazines and cyclosporin (Eytan and Kuchel, 1999). However, most of these first generation modulators required high doses of drugs to reverse MDR and were associated with unacceptable toxic effects (Tan *et al.* 2000),

limiting their effective clinical use. In addition, the modulation of P-gp in normal tissues can affect the pharmacokinetics and, thus, the toxicity of the associated chemotherapeutic agents. Second and third generation MDR inhibitors include PSC 833, S9788, GF120918 and valspodar. Limitations to the use of these agents still exist and include multiple and redundant cellular mechanisms of resistance, alterations in pharmacokinetics of cytotoxic agents, and clinical toxicities (Tan *et al.* 2000). However, Phase I/II trials with valspodar have shown that this new agent can be safely administered in combination with different chemotherapy regiments after dose adjustments of cytotoxic drugs that are P-gp substrates, and reversal has been demonstrated in patients with AML (Covelli, 1999).

The effects of PSC 833, verapamil and S9788 have been studied singly and in combination by various groups (Merlin *et al.* 2000; Moins *et al.* 2000). Moins *et al.* found a strong MDR reversing effect of S9788 which appears specific to P-gp and an additive effect between verapamil and PSC 833, suggesting a better therapeutic efficiency if used in well defined combinations. Similarly, Merlin *et al.* reported a synergistic effect with a combination of S9788 and PSC 833 and suggested that this might offer alternative ways to decrease the toxicity generated by high-dose P-gp blockers without altering the efficacy of the resistance modulation.

Although the mechanism of action of these agents is not completely clear, some may act by binding to P-gp and preventing drug efflux, thereby causing intracellular accumulation of drug, while others may not affect efflux but instead cause redistribution of drug within the cell.

While the above modulators are being actively studied some cytokines have also been found to modulate the activity of a number of chemotherapeutic agents. IFN-α can

modulate the actions of bleomycin (Viano *et al.* 1989), doxorubicin (Hoff *et al.* 1986; Scala *et al.* 1991), methotrexate (Welander, 1987), mitomycin C (Sklarin *et al.* 1988), and vinca alkaloids (Sidkey *et al.* 1987). One mechanism by which cytokines may modulate this activity is via the possible reversal of MDR. In 1991 Scala *et al.* demonstrated the functional reversal of the MDR1 phenotype by IFN-α. Pre-treatment of multidrug resistant LoVo/Dx cells for either 48 or 120h led to a reduction of the IC_{50} dose for doxorubicin. This change was found not to be due to effects on the cell cycle or change in MDR1/P-gp expression, but rather an increased accumulation of doxorubicin in the cells treated with IFN-α, *i.e.* by affecting the function of P-gp. IFN-α, therefore, appeared to be able to restore chemosensitivity of the LoVo/Dx cell line through modulation of the MDR1 phenotype. The authors suggested that this was achieved by a direct mechanism in which IFN-α competes for P-gp binding and, as IFN-α is well tolerated, there may be a rationale for its clinical use in chemotherapy schedules for patients with drug-resistant tumours (Scala *et al.* 1991).

In addition to the above study, a more recent paper reported on the effectiveness of recombinant human interferon-alpha-2a (rHuIFN-α-2a) in enhancing the reversal of multidrug resistance caused by the monoclonal antibody (MAb) MRK-16 (Fogler *et al.* 1995). The authors used a retrovirus-infected human colon tumour expressing the human MDR1 gene (HT-29^{mdr1}) and demonstrated that the reversal of vincristine resistance, mediated by the anti-P-gp MAb, MRK-16, could be potentiated both *in vitro* and *in vivo* by non-toxic doses of rHuIFN-α-2a. The mechanism by which rHuIFN-α-2a achieved this remains unclear, but it has been demonstrated that IFNs can exert an additive or synergistic potentiation of the cytotoxic activity of structurally and mechanistically unrelated chemotherapeutic agents, including multidrug-resistant and non-multidrug-resistant phenotype drugs (Wadler and Schwartz, 1990).

1.6   RATIONALE BEHIND STUDY

1.6.1   B-CLL AND DRUG RESISTANCE

The dysregulation of apoptosis in B-lymphocytes is one physiological problem presented in B-CLL. Another is the ability of tumour cells to survive exposure to anticancer agents, as this produces a great obstacle to successful cancer chemotherapy in this disease.

Of particular importance to the phenomenon of MDR is the expression of the MDR1 gene (section 1.5.3, p47). Translation of this gene results in a 170KD transmembrane protein, P-gp that increases cellular resistance to chemotherapeutic agents such as daunorubicin, colchicine, and vinblastine. Even with the introduction of combination treatments for CLL (section 1.2.1.5.1.2, p20), there is an emerging resistance to a variety of structurally, chemically and phase-specific unrelated drugs used in CLL treatment regimens such as vinca alkaloids and anthracyclines. This resistance may be related to MDR1 gene expression and, although the expression of this gene is more commonly associated with MDR in ALL and AML, some groups have also reported an increase in the MDR1 gene mRNA in a subset of patients with CLL (Arai *et al.* 1997; Michieli *et al.* 1991; Sonneveld *et al.* 1992; Webb *et al.* 1998). Other studies have reported that approximately 40% of patients with B-CLL have increased levels of the MDR1 gene (Sparrow *et al.* 1993), while several authors have described a large majority of patients expressing P-gp, either before or after treatment (Michieli *et al.* 1991; Shustik *et al.* 1991). Recently, Svoboda-Beusan *et al.* (2000) have published a study on the relevance of P-gp expression to the treatment response of B-CLL. The authors found that P-gp was expressed in patients and that this expression decreased in patients treated with chlorambucil while expression remained unchanged or even increased in those patients who did not respond to therapy.

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Subsequently, they concluded that sequential, follow-up P-gp expression values correlated with treatment response, suggesting that P-gp expression in this disease was relevant to the treatment response. In contrast to this, however, some groups have reported that P-gp-associated MDR is not prevalent in B-CLL and have failed to detect increased levels of the MDR1 gene mRNA or P-gp (Ribrag *et al.* 1996).

A related gene, but of an entirely different sequence, is the MDR3 gene also reported to be overexpressed, particularly in advanced disease (Herweijner *et al.* 1990; Larkin *et al.* 1999a; Sonneveld *et al.* 1992). This overexpression is peculiar to B-CLL lymphocytes as, unlike MDR1, it is not noted in normal cells. Previous reports concluded that the MDR3 gene product had a limited role in MDR (Marie, 1995), however, as function of the MDR3 gene product (as an efflux mechanism) has now been described (Arai *et al.* 1997), it is possible that the MDR3 gene may play a role in the pathogenesis or clonal evolution of the disease.

1.6.1.1 Cytokines and Drug Resistance

Some cytokines have been found to modulate the activity of a number of chemotherapeutic agents. One mechanism by which cytokines may modulate this activity is via the reversal of MDR. In 1991 Scala *et al.* demonstrated the functional reversal of the MDR1 phenotype by IFN-α as described above. The authors suggested that the increased chemosensitivity was achieved by a direct mechanism in which IFN-α competes for P-gp binding. As IFN-α is well tolerated, and more common P-gp modulators, such as verapamil, are not well tolerated at the necessary physiological concentrations for P-gp modulation, there may be a rationale for its use clinically in patients with drug-resistant tumours (Scala *et al.* 1991).

The dysregulation of apoptosis may have an important role to play in the development of B-CLL and may present therapeutic opportunities, however, the suggestion by Sparrow *et al.* (1993) that the overexpression of resistance genes could lead to clonal evolution of the disease is also of interest. Add to this hypothesis the problem of MDR in B-CLL and the need to modulate drug resistance mechanisms becomes more pertinent.

The conflicting results surrounding the role of MDR1 and P-gp in B-CLL MDR suggested that continued investigation into P-gp-associated MDR was warranted, especially in light of the favourable reports of IFN-α being a well tolerated resistance modulator *in vitro* (Scala *et al.* 1991).

1.7   AIMS

1] To determine whether or not P-glycoprotein is expressed in B-CLL patients.

2] To determine whether or not P-glycoprotein is functional in B-CLL patients.

3] To determine whether the function of P-glycoprotein in B-CLL can be modulated by IFN-α.

1.8   DESIGN OF EXPERIMENTAL APPROACH

To study P-gp expression and function in B-CLL patients, extraction and subsequent *in vitro* cell culture of B-lymphocytes was required. The determination of P-gp expression on the cell surface would then be studied by monitoring the presence of the protein using a monoclonal antibody as well as determining MDR1 mRNA expression.

Function of the protein has characteristically involved the use of fluorescent P-gp substrates such as rhodamine 123 and anthracycline drugs, *e.g.* daunorubicin as well as P-gp modulators such as verapamil (section 1.5.3.6.3). Although the use of anthracycline drugs is associated more with the treatment of AML, these agents are still used in some treatment regimens for B-CLL (section 1.2.1.5.1) and there has been increased interest in the use of the newer, more lipophilic, anthracyclines for treatment in B-CLL, *e.g.* idarubicin (Gahn *et al.* 2000). The potential use of anthracyclines in B-CLL treatment coupled to the fact that they are P-gp substrates and fluorescent (and therefore easily monitored) suggested their use in studying the role of P-gp in B-CLL. However, anthracyclines are not very stable, being prone to photodegradation as well as changes in pH, and, in the presence of cells, this would mean that degradation products as well as metabolites would co-inhabit the cell culture environment. As the metabolites of some anthracycline drugs are also subject to P-gp action, the presence of degradation products with similar properties was of concern as it could potentially interfere with the interpretation of results of P-gp functionality experiments.

This concern has not been addressed to any great extent in previous P-gp studies and few studies have looked at the stability of anthracycline drugs in cell culture environments. However, this potential instability was thought to be an important factor to consider during subsequent P-gp functional analysis. Therefore, the first experimental section of this thesis addresses the stability of the anthracycline, daunorubicin, *in vitro* and determines the final cell culture conditions employed in the following two experimental sections examining P-gp expression and functionality.

2. ANTHRACYCLINE STABILITY IN THE CELL CULTURE SYSTEM

2.1   INTRODUCTION

Cell culture systems have been widely used to investigate various aspects of the haematological malignancies. To carry out these studies, robust cell culture systems are necessary, in addition to which, quick, robust and reliable analytical methods are required.

The use of anthracyclines for *in vitro* analysis can pose potential problems due to their reported instability under certain conditions (Beijnen *et al.* 1986; Bosanquet, 1986). Many different chemical and physical parameters including temperature, pH, culture media, and protein binding influence this stability and these parameters have been analysed by a variety of techniques, *e.g.* high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE).

2.1.1 HPLC

Chromatography is a separation method that relies on differences in the partitioning behaviour of a compound between the flowing mobile phase, *i.e.* solvent/buffer system, and a stationary phase, *i.e.* column, to separate the components in a mixture. The column holds the stationary phase, which is usually adsorbed onto a silica support, and the mobile phase carries the sample through it. Sample components that partition strongly onto the stationary phase spend a greater amount of time on the column and are separated from the components that stay predominantly in the mobile phase and pass through the column more quickly. As the components elute from the column they can be quantified by a detector and/or collected for further analysis.

High-performance liquid chromatography (HPLC) is a variation of liquid chromatography that utilises high-pressure pumps to increase the efficiency of the separation of compounds that are dissolved in solution. The basic instrumentation consists of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector (Fig. 2.1).

Fig. 2.1 Schematic plan of an HPLC system
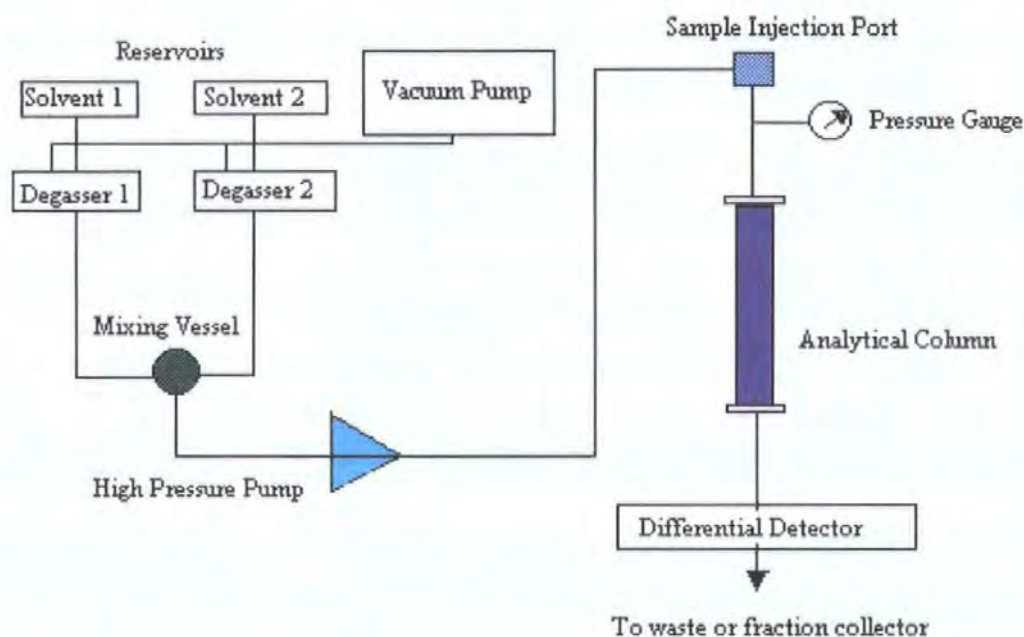
A sample mixture is injected into the system and is carried to the analytical column by the mobile phase. The different components of the mixture are separated as they pass through the column, and components in the column effluent are recorded by various means, *e.g.* UV-VIS absorption at a set wavelength.

The resulting record is normally in the form of a chromatogram showing the chromatography over a certain time period (Fig. 2.2). As compounds are retained during chromatography, the time it takes for a component to move through the system is called the retention time.

Fig. 2.2 100ng/ml daunorubicin analysed by HPLC
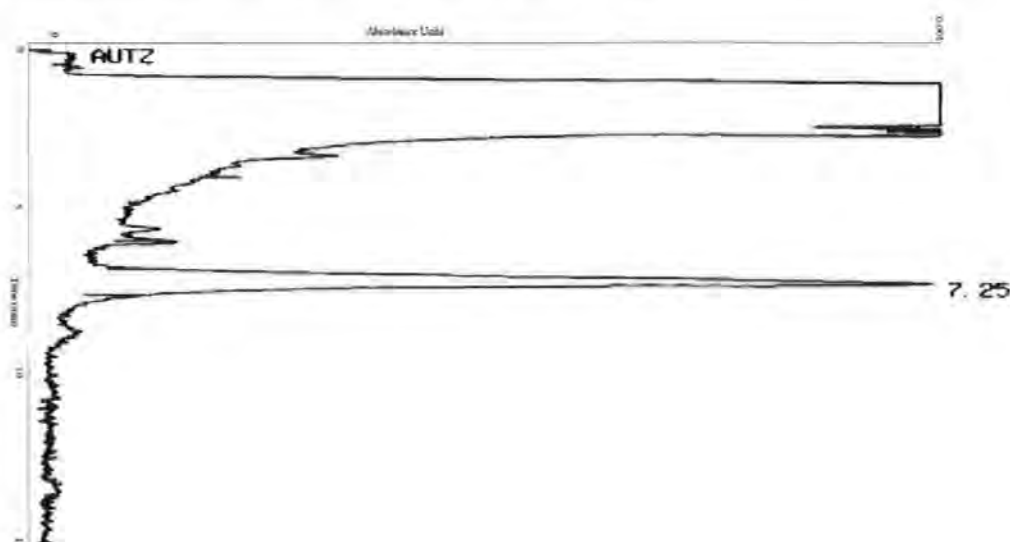
Fig. 2.2 100μl of 100ng/ml daunorubicin in RPMI culture medium was injected (full loop volume) and analysed for 15min at a flow rate of 1ml/min. Detector sensitivity was set at 0.001 absorbance units full deflection. Detection occurred at 254nm. Daunorubicin was detected at a retention time of 7.25min.

HPLC has been the predominant method of analysis for investigation into the stability of anthracycline drugs. Capillary Electrophoresis (CE), however, is another method by which anthracycline drugs may be analysed.

2.1.2 CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) is a family of related techniques that employ narrow-bore (20-200μm internal diameter) capillaries to perform high efficiency separations of both large and small molecules (Fig. 2.3).

Fig. 2.3 Schematic plan of a CE system
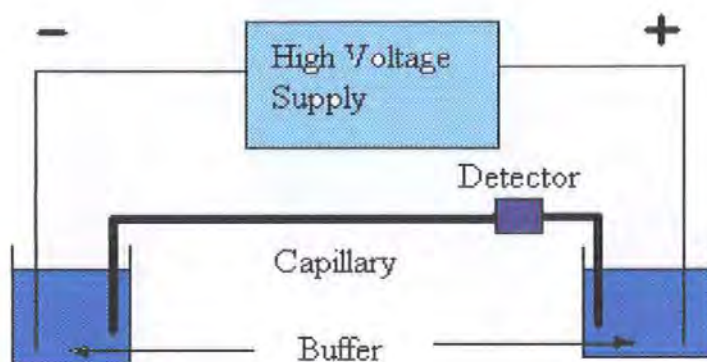
Performing electrophoresis in small-diameter capillaries allows the use of very high electric fields because the small capillaries efficiently dissipate the heat that is produced. Increasing the voltage across the column produces very efficient separations and reduces separation times. The resulting separation is a combination of traditional polyacrylamide gel electrophoresis (PAGE) and modern HPLC.

This technique not only requires small amounts (10-30μl) of sample but also consumes limited quantities of reagents & is applicable to a wider selection of analytes compared to other analytical separation techniques. In addition, the simplicity and sensitivity of the technique provides a good way to analyse intracellular and extracellular drug concentrations with the minimum of sample preparation. The absence of conventional sample preparation techniques such as filtration or precipitation means that a more accurate impression of a complex culture system may be obtained.

2.1.3   DRUG STABILITY IN CELL CULTURE SYSTEMS

Many similar cell culture systems have been used to investigate anthracycline resistance in B-CLL, but these investigations do not seem to have considered the stability of the anthracycline in the culture environment. This is an important consideration as the toxic effect of the anthracyclines is influenced by their concentration, which, in turn, is related to drug stability. In addition to this, the instability of a drug may influence conclusions derived from experiments; resulting degradation and metabolic products could exert an array of effects making it difficult to attribute a particular effect to any individual component.

Even though daunorubicin is not normally included in the regimens used to treat CLL, many investigations into P-gp function have used it as a model P-gp substrate (Ayesh *et al.* 1996; Beck *et al.* 1996; Lizard *et al.* 1995; Merlin *et al.* 2000; Roovers *et al.* 1999; Wang *et al.* 2000). Therefore, this anthracycline was chosen as one P-gp substrate with which to monitor P-gp function with the intention of continuing this line of study with other more relevant anthracyclines such as doxorubicin and idarubicin given sufficient time. Consequently, this chapter discusses the stability of daunorubicin under different test conditions with a view to optimising a final cell culture system for further investigation into P-gp expression and function.

2.2   MATERIALS

Unless otherwise stated, materials used in this study were of at least analytical grade and obtained from Sigma-Aldrich Company Ltd., Poole, UK.

All water used was 3MΩ water, filtered and deionised to 18MΩ by an Elga UHQ II water purifier (Elga Ltd., Buckinghamshire, UK).

2.2.1   GENERAL MATERIALS

Equipment

- 15ml polypropylene tubes (Life Sciences Int. (UK) Ltd., Hanks, UK)

- 5ml polypropylene tubes (Sarstedt Ltd., Leicester, UK)

- Autosampler vials (HPLC Technology Company Ltd., Cheshire, UK)

- EBA 12R Centrifuge (Hettich, TuHlingen, Germany)

Reagents

- 3MΩ Sterile Water (Baxter Healthcare Ltd., Norfolk, UK).

- Daunorubicin (Rhône-Poulenc Rorer, Vitry-Alfortville, France).

- Disodium Hydrogen Orthophosphate Dihydrate (Fisher Scientific, Leicestershire, UK).

- DMEM buffered (with sodium bicarbonate & 25mM HEPES) (Sigma-Aldrich Company Ltd., Poole, UK).

- Fetal Bovine Serum (Sigma-Aldrich Company Ltd., Poole, UK).

- HPLC Grade Methanol (Fisher Scientific, Leicestershire, UK).

- Hydrochloric Acid [10M] (BDH, Poole, UK).

- Orthophosphoric Acid (Fisher Scientific, Leicestershire, UK).

- Potassium Hydroxide (BDH, Poole, UK).

- RPMI 1640 unbuffered (with sodium bicarbonate) (Sigma-Aldrich Company Ltd., Poole, UK).

- RPMI 1640 buffered (with sodium bicarbonate & 25mM HEPES) (Sigma-Aldrich Company Ltd., Poole, UK).

- Sodium Hydroxide (Fisher Scientific, Leicestershire, UK).

2.2.2   HPLC MATERIALS

Equipment

- C1 Filtration Units (Sartorius Ltd., Surrey, UK).

- ConstaMetric 3000 Pump (Milton Roy/LDC., Staffordshire, UK)

- Integrator (Milton Roy/LDC., Staffordshire, UK)

- Microfilters - Minisart [$0.2\mu m$] (Sartorius Ltd., Surrey, UK).

- SpectroMonitor 3000 UV Detector (Milton Roy/LDC., Staffordshire, UK)

- Varian MicroPak Column SP-C18-5 (Varian Ltd., Surrey, UK)

Reagents

- HPLC grade Acetonitrile (Fisher Scientific, Leicestershire, UK).

2.2.3   CAPILLARY ELECTROPHORESIS MATERIALS

Equipment

- $375\mu m$ OD, $100\mu m$ ID Capillary Tube (Dionex UK Ltd., Surrey, UK).

- $375\mu m$ OD, $75\mu m$ ID Capillary Tube (Dionex UK Ltd., Surrey, UK).

- AI-450 CE Software (Dionex UK Ltd., Surrey, UK).

- Dionex CE System (Dionex UK Ltd., Surrey, UK).

Reagents

- Boric Acid (Thornton & Ross, Huddersfield, UK).

- Disodium Tetraborate (Fisons Scientific, Loughborough, UK).

- Absolute ethanol (Hayman Ltd., Essex, UK).

- Hydrochloric Acid (BDH, Poole, UK).

- Medical Air (BOC Gases, Manchester, UK).

- Medical Helium (BOC Gases, Manchester, UK).

- Propan-2-ol (Fisher Scientific, Leicestershire, UK).

- Sodium Dodecyl Sulphate (Fisher Scientific, Leicestershire, UK).

2.3   GENERAL METHODS

2.3.1   METHOD DEVELOPMENT

As the drug stability analysis was intended for the optimisation of a cell culture environment, a robust system was needed which could give reliable analysis and in addition, could reflect more closely the culture environment at any instant in time being analysed. It was realised that future *in vitro* cell culture experimentation would require manipulation of relatively small sample volumes (2ml maximum) and complex cell culture conditions, *i.e.* inclusion of culture constituents such as glutamine and foetal bovine serum (FBS). It was therefore imperative that an analytical method requiring minimum sample extraction be used, thus decreasing the risk of altering any equilibrium states between the solubilised drug and medium components. This prompted the use of CE as the preferred method of choice.

Initially the use of simple capillary zone electrophoresis (CZE) was attempted with a method developed by Dionex (suppliers of the capillary electrophoresis system). CZE is the simplest form of CE and was employed to detect 50μg/ml of an aqueous daunorubicin solution using a 100mM sodium dihydrogen orthophosphate buffer (pH 4.2) as the mobile phase. The sample was injected by gravity injection (100mm for 30s) and detected using UV absorbance at a wavelength (λ) of 234nm with a run time of 25min. Dionex had previously analysed varying concentrations of daunorubicin using this method and achieved a lower detection limit of 1μg/ml.

The initial attempts at detection were unsuccessful and it was theorised that detection was not being performed at the optimum absorption wavelength. An absorption spectrum

showed maximum absorbency at 254nm. Continued analysis at this λ did not result in drug detection.

Conditioning of the internal capillary surface was then advised both before detection and as a general maintenance process using the following procedure: 0.1M sodium hydroxide was washed through the capillary for 10min, then water for 5 min, and finally run buffer for 10 min. The procedure was important to ensure that the surface of the capillary was fully and uniformly charged, to minimise changes in migration times. This, however, also proved unsuccessful showing that the difficulty in detection did not seem to be related to interference with compound migration due to a non-uniformly charged capillary surface.

To eliminate the possibility of capillary problems a new capillary was made to recommended specifications (Length = 70cm, internal diameter = 75μm) and an aqueous daunorubicin sample injected again. On optimisation of experimental parameters such as current & power (current = 15kV & power = 5000mW) there was a detectable daunorubicin peak (10μg/ml) but on further investigation it was apparent that detection of concentrations less than 5μg/ml was not possible.

In view of the difficulties detecting aqueous solutions of daunorubicin with capillary electrophoresis, HPLC was considered as an alternative method. However, method development for CE was continued as this method was thought to be more practical in the long run, *i.e.* it was anticipated that HPLC would pose problems when trying to determine protein binding as a clean-up procedure would have to be employed to prevent column contamination, risking a disturbance in any potential equilibrium.

Further development of the technique of CE was attempted by using electrokinetic injection and micellar electrokinetic capillary chromatography (MECC), however, significant limitations still occurred, as described below:

1] It had been observed that, when diluted in the culture medium RPMI, the daunorubicin peak was considerably distorted. Although electrokinetic injection enabled increased sample concentration in the capillary leading to an increased ability to detect lower concentrations of daunorubicin, it did not overcome the problem of peak distortion.

2] The use of MECC enabled improved detection of daunorubicin in water and RPMI, however, this technique was unsuitable for the quantitation of protein binding as MECC requires the use of a detergent - the most common is sodium dodecyl sulphate (SDS). The analysis of a protein containing sample would be impossible without the detergent denaturing the proteins in the solution. This would effectively destroy any equilibrium between free drug and protein bound daunorubicin, making accurate quantitation impossible.

In view of all the individual problems encountered while attempting to optimise a CE technique, the technique of HPLC was explored as an alternative method of analysis. The mobile phase for HPLC analysis was an adaptation of the buffer used for CE analysis – after modification the resulting phase was acetonitrile (CH_3CN):100mM sodium dihydrogen orthophosphate (NaH_2PO_4) pH 4.2, (30:70, v/v). Detection of the drug solution was achieved using UV absorbance at λ 254nm. In comparison to CE, HPLC was found to have a greater ability in detecting lower concentrations (section 2.3.4, p87) and this ability was enhanced when using fluorescence detection rather than UV absorbance. This was important as it was expected that drug concentrations in the ng/ml range would be achieved

in cell culture environment and therefore such concentrations were used for stability determinations.

In consideration of the increased sensitivity of HPLC and taking into account the problems encountered while attempting to optimise a CE technique, it was decided that HPLC gave greater flexibility of analysis for the particular conditions required in this investigation and this technique was further validated (section 2.3.4, p87).

For the analysis of protein binding using HPLC, attempts were made at separating the free-drug fraction from the assumed protein-adsorbed drug fraction using, *e.g.* protein precipitation with ammonium chloride and solvent extraction. However, on further analysis with HPLC, daunorubicin was found to be unstable under the required conditions and it was thought more efficient to use an ultrafiltration method.

As the majority of anthracycline-protein binding has been reported to occur with large proteins such as albumin (Trynda-Lemiesz and Kozlowski, 1996) the filtration units used had a cut-off size of 20 000kD, *i.e.* lower than the majority of proteins found in the normal circulation and in cell culture systems. This not only resulted in a protein-free supernatant but the technique of filtration has also been reported to afford the least intrusive method of protein separation and thus minimises interference with any established protein-drug equilibria (Whitlam and Brown, 1981; Yanagisawa *et al.* 1998).

2.3.2 PREPARATION OF GENERAL SOLUTIONS AND MEDIA

2.3.2.1 General Solutions

Phosphate buffered Saline (PBS)

This was prepared by mixing PBS A (0.5M disodium hydrogen orthophosphate dihydrate) and B (0.5M sodium dihydrogen orthophosphate) in a ratio of 315:47ml to give a 0.5M phosphate buffer solution. 8.06g of sodium chloride (0.138M final concentration) was added to 20ml of this buffer, as was 0.2g of potassium chloride (0.0027M final concentration). The mixture was altered to a pH of 7.2 with 1M HCl and made up to 1 Litre with 18MΩ water. The resultant solution, *i.e.* PBS was stored at 4°C. Solutions A & B were made up with 18MΩ water in 500ml glass volumetric flasks as follows:

PBS A $Na_2HPO_4.2H_2O$ 44.5g/500ml

PBS B $NaH_2PO_4.2H_2O$ 39g/500ml

HPLC Mobile Phase

This was prepared by mixing acetonitrile (CH_3CN) with 100mM sodium dihydrogen orthophosphate solution (pH 4.2) at a ratio of 30:70, v/v.

100mM sodium dihydrogen orthophosphate was prepared by dissolving : 15.6g in 1L of 18MΩ water. The solution was adjusted to pH 4.2 with orthophosphoric acid (85% w/v) and the solution degassed for 5 min using a vacuum pump. The final solution was stored at 4°C until required.

<u>Daunorubicin Standard</u>

5-15mg of daunorubicin hydrochloride powder was weighed into a dry, pre-weighed, 25ml, silanised glass volumetric flask in a Class II cabinet (designated for cytotoxic handling) observing good cytotoxic handling procedures at all times. The volumetric flask was weighed again to determine the exact amount of daunorubicin hydrochloride powder transferred. The daunorubicin hydrochloride was made up to volume with 18MΩ water. This stock solution (*e.g.* 320µg/ml) was stored at 4°C (in the dark) and was found to be stable for 1 month. An example calculation of the final daunorubicin concentration is shown in Appendix A.

2.3.2.2 Cell Culture Media

<u>Dulbecco's Modified Eagles Medium (DMEM)</u>

This was purchased directly as an endotoxin tested, sterile-filtered solution, packaged in PETG plastic bottles buffered to pH7.2. During stability experimentation, it was used as a neat solution supplemented with 2mM L-glutamine (sections 2.4.1.3 & 2.6, p94 & p117).

<u>RPMI 1640</u>

This was purchased as an unbuffered and buffered with 25mM HEPES (pH 7.2), endotoxin tested, sterile-filtered solution, packaged in borosilicate glass bottles, and during the stability studies was used neat or supplemented with 2mM L-glutamine (sections 2.4.1.3, 2.4.1.4 & 2.6, p94, 95 & 117, respectively).

2.3.3   UV-VISIBLE ABSORBANCE INVESTIGATION

Future investigation required the comparison of solutions of daunorubicin dissolved in both water (standard solution) and any prospective cell culture growth medium. For this reason a spectral analysis was performed for daunorubicin in H_2O and daunorubicin in RPMI/Mobile phase to obtain the optimum detection wavelength for the daunorubicin during analysis using HPLC.

2.3.3.1 Daunorubicin Absorbance in Water (H_2O)

A disposable plastic cuvette, containing an aqueous daunorubicin solution at a concentration of 1µg/ml, was inserted into a Pye Unicam SP8-100 Series UV-VIS Spectrophotometer and the absorption spectra obtained over a wavelength range of 190 - 600 nm.

2.3.3.2 Daunorubicin Absorbance in RPMI/Mobile Phase

A disposable plastic cuvette, containing a daunorubicin solution in unbuffered RPMI/HPLC mobile phase, at a concentration of 1µg/ml, was inserted into a Pye Unicam SP8-100 Series UV-VIS Spectrophotometer and the absorption spectra obtained over a wavelength range of 190 – 600nm.

2.3.4   HPLC VALIDATION

HPLC validation involved the use of the following method: the HPLC system was equilibrated with mobile phase (section 2.3.2.1, p85) for 1hr. 400µl of daunorubicin sample

solution was loaded into a 100μl loop and injected onto the HPLC system, *i.e.* full loop volume. The sample was run at a flow rate of 1ml/min for 10-15min and daunorubicin was detected at 254nm at 0.001 AUFs (10-100ng/ml) or 0.01 AUFs (700-1300ng/ml) (Fig. 2.2, p75).

This method was validated in accordance with Bressolle *et al.* (1996) and Karnes *et al.* (1991) as described below. Validation included measurements into the specificity, selectivity, precision and accuracy of the method, construction of calibration plots (10 - 100ng/ml & 700-1300ng/ml), determination of limit of quantification (LOQ) and a daunorubicin stability analysis by forced degradation.

2.3.4.1 Specificity

For investigation into the specificity of the method, drug free samples of H_2O and unbuffered RPMI were injected onto the HPLC system. A sample of RPMI was then spiked with a known concentration of daunorubicin and analysed in a similar fashion. Selectivity was determined by forcibly degrading a sample of daunorubicin under varying conditions and injecting the resulting samples for HPLC analysis – stability of 5μg/ml daunorubicin in H_2O and in RPMI was determined using the method outlined in Appendix B (section 2.3.4.5, p90).

2.3.4.2 Calibration Plots of Daunorubicin in unbuffered RPMI incorporating 100ng/ml & 1μg/ml

Dilutions of a stock daunorubicin solution were made over a range of 10 -100ng/ml & 700 – 1300 ng/ml. Each dilution was made in a 10ml silanised, glass volumetric flask using

18MΩ water and stored at 4°C until required for assay (within 24h). 100μl of each solution from each concentration range was injected in triplicate for analysis by HPLC. Average peak area values were used to construct calibration curves, the equation of each line being calculated by linear regression analysis.

2.3.4.3 Limit of Quantification

Dilutions of a stock daunorubicin solution were made over a range of 10 -100 ng/ml. Each dilution was made in a 10ml silanised volumetric flask using 18MΩ water and kept at 4°C until required for analysis. 100μl of each solution was analysed in triplicate for analysis by HPLC.

2.3.4.4 Precision/Accuracy Determination

10ng/ml, 100ng/ml & 1000ng/ml solutions of daunorubicin were made in the culture medium RPMI (unbuffered) in silanised 20ml volumetric flasks and kept at 4°C until required for analysis.

A sample from each solution was removed and 100μl injected for HPLC analysis. This process was repeated seven times over the course of 3-4 hours & between days. The coefficient of variation (C.V.) for each concentration was calculated as outlined below and reported as percentage: -

$$\text{Coefficient of Variation} = \frac{\delta_{n-1}}{X^*} \times 100\%$$

Where, X^* = mean

and δ_{n-1} = standard deviation

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2.3.4.5 Stability Indication/Selectivity

Stability indication studies were performed on daunorubicin solutions ($5\mu g/ml$) in H_2O and in unbuffered RPMI using six silanised 10ml glass volumetric flasks. The stability indication studies were performed under varying conditions (as outlined in Appendix B and Fig. 2.10) to determine the ability of HPLC analysis to separate the analyte from degradation products.

2.3.5   EVAPORATION

As final cell culture experiments would involve the addition of an anthracycline drug solution into a liquid environment, it was postulated that evaporation from the cell culture plate during incubation could lead to erroneous drug concentration quantitation.

Evaporation at 37°C incubation was therefore quantified by recording the weights of duplicate 24-well culture plates, each filled with 24ml of an aqueous daunorubicin solution, over time. The percentage weight loss was then calculated.

2.4   EXPERIMENTAL METHODS

2.4.1   STABILITY OF DAUNORUBICIN UNDER CELL CULTURE CONDITIONS

An investigation into the stability of daunorubicin under different culture conditions was required. The investigation was conducted by varying experimental parameters.

2.4.1.1 Effect of Temperature & Concentration

2.4.1.1.1   Stability of Daunorubicin in H_2O and unbuffered RPMI at Different Temperatures

A comparison of the stability of daunorubicin dissolved in both H_2O (control) and RPMI (proposed culture condition) was made at different temperatures.

A stock solution of daunorubicin, *e.g.* 38.2μg/ml, was diluted in H_2O and RPMI to give a final concentration of 100ng/ml. The respective solutions were made in 25ml silanised glass volumetric flasks and subsequently transferred to 24-Well Culture plates (in triplicate with 2ml per well, *i.e.* 4 wells per plate).

The plates were incubated in the dark at three temperatures, *i.e.* 4, 25, and 37°C. Plates incubated at 4°C were incubated for 25.6h and sampled at 0, 2, 4, 6, 25.6 h for HPLC analysis. At 25°C, plates were incubated for 4 h and sampled at 0, 2 and 4h, while at 37°C, the plates were incubated for 2h and sampled regularly (under darkened conditions) every 20min for analysis using HPLC. All samples were placed on ice until analysis to prevent any further temperature-related degradation.

2.4.1.1.2   Stability of Daunorubicin in H_2O and unbuffered RPMI at Varying Concentrations

A comparison of the stability of daunorubicin dissolved in both H_2O (control) and unbuffered RPMI (proposed culture condition) was made at varying concentrations.

A stock solution of daunorubicin, *e.g.* 38.2µg/ml, was diluted in H_2O and RPMI to give final concentrations of 100, 500, & 1000ng/ml. The respective solutions were made in 25ml silanised glass volumetric flasks and subsequently transferred to 24-Well Culture plates (in triplicate with 2ml/well) and stored at 37°C in the dark. The plates were incubated for 2 hours and samples taken regularly every 20min (100ng/ml) and 30min (500 and 1000ng/ml), for analysis using HPLC. All samples were placed on ice (under darkened conditions) until analysis to stop any further temperature-related degradation.

2.4.1.2 Effect of pH

As it has been reported that the maximum stability for daunorubicin is achieved between a pH of approximately 4.5 – 7.5 (Poochikian *et al.* 1981), investigations were undertaken to examine the effect of differing the pH of the solution on the stability of the drug and determine the drug loss (if any) due to adsorption and degradation.

Preliminary results demonstrated that the pH of the culture medium RPMI (unbuffered) in a 5% CO_2 incubator could change substantially in a short period of time. Subsequently the proposed culture medium (RPMI 1640) was buffered to a pH of 6, 7, and 7.5 (keeping the ionic strength of each solution constant) and these were compared to an unbuffered solution of RPMI (pH 7 at start of incubation and pH 5.4 at end of incubation).

A stock solution of daunorubicin, *e.g.* 38.2µg/ml, was diluted to a concentration of 100ng/ml in the different buffered and unbuffered solutions in 25ml silanised volumetric flasks. These solutions were transferred to 24-Well Culture plates (in triplicate with 2ml/well) and incubated at 37°C for 0.5 hours in the dark. Incubation for each solution was staggered by 45min to enable immediate HPLC analysis for each pH after sampling at 0.5

hours. All samples were placed on ice (under darkened conditions) to stop any further temperature-related degradation.

In addition to investigating the effect of differing pHs on drug stability, this experiment was designed to distinguish between drug loss due to degradation and adsorption. As a result, the design of this experiment was slightly more complex (see below) than previous analyses and, as prior investigation into the stability of 100ng/ml of daunorubicin in unbuffered RPMI at $37^{\circ}C$ showed drug loss to occur within the first 0.5h of incubation (Fig. 2.13, p109), this short period of incubation was thought to provide a sufficiently informative and convenient time frame for further study. Therefore, an incubation period of 0.5h was used for continued stability analysis.

To distinguish between drug loss due to degradation or adsorption, drug adsorbed to the culture plate was isolated by extracting bound drug from the side of the culture well using an organic solvent, *i.e.* by the gentle pipetting of 2ml of methanol along the side of the well (in darkened conditions) before quantification by HPLC analysis. This quantified amount was incorporated into the described results by adding it to the daunorubicin concentration remaining after incubation at $37^{\circ}C$ for 0.5h, *i.e.* compensating for adsorbed drug. The resulting value was termed '0.5 hours no adsorption' (Table 2.3, p112).

Degradation was assumed to be occurring if, after compensating for adsorbed drug, the resulting daunorubicin concentration was different to the value at T_0 (Table 2.3). The difference between the value for T_0 and the value for '0.5 hours no adsorption' was calculated and described as 'Amnt lost to assumed degradation' (section 2.5.2.2, p112).

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2.4.1.3 Effect of Growth Media

To optimise the cell culture system further, and considering that sodium bicarbonate is a weak buffer compared to HEPES, an investigation was made between HEPES buffered RPMI 1640, HEPES buffered DMEM and PBS (all at pH 7.2) supplemented with 2mM L-glutamine, to test which medium afforded the greatest drug stability. The control solution was daunorubicin in H_2O.

A stock concentration of daunorubicin, *e.g.* $38.2\mu g/ml$, was diluted in the different media (in 25ml silanised volumetric flasks) to give a final concentration of 100ng/ml. The respective solutions were subsequently transferred to 24-Well Culture plates (in triplicate with 2ml/well) and incubated at 37°C. Incubation for each media type was staggered by 45min to enable immediate analysis for each culture medium after sampling at 0.5 hours. All samples were placed on ice to stop any further temperature-related degradation.

Drug adsorbed to the culture plate was isolated by extracting bound drug from the side of the culture well using an organic solvent, *i.e.* gentle pipetting of 2ml of methanol along the side of the well before quantifying by HPLC analysis. This quantified amount was incorporated into the described results by adding it to the daunorubicin concentration remaining after incubation at 37°C for 0.5h, *i.e.* compensating for adsorbed drug. The resulting value was termed '0.5 hours no adsorption' (Table 2.4, p114).

Degradation was assumed to be occurring if, after compensating for adsorbed drug, the resulting daunorubicin concentration was different to the value at T_0 (Table 2.4, p114). The difference between the value for T_0 and the value for '0.5 hours no adsorption' was calculated and described as 'Amnt lost to assumed degradation' (section 2.5.2.3, p113).

2.4.1.4 Effect of added Protein

It was hypothesised that there would be some protein binding of daunorubicin to any FBS in the RPMI culture medium. As the degree of binding was unknown and the experimental method only produced small sample volumes, a higher concentration of daunorubicin – 1μg/ml – was used during this experimentation thus increasing the chances of detection and quantification even in the event of high protein binding.

The samples were split into two groups. The control group consisted of three HEPES-RPMI solutions + 2mM L-glutamine devoid of FBS being spiked with 200μl of a 10μg/ml stock solution of daunorubicin to a final concentration of 1μg/ml (final volume 2ml). The experimental group consisted of three RPMI solutions + 2mM L-glutamine containing 10% (v/v) FBS being spiked with 200μl of a 10μg/ml stock solution of daunorubicin to a final concentration of 1μg/ml (final volume 2ml).

These solutions were incubated for 15min, at 37°C in 3 individual 5ml polypropylene tubes (in the dark) before each being transferred to 3 individual C1 filtration units (section 2.3.1, p81) and centrifuged at 540g for 15min, leaving a protein-free supernatant.

The filtration units were not made of polypropylene and, by using methanol to extract any adsorbed daunorubicin, it was found that daunorubicin adsorbed to the inside surfaces as well as the filtration membrane thus increasing the possibilities of an erroneous result. To overcome this factor the process of spiking and centrifuging the RPMI solutions was repeated 5 times to saturate the filtration units. This required that each 5ml polypropylene tube have a corresponding filtration unit. When the spiked RPMI solution had been incubated for the allotted time in the 5ml polypropylene tube, it was transferred to its

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corresponding C1 filtration unit, which was then centrifuged. The same polypropylene tube was filled again with 2ml of RPMI then spiked and incubated. At the end of this incubation period the solution was transferred to the same, previously allotted C1 filtration unit. This procedure was repeated 5 times with all six 5ml polypropylene tubes *i.e.* 3 control and 3 experimental, until saturation of each C1 filtration unit. The supernatant obtained after the final centrifugation of each C1 filtration unit was analysed by HPLC.

The above was repeated at the following incubation times, 20, 30 and 60min. The difference between the control and experimental samples at each time point was calculated in triplicate and expressed as a percentage of the control, and the average value plotted for each time point.

2.4.2 STATISTICAL ANALYSIS

All statistics presented in this chapter were calculated using ANOVA with a significance level ($P < 0.05$).

2.5   RESULTS

2.5.1   METHOD DEVELOPMENT RESULTS

2.5.1.1 Absorbance

The absorption spectrum for daunorubicin in mobile phase was taken to optimise the detection wavelength for HPLC analysis. The spectrum showed maxima occurring at 254nm, 290nm, & 480nm. Figure 2.4 (spectrum for daunorubicin in mobile phase) depicts the profile found.

Fig. 2.4 Absorption spectrum for daunorubicin in HPLC mobile phase.
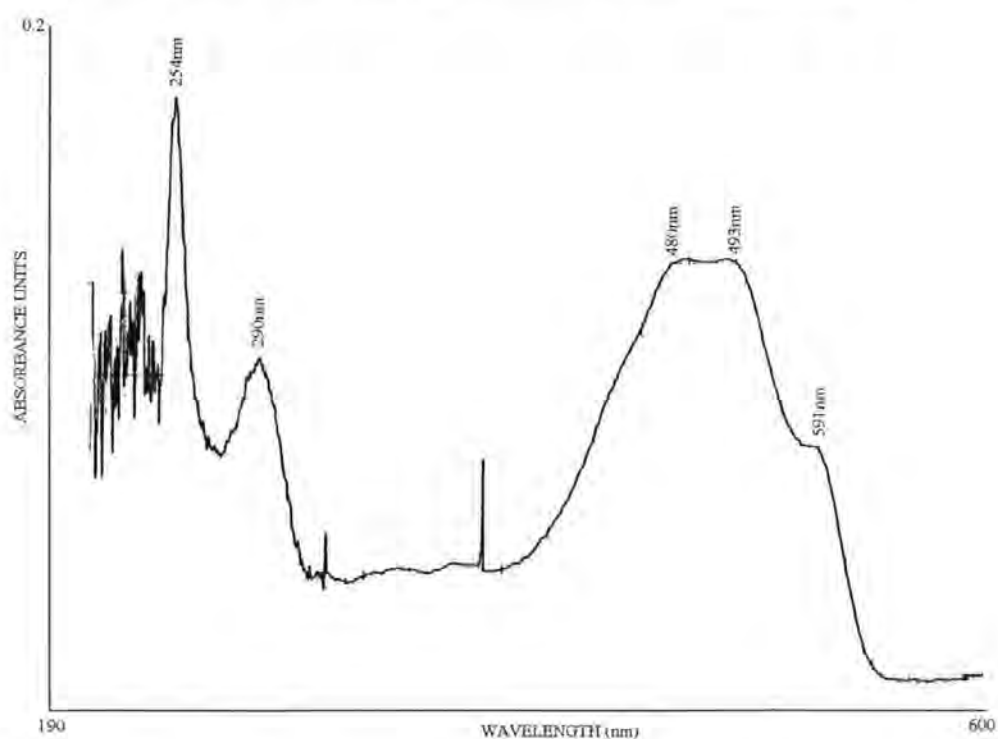
Fig. 2.4 10μl of a 100μg/ml solution of daunorubicin in unbuffered RPMI was diluted to 1μg/ml in 990μl of HPLC mobile phase and its absorbance spectrum analysed between 190nm – 600nm and the absorbance maxima noted.

The peak at 254nm gave the highest absorbance reading and subsequently this wavelength was used for HPLC analysis.

2.5.1.2 HPLC Validation

2.5.1.2.1   Specificity/Selectivity

The chromatograms comparing the blank H_2O & medium (Fig. 2.5 & Fig. 2.6 respectively) with that of medium spiked with daunorubicin (Fig. 2.7) demonstrate the specificity of the system. The method unequivocally produces a response for this single analyte in the presence of endogenous compounds such as are found in RPMI culture medium.

Fig. 2.5 Injection of Blank H_2O
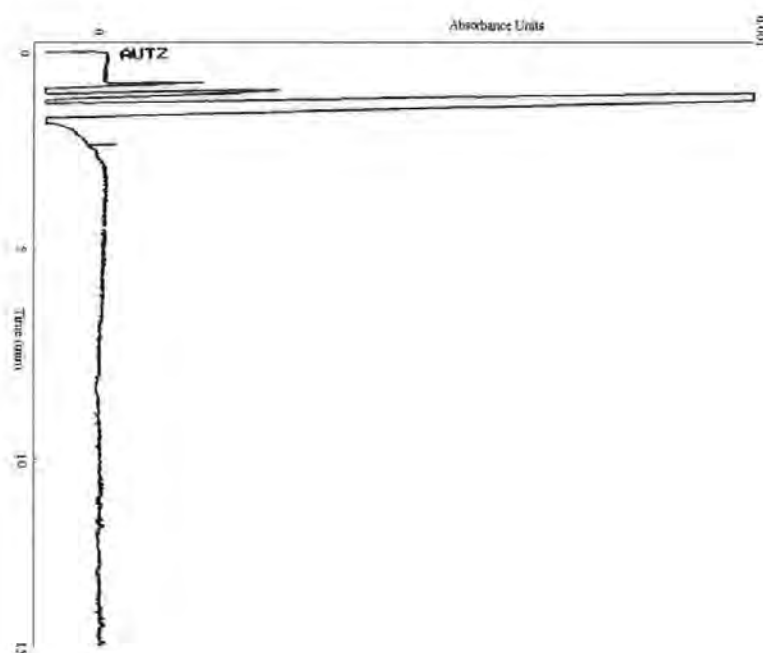
Fig. 2.5 100µl of H_2O was injected (full loop volume) onto the HPLC system and analysed for 15min at a flow rate of 1ml/min. Detector sensitivity was set at 0.001 absorbance units full deflection. Detection occurred at 254nm. A peak at 7.25min was not detected.

Fig. 2.6 Injection of Blank RPMI
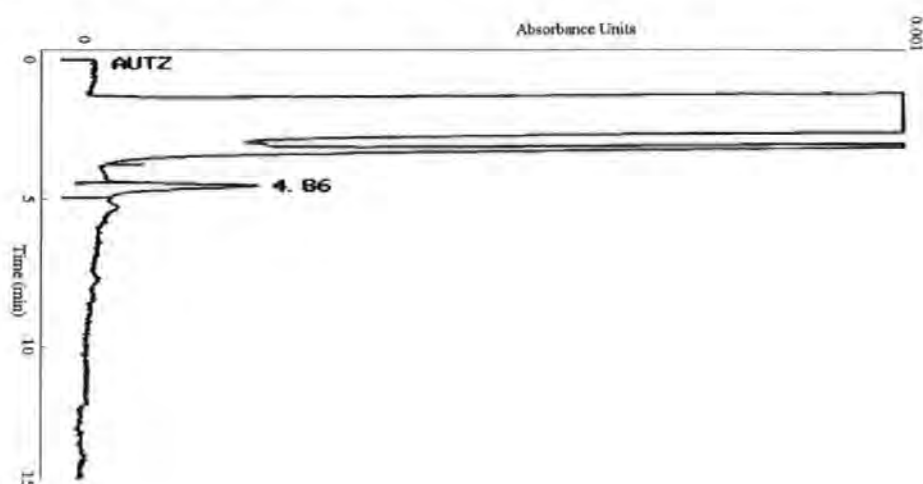
Fig. 2.6 100µl of unbuffered RPMI was injected (full loop volume) onto the HPLC system and analysed for 15min at a flow rate of 1ml/min. Detector sensitivity was set at 0.001 absorbance units full deflection. Detection occurred at 254nm. A peak at 7.25min was not detected.

Fig. 2.7 Injection of RPMI + daunorubicin
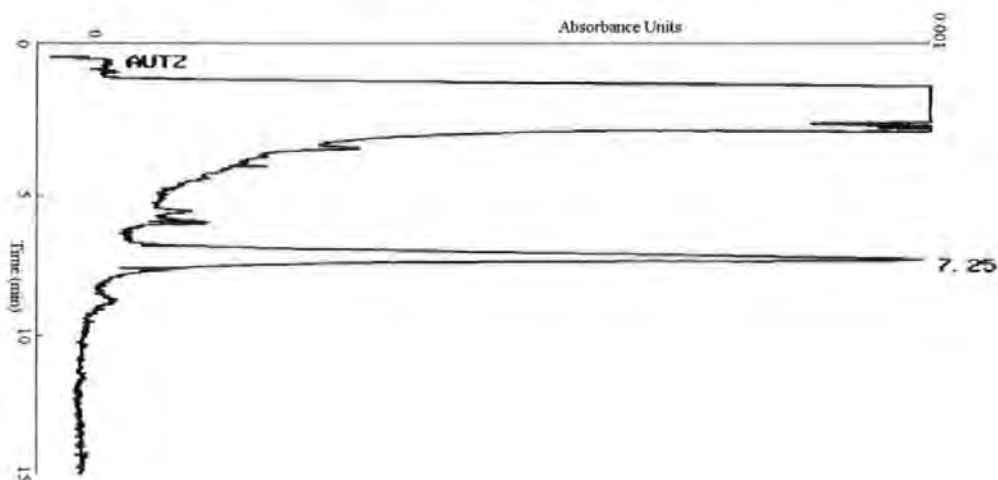
Fig. 2.7 100µl of an unbuffered sample of RPMI containing 100ng/ml daunorubicin was injected (full loop volume) onto the HPLC system and analysed for 15min at a flow rate of 1ml/min. Detector sensitivity was set at 0.001 absorbance units full deflection. Detection occurred at 254nm. A peak was detected at a retention time of 7.25min.

The selectivity of the system is shown under the section 2.5.1.2.5 (p103).

2.5.1.2.2   Calibration Plot

Calibration plots prepared using spiked unbuffered RPMI were linear over the range of: (a) 10 - 100ng/ml, with a regression equation of $y = 4533.2x + 5926.5$, $r^2 = 0.99$, $n = 6$, and, (b) 700 – 1300ng/ml, with a regression equation of $y = 454.18x - 12266$, $r^2 = 0.998$, $n = 7$ (Figs. 2.8 & 2.9).

Fig. 2.8 Calibration plot for 10 – 100ng/ml daunorubicin in unbuffered RPMI
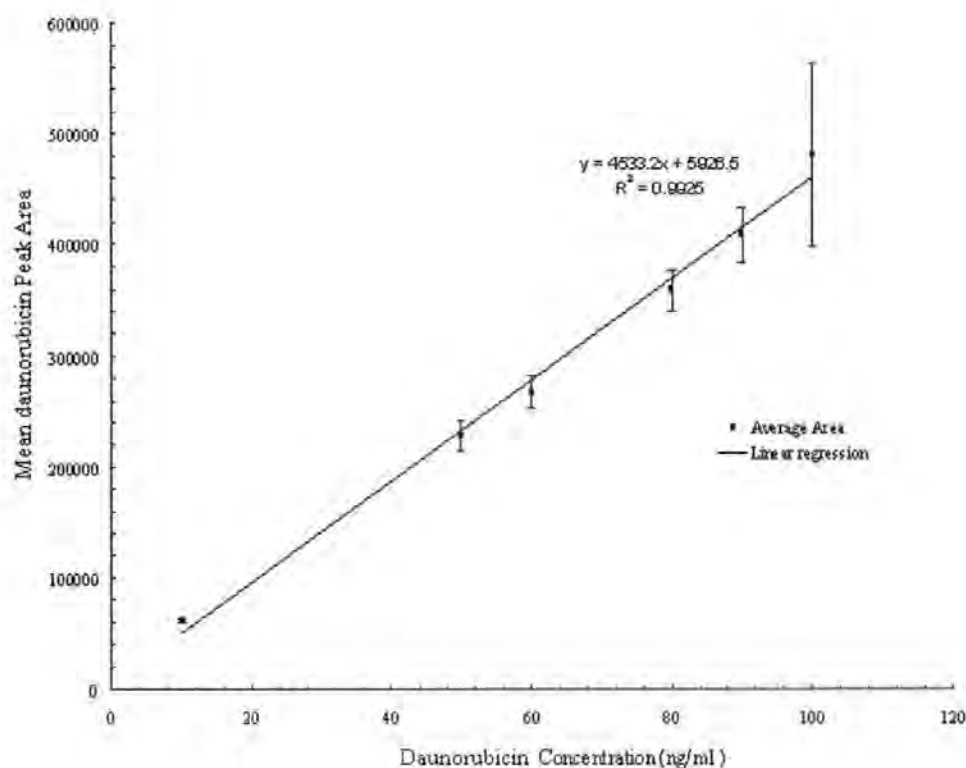
Fig. 2.8 Dilutions of daunorubicin were made in unbuffered RPMI over a range of 10-100ng/ml in silanised, glass volumetric flasks. Each dilution was injected in triplicate for analysis by HPLC and the average peak area values were used to construct a calibration curve. Regression analysis gave a linear equation of $y = 4533.2x + 5926.5$, $r^2 = 0.99$.

Fig. 2.9 Calibration plot for 700 - 1300ng/ml daunorubicin in RPMI
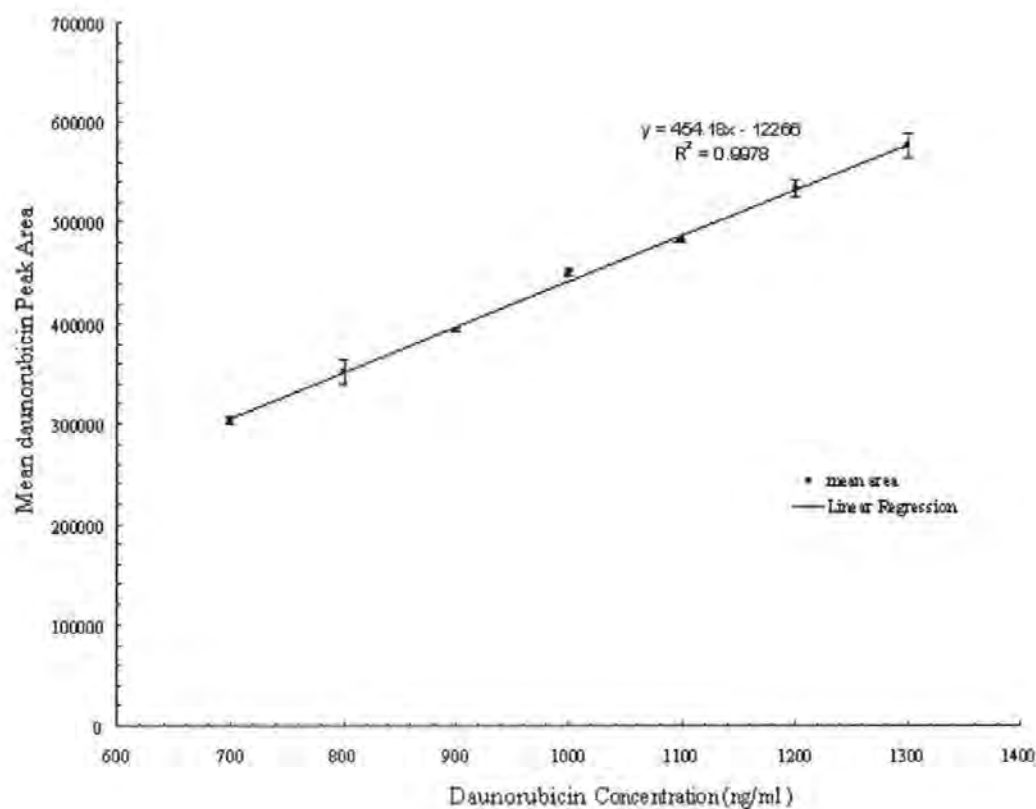
Fig. 2.9 Dilutions of daunorubicin were made in unbuffered RPMI over a range of 700-1300ng/ml in silanised, glass volumetric flasks. Each dilution was injected in triplicate for analysis by HPLC and the average peak area values were used to construct a calibration curve. Regression analysis gave a linear equation of $y = 454.18x - 12266$, $r^2 = 0.998$.

The concentration of daunorubicin in RPMI was calculated using the linear regression equation appropriate for the daunorubicin concentration. Table 2.1 (p103) shows the calibration data: the theoretical concentrations of daunorubicin in spiked RPMI and the corresponding observed concentrations determined from the calibration plot.

2.5.1.2.3 Limit of Quantification

Karnes *et al.* (1991) suggest that the LOQ is defined as the,

"..concentration above which quantitation is possible within a certain pre-set level of certainty."

Karnes *et al.*, further conclude that the value cannot be accurately calculated but is estimated to be; $LOQ = K_{S_b}/S$, where K is a factor indicating the desired precision at the lower limit, s_b represents the standard deviation of the blank measurement, and S is defined as the slope of the calibration curve.

Practical validation can be accomplished by defining the LOQ as the concentration of the lowest standard and demonstrating an acceptable standard error at lowest concentration. The lowest standard able to be accurately quantitated and distinguished in the RPMI culture medium was found to be 10ng/ml (Table 2.1, p103).

2.5.1.2.4 Precision/Accuracy Determination

Accuracy and precision of daunorubicin in unbuffered RPMI at 10, 100 & 1000ng/ml are presented in Table 2.1:

C^a (ng/ml)	Mean daunorubicin Peak Area	C^b (ng/ml)	Daunorubicin Assay % Of Theoretical	Within-Run Precision C.V. (%)	Between-Run Precision C.V. (%)
Calibration Standards					
10	61748.5	12.3	123.0	12.7 (N=6)	0.4 (N=2)
50	227756.2	48.9	97.8		
60	266706.4	57.6	96.0		
80	358634.0	77.8	97.2		
90	408426.2	88.8	98.7		
100	480247.4	104.6	104.6	3.0 (N=7)	4.3 (N=7)
700	303867.5	696.1	99.4		
800	352087.5	802.2	100.3		
900	393836.0	894.1	99.3		
1000	450678.5	1019.3	101.9	1.8 (N=7)	0.9 (N=3)
1100	481612.0	1087.4	98.9		
1200	534310.5	1203.4	100.3		
1300	577031.5	1297.5	99.8		

Table 2.1 Accuracy and precision of the HPLC method [a] Theoretical concentrations of standard solutions of daunorubicin in unbuffered RPMI. [b] Mean concentration of daunorubicin calculated for standards in RPMI calculated from the linear regression equations: $y = 4533.2x + 5926.5$ (10 -110ng/ml) and $y = 454.18x - 12266$ (700 – 1300ng/ml).

2.5.1.2.5   Stability Indication/Selectivity

A stability indication study was performed on 5μg/ml daunorubicin to determine the presence of potential degradation products by forced degradation under various conditions (Appendix B). Resulting chromatograms are shown in Fig. 2.10 (A-F).

Stability indication investigations on daunorubicin at elevated temperature revealed a complete loss of drug under the basic and acidic conditions studied as well as complete loss when subjected to elevated temperature in the culture medium RPMI. Incubation with water gave a 13% loss while incubation with H_2O_2 gave a 78.2% loss. The HCl and H_2O_2 - degraded solutions revealed the presence of degradation products at retention times of 5.69, 8.03 min and 6.23, 5.65, 5.39 min, respectively. The NaOH, RPMI & H_2O solutions did not reveal the presence of any degradation products. This assay was considered to be stability indicating.

Fig. 2.10 Stability indicating chromatograms for 5µg/ml daunorubicin exposed to various conditions

[a]

[b]

[c]

[d]

[e]

[f]
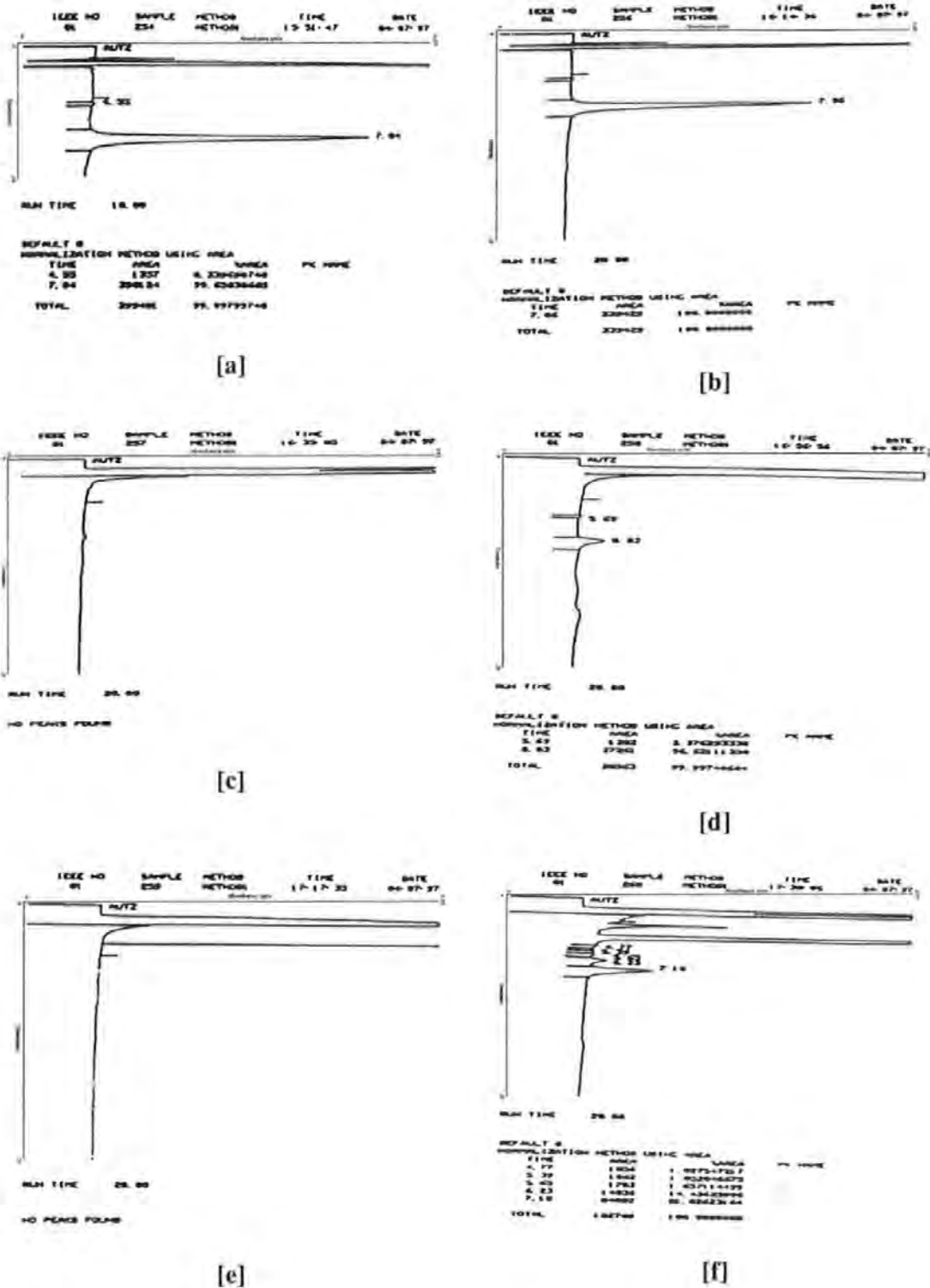
Fig 2.10 [a] control [b] boiling control [c] boiling in RPMI [d] boiling in HCl [e] boiling in NaOH [f] boiling in H_2O_2.

2.5.1.3 Evaporation Effects

It was thought that at 37°C some evaporation might occur from the culture plates creating problems for the quantitation of drug concentration in future studies. Substantial evaporation could mean an increase in the concentration of the drug solution leading to erroneous quantitation. On incubation of a daunorubicin solution at 37°C in a 24-well culture plate some evaporation did occur over a 24 hour period, however, the results showed that at 37°C, there was a less than 0.5% drop in weight in the first 9 hours of incubation (Table 2.2).

Time (h)	% Weight loss 37°C
0	0
6	0.3
9	0.4
15	1.7
18	2.0
24	2.7

Table 2.2 Percent (%) weight loss of 24-well culture plates due to evaporation.

Although drug exposure times have varied in the literature, it was thought that 9 hours would be ample time for future *in vitro* experiments. As such, the small decrease in weight relating to this time period indicated that evaporation would not be a major factor for consideration in drug quantitation.

2.5.2 EXPERIMENTAL RESULTS

The effect of different parameters on Daunorubicin stability was investigated and recorded.

2.5.2.1 Effect of Temperature and Concentration

2.5.2.1.1 Effect of different temperatures on stability of daunorubicin in water (H₂O) and unbuffered RPMI

It was speculated that at lower drug concentrations (some of which would be applicable to future work) any effects on drug stability would be more noticeable and, therefore, the majority of the results presented use daunorubicin at a concentration of 100ng/ml.

At 4, 25, and 37°C (Figs. 2.11 – 2.13) the daunorubicin concentration fell substantially within a short period of time (a few hours) when dissolved in both H₂O and unbuffered RPMI. Over a period of 2h daunorubicin loss was 45.3%, 57.0% and 49.8%, when incubated at 4°C, 25°C and 37°C, respectively in unbuffered RPMI.

Fig. 2.11 Stability of 100ng/ml daunorubicin in water and unbuffered RPMI at 4°C.
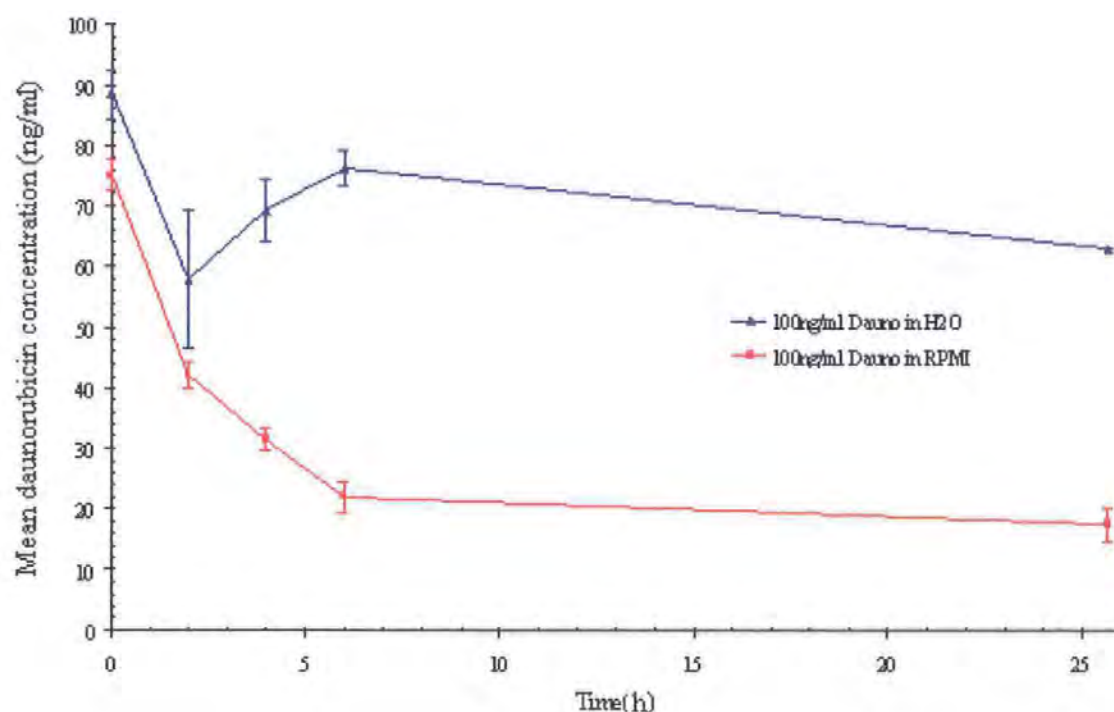
Fig. 2.11 2ml solutions of 100ng/ml daunorubicin in water or unbuffered RPMI were incubated (in triplicate) in a 24-well culture plate at 4°C for 25.6h and sampled at 0, 2, 4, 6, & 25.6h for HPLC analysis. In both water and RPMI the concentration of daunorubicin decreased over time. Drug loss was significantly greater in RPMI when analysed by ANOVA ($P < 0.05$). In each case $N = 3$, where N = the number of separate experiments conducted.
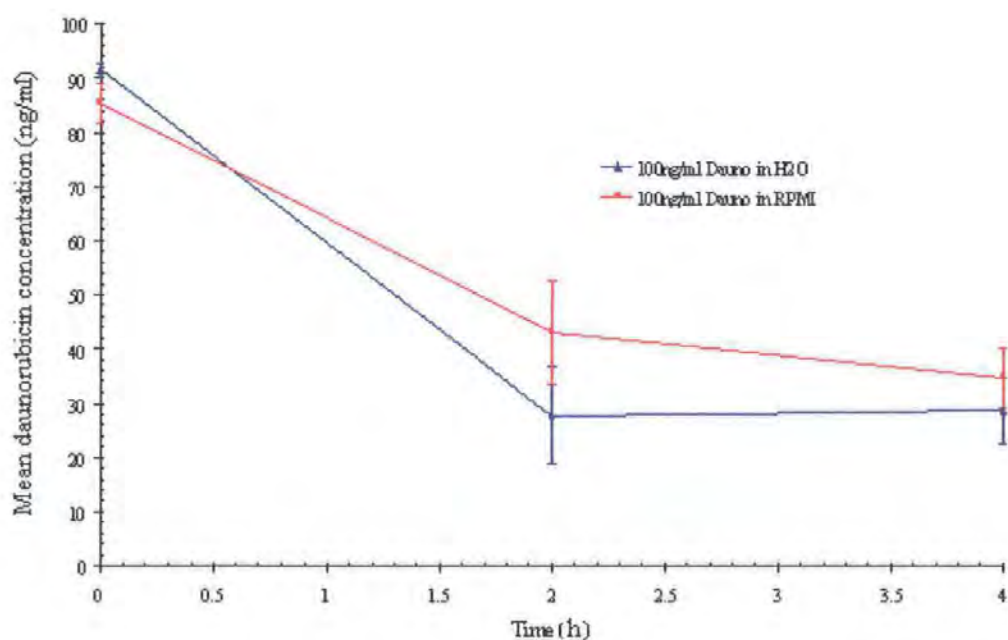
Fig. 2.12 2ml solutions of 100ng/ml daunorubicin in water or unbuffered RPMI were incubated (in triplicate) in a 24-well culture plate at 25°C for 4h and sampled at 0, 2, & 4h for HPLC analysis. In both water and RPMI the concentration of daunorubicin decreased over time. Drug loss was not significantly different between the two diluents when analysed by ANOVA ($P > 0.05$). In each case $N = 3$, where $N =$ the number of separate experiments conducted.

Fig. 2.13 Stability of 100ng/ml daunorubicin in water and unbuffered RPMI at 37°C.

Fig. 2.13 2ml solutions of 100ng/ml daunorubicin in water or unbuffered RPMI were incubated (in triplicate) in a 24-well culture plate at 37°C for 2h and sampled every 20min for HPLC analysis. In both water and RPMI the concentration of daunorubicin decreased over time. Drug loss was significantly greater in RPMI when analysed by ANOVA (P < 0.05). In each case N = 3, where N = the number of separate experiments conducted.

2.5.2.1.2   Effect of different concentrations of daunorubicin in water (H$_2$O) and unbuffered RPMI

At 37°C, when Daunorubicin was dissolved in either H$_2$O (Fig. 2.14) or unbuffered RPMI (Fig. 2.15), the stability of the drug was always 100<500<1000ng/ml, in order of increasing stability.
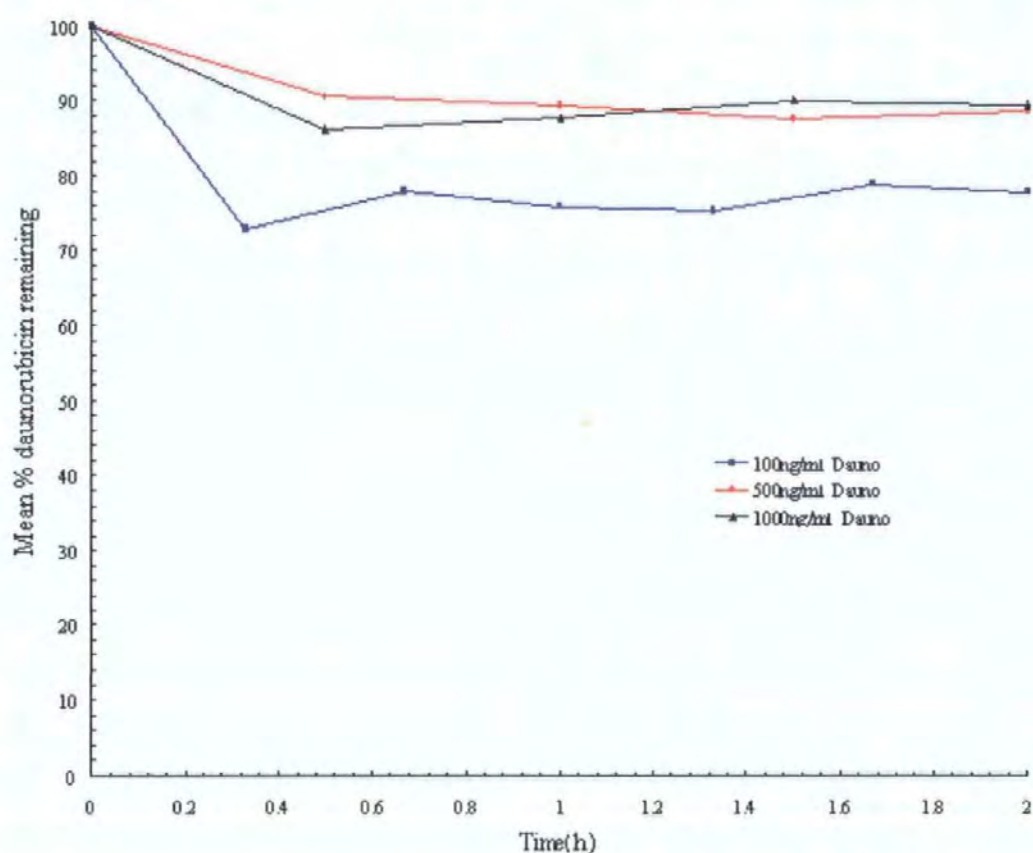
Fig. 2.14 2ml solutions of daunorubicin (100, 500, & 1000ng/ml) in water were incubated (in triplicate) in a 24-well culture plate at $37^{\circ}C$ for 2h. Samples were taken at regular intervals for HPLC analysis. In all three cases, daunorubicin concentration decreased over time. Drug loss at a concentration of 100ng/ml was significantly greater compared to 500ng/ml or 1000ng/ml when analysed by ANOVA ($P < 0.05$). In each case $N = 3$, where $N =$ the number of separate experiments conducted.
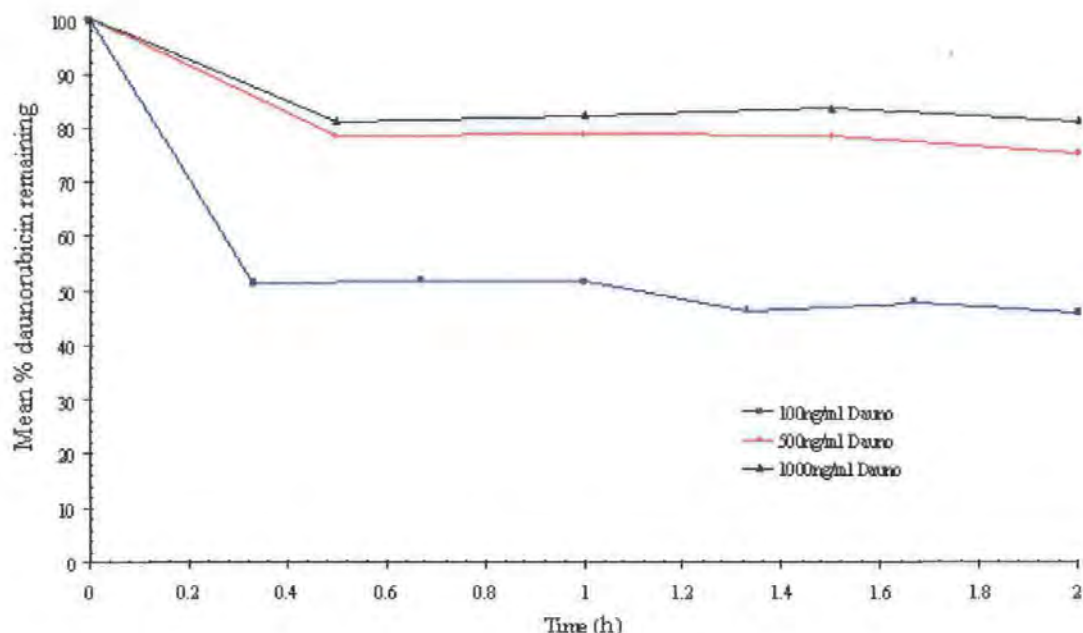
Fig. 2.15 2ml solutions of daunorubicin (100, 500, & 1000ng/ml) in unbuffered RPMI were incubated (in triplicate) in a 24-well culture plate at 37°C for 2h. Samples were taken at regular intervals for HPLC analysis. In all three cases, daunorubicin concentration decreased over time. Drug loss at a concentration of 100ng/ml was significantly greater compared to 500ng/ml or 1000ng/ml when analysed by ANOVA (P < 0.05). In each case N = 3, where N = the number of separate experiments conducted.

As future experiments were to be conducted at 37°C the loss of drug at this temperature became the main focus of the subsequent stability studies where an attempt was made to differentiate between adsorption and degradation during the experimentation.

2.5.2.2 Effect of pH

A comparison was made of the stability of 100ng/ml of daunorubicin in RPMI buffered to different pH values (Table 2.3 and Fig.2.16).

Sample	Absolute Values of daunorubicin (ng/ml) +/- Standard Error						
	Standard	0 hour (T_0)s	0.5 hours no adsorption	0.5 hours adsorption	Amnt lost instantly	Amnt lost to adsorption	Amnt lost to assumed degradation
Unbuffered RPMI	100	79.0 +/- 0.7	72.8 +/- 2.1	56.4 +/- 2.1	21.0 +/- 1.3	16.4 +/- 2.4	6.2 +/- 2.1
RPMI pH 6	100	69.6 +/- 1.7	73.4 +/- 1.4	61.5 +/- 1.6	30.4 +/- 1.4	11.9 +/- 1.3	0
RPMI pH 7	100	65.6 +/- 2.6	19.0 +/- 1.9	17.4 +/- 1.5	34.4 +/- 2.1	1.7 +/- 0.1	46.6 +/- 1.3
RPMI pH 7.5	100	57.1 +/- 2.6	8.5 +/- 1.7	6.6 +/- 0.6	42.9 +/- 1.9	1.9 +/- 0.7	48.6 +/- 2.0

Table 2.3 Stability of 100ng/ml daunorubicin in unbuffered RPMI and in RPMI buffered to pH 6, 7 and 7.5.

Fig. 2.16 Stability of 100ng/ml daunorubicin in unbuffered RPMI and in RPMI buffered to pH 6, 7 and 7.5 at 37°C.
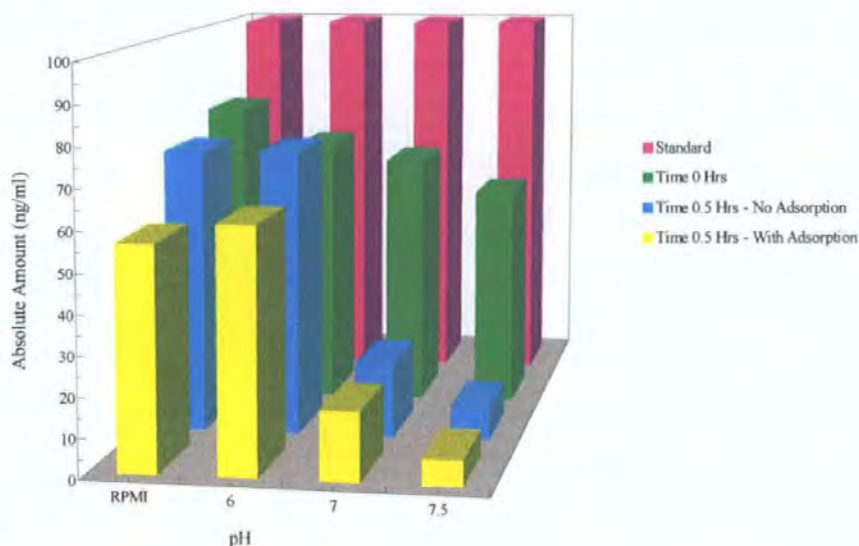
Fig. 2.16 2ml solutions of 100ng/ml daunorubicin in unbuffered and buffered RPMI (pH 6, 7, & 7.5) were incubated (in triplicate) in a 24-well culture plate at $37^{\circ}C$ for 0.5h. After 0.5h, drug loss was observed when compared to T_0. Significantly greater drug loss occurred at pH 7 & 7.5 when analysed by ANOVA ($P < 0.05$).

Greatest stability was observed at a pH of 6.0 where decreased concentration was due to an instant drug loss (see later discussion) and adsorption (11.9ng/ml). At pH 7 and 7.5, however, drug loss was mainly due to degradation (46.6ng/ml and 48.6ng/ml, respectively).

2.5.2.3 Growth Media

A comparison was made of the stability of 100ng/ml of daunorubicin in different culture media (Table 2.4 and Fig.2.17).

Sample	Absolute Values of daunorubicin (ng/ml) +/- Standard Error						
	Standard	0 hours (T$_0$)	0.5 hours no adsorption	0.5 hours adsorption	Amnt lost instantly	Amnt lost to adsorption	Amnt lost to assumed degradation
H$_2$O	100	100.4 +/- 2.1	89.4 +/- 1.8	82.5 +/- 1.7	0.6 +/- 1.0	7.0 +/- 2.2	10.9 +/- 1.7
Buffered PBS + 2mM L- glutamine	100	77.8 +/- 2.8	75.2 +/- 1.3	61.3 +/- 0.5	22.2 +/- 1.2	13.9 +/- 1.2	2.6 +/- 0.8
Buffered RPMI + 2mM L-glutamine	100	79.0 +/- 0.4	72.8 +/- 1.9	56.4 +/- 1.1	21.0 +/- 0.9	16.4 +/- 0.6	6.2 +/- 1.2
Buffered DMEM + 2mM L- glutamine	100	67.6 +/- 5.8	62.8 +/- 2.1	49.5 +/- 11.0	32.4 +/- 1.3	13.3 +/- 0.9	4.8 +/- 1.4

Table 2.4 Stability of 100ng/ml daunorubicin in water, PBS, HEPES-DMEM and HEPES-RPMI buffered to pH 7.2, supplemented with 2mM L-glutamine

Fig. 2.17 Stability of 100ng/ml daunorubicin in water, PBS, HEPES-DMEM and HEPES-RPMI buffered to pH 7.2, supplemented with 2mM L-glutamine.
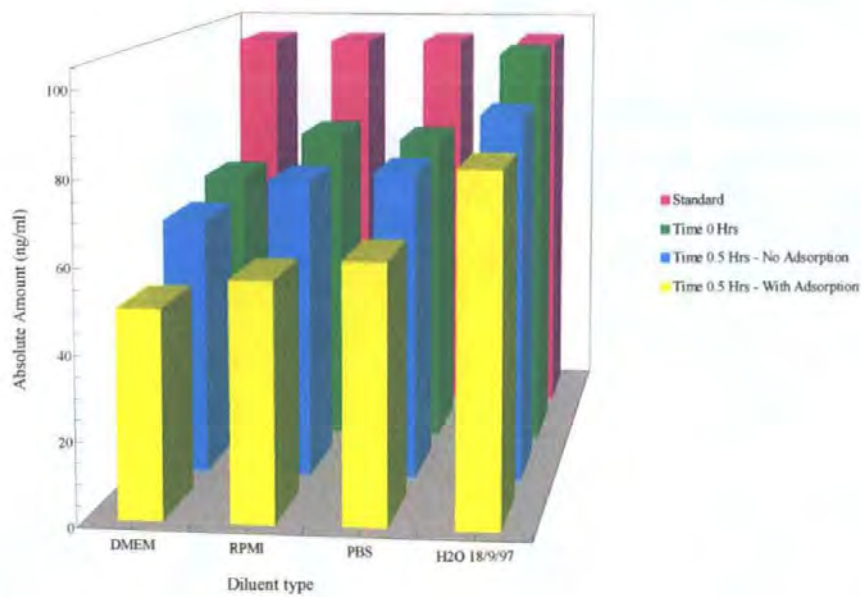
Fig. 2.17 2ml solutions of 100ng/ml daunorubicin in H$_2$O and buffered PBS, RPMI, & DMEM (pH 7.2) were incubated (in triplicate) in a 24-well culture plate at 37°C for 0.5h. After 0.5h, drug loss was observed when compared to T$_0$. Greatest drug loss occurred with DMEM > RPMI > PBS > H$_2$O.

Drug loss between the different buffered diluent types was 17.5ng/ml, 38.7ng/ml, 43.6ng/ml and 50.5ng/ml after 0.5h incubation for H_2O, PBS, RPMI and DMEM, respectively. In PBS, RPMI and DMEM there was an instantaneous drug loss with further loss occurring upon incubation. Loss during incubation occurred by both degradation and adsorption to the culture plate.

2.5.2.4 Protein Binding

The extent of protein binding on a 1μg/ml sample of daunorubicin in HEPES-RPMI + 2mM L-glutamine + 10% (v/v) FBS was analysed over time and the data is presented in Fig. 2.18.

Protein binding after 60min incubation led to a 16.9% loss in free drug.

Fig. 2.18 Protein binding at 37°C for a 1µg/ml solution of daunorubicin in buffered RPMI
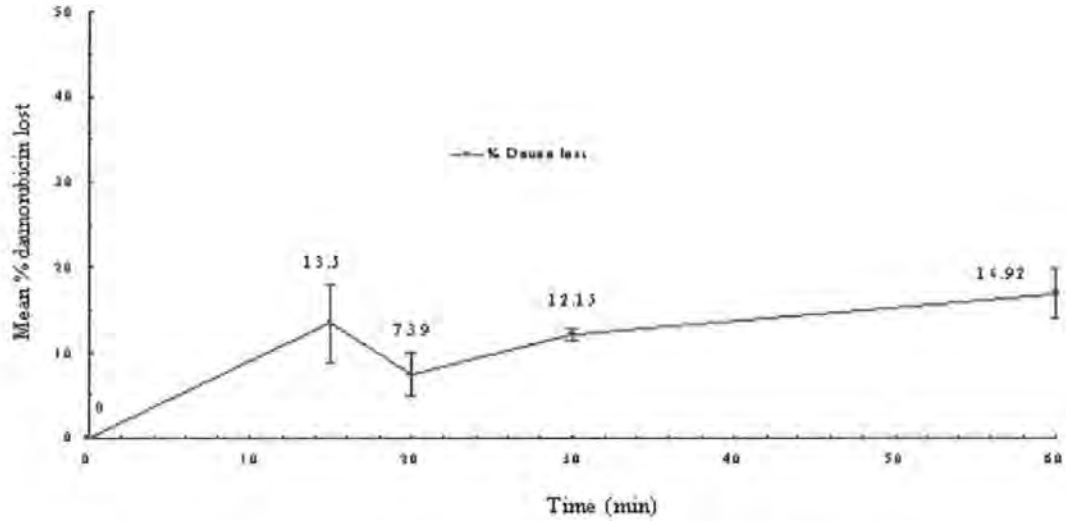
Fig. 2.18 A 2ml solution of 1µg/ml daunorubicin in buffered RPMI + 10% FBS was incubated (in triplicate) in a 15ml polypropylene tube at 37°C for 15min. A control solution (devoid of FBS) was incubated simultaneously to the experimental solution containing FBS (also in triplicate). At the end of the 15min incubation the samples were centrifuged in filtration units to separate free drug from the protein-bound fraction. The amount of free drug for control and experimental samples was determined by HPLC analysis. The difference between the control and experimental samples was calculated and expressed as a percentage of the control. The analysis was repeated for incubation times of 20, 30 and 60min and the average values plotted for each time point.

2.6 DISCUSSION

As it was known at the start of this investigation that study of anthracycline resistance in B-CLL would involve cell culture work, the initial aim was to set up a robust and stable cell culture system with which to examine this resistance. For this reason the development of a rapid, reliable, and robust analytical system was a priority to facilitate studies on the effects of different temperatures, concentrations, pH and media on drug stability, as well as the effects of protein addition. This information would then be used to design the final cell culture system for P-gp analysis in patient B-lymphocytes.

The final, validated, HPLC analytical method (section 2.5.1.2, p98) was used to investigate the effect of differing temperatures on 100ng/ml daunorubicin in H_2O and RPMI (the culture medium most commonly used in *in vitro* experiments involving B-CLL patient cells). In RPMI, the seemingly greater drug loss at 25°C was attributed to pH effects *i.e.* the drug loss after incubation at 37°C was less than expected because of a favourable pH change to more acidic conditions due to the 5% CO_2 environment in the incubator (Figs. 2.11, 2.12 & 2.13, pages 107, 108 & 109). Continued investigation examined drug stability at 100, 500 and 1000ng/ml and reaffirmed that the effects on drug stability are exacerbated at lower drug concentrations, *i.e.* a loss in drug will be more significant at low drug concentrations than at high concentrations (Figs. 2.14, p110 & 2.15, p111). In both cases, *i.e.* stability investigation at varying temperatures and drug concentrations, drug loss appeared to be greater when the drug was dissolved in RPMI, suggesting an added interaction which decreased stability - see later discussion.

Daunorubicin is a photoreactive compound (Wood *et al.* 1990a). Photolysis of this anthracycline has been postulated to result in the formation of aglycones leading to an

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overall decrease in parent drug concentration (Gray & Phillips, 1981). Therefore it was suspected that photodegradation may be partially responsible for the observed drug loss, but as all experiments were conducted in darkened conditions (as many lights as possible were turned off in the laboratory and drug solutions were wrapped in aluminium foil at all times), loss through photodegradation would have been kept to a minimum. The observed drug loss, therefore, suggested either non-photolytic degradation of the drug - especially at 37°C where approximately 50% of the drug is lost if in RPMI - or other processes leading to drug loss.

Daunorubicin has been reported to adsorb to all materials except for polypropylene and silanised glass (Bosanquet, 1986). Further investigation determined that adsorption onto the polyethylene culture plate was partially responsible for the observed drug loss. To overcome this problem, culture plates used in future experiments would have had to be made of polypropylene. Although polypropylene 96-well plates could be acquired the majority of the experiments needed to be performed in 24-well plates to grow a sufficient density of cells with which to work. As 24-well plates were not available in polypropylene the problem of adsorption would be a factor in the remainder of the experiments and was therefore quantified (Tables 2.3 & 2.4, p112 & p114).

Drug loss due to adsorption could be quantified and would not have interfered with continued P-gp analysis. However, if drug loss was also due to degradation, then the presence of these degradative products could interfere when analysing results from P-gp function and modulation investigations. Previously published stability data (Wood *et al.* 1990b) suggests that a degradative loss of drug is not to be expected at lower temperatures such as 4°C. At 37°C, under the experimental conditions used in this study, degradation products were detectable and therefore drug loss due to degradation was also determined

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(sections 2.4.1.2, p92 & 2.4.1.3, p94) and considered in the investigations regarding P-gp function and modulation (section 4.6, p236).

Continued analysis into the effects of pH demonstrated that drug adsorption and degradation at $37^{\circ}C$ was pH dependent (Table 2.3, p112). The greatest loss occurred at pH 7 - 7.5 and was predominantly due to degradative processes. This was of concern as, in general, this is the optimum pH for cell growth - see later discussion. It was also noted that the RPMI used for analysis until this point had been unbuffered and devoid of additional culture components such as L-glutamine. However, cell culture media is commonly buffered to within tight limits (pH 7.2-7.4) to facilitate optimum cell growth and also to mimic *in vivo* conditions as much as possible. In addition, L-glutamine is commonly required for cell growth. For this reason HEPES buffered RPMI (pH 7.2) was supplemented with L-glutamine to a final concentration of 2mM and used in a study on the stability of 100ng/ml daunorubicin over 2h at $37^{\circ}C$ (Fig. 2.19).

The stability of 100ng/ml daunorubicin did not differ significantly between the unbuffered and HEPES buffered, 2mM L-glutamine RPMI (Fig. 2.13, p109 & 2.19, p120). The HEPES buffered RPMI + 2mM L-glutamine was therefore used for continued investigation into drug stability in different media (also buffered and supplemented with 2mM L-glutamine).

It was found that drug loss at $37^{\circ}C$ occurred by adsorption and degradation to varying degrees with differing culture media (Table 2.4, p114), however, in contrast to the effects of varying pH, the observed drug loss was predominantly due to adsorptive processes. Greatest drug loss was observed in buffered DMEM (another commonly used culture medium) and, as it was unlikely that cells would tolerate culture in water or PBS, it was

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thought that the supplemented, buffered RPMI provided the optimal culture medium for continued analysis of P-gp expression, function and modulation in patient B-lymphocytes.

Fig. 2.19 Stability of 100ng/ml daunorubicin in unbuffered RPMI and RPMI buffered with HEPES and supplemented with 2mM L-glutamine
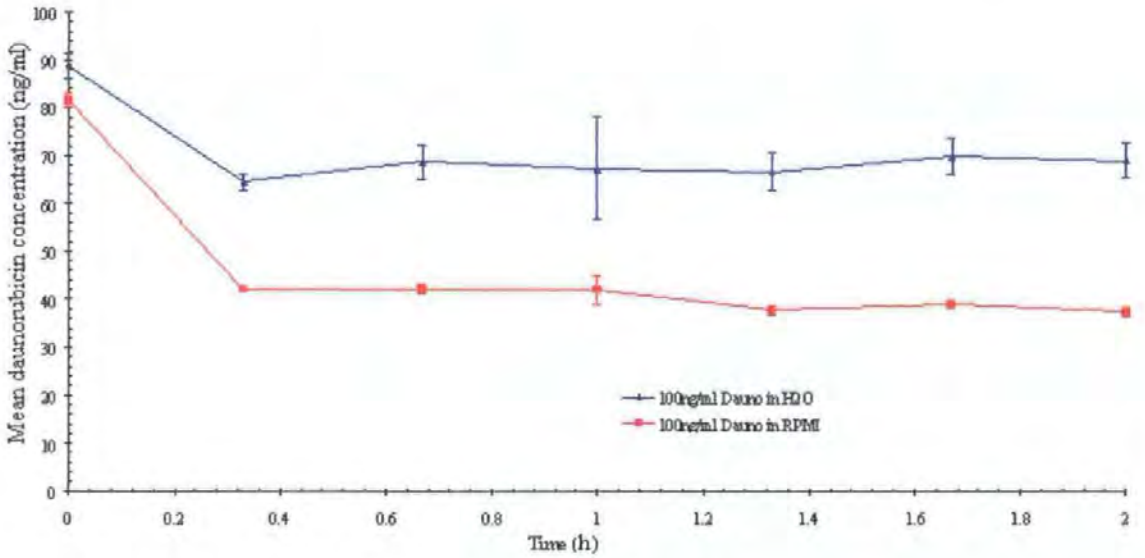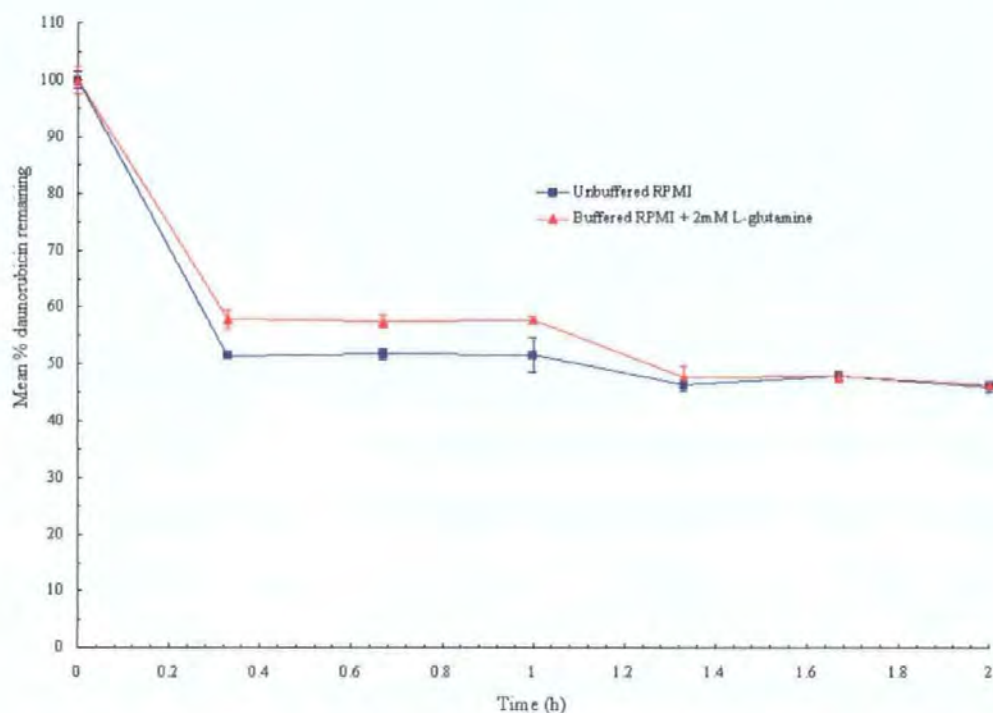

Fig. 2.19 2ml solutions of 100ng/ml daunorubicin, in unbuffered and buffered (pH 7.2) RPMI, were incubated (in triplicate) in a 24-well culture plate at 37°C for 2h. Samples were taken at regular intervals for HPLC analysis. In each case daunorubicin concentration decreased over time. Drug loss was not significantly different between the two diluents when analysed by ANOVA (P > 0.05).

Further to the effects of temperature, concentration, culture media & pH, protein binding was also evident in the experimental system (Fig. 2.18, p116). A large majority of culture systems add foetal bovine serum (FBS) to the growth medium to help sustain cell growth,

indeed many cells require its presence. The anthracyclines, however, have been reported to bind to albumin (Trynda-Lemiesz and Kozlowski, 1996) and therefore could also bind to other proteins. The experimental system (RPMI + 2mM L-glutamine + 10% (v/v) FBS) showed there to be protein binding, the immediate effect being a decrease in the availability of free drug for cellular interaction. It was also considered possible that any protein/drug complex could exert an individual effect on P-gp, further complicating the cell culture scenario.

In addition all investigations revealed an initial, seemingly instantaneous, loss when the drug was dissolved in diluents other than the control (water). This instantaneous loss was measured by spiking two solutions, water and the diluent of interest, with a known concentration of daunorubicin, mixing, and immediately removing a sample for HPLC analysis prior to any experimental incubation. As all drug solutions were made in silanised, glass volumetric flasks wrapped in aluminium foil, this instantaneous drug loss could not be attributed to either adsorption onto the walls of the glass volumetric flask or photodegradation. Although this phenomenon still remains unexplained it may be that a physico-chemical interaction between the drug and a media constituent could be responsible, *e.g.* the drug-media constituent conjugate could elute at a different time than expected. Indeed the amino acid content of the RPMI or DMEM could have been interacting with the sugar moiety of the daunorubicin, although this affords no explanation for the loss seen in PBS.

Using the results generated from the stability investigations as well as considering future cell culture requirements, HEPES-buffered RPMI was the medium of choice for future cell culture work. This decision took into account the drug loss occurring with RPMI as well as the probability of cell survival in any of the other diluents. While it was also tempting to

use the results to decide the incubation period and concentration of daunorubicin to be used in future experiments, it was realised that these limiting conditions would only reflect the state of the cell environment at one instant in time. It was acknowledged that the investigative system would have to balance optimal drug stability conditions with cell survival conditions as well as experimental requirements, and therefore the attempt to quantitate the drug loss processes occurring at any one instant in time, in a system devoid of cellular material, were deemed to be impractical. This, however, did not detract from the importance of qualifying the processes that were occurring in the proposed cell culture system.

This importance was emphasised when investigating the effect of pH on drug stability. Daunorubicin is most stable between a pH of 4.5 - 6.5 (Poochikian *et al.*, 1981). The unbuffered RPMI (containing only sodium bicarbonate) reflected this increased drug stability at acidic pHs (Table 2.3, p112 and Fig. 2.16, p113). This was attributed to a decrease in pH from 7.0 at the start of the incubation to 5.4 at the end of incubation. With the buffered RPMI solutions, the greatest daunorubicin stability was observed at an acidic pH of 6 and any loss, additional to the instant loss observed, was attributed solely to adsorption. At a higher pH of 7 or 7.5, however, the majority of the drug was lost to degradation, having serious implications for future cell work.

This dilemma of which pH to choose for future experimental work was apparent when considering optimum cell growth conditions. A decrease in pH, although beneficial for drug stability, provides a hostile environment for most cells cultured *in vitro*. In addition, physiological pH is regulated between the very tight limits of 7.3 - 7.4. Subsequently, future *in vitro* work, if trying to replicate the *in vivo* situation as closely as possible, would have to keep the pH of the culture environment as close to *in vivo* pH values as possible.

Any deviation from these limits could mean an alteration in the normal physiology of the patient B-lymphocytes and therefore an alteration in the resistance of the cell. However, greatest drug loss was observed at physiological pH and this factor was of extreme importance because it emphasised that, along with the parent anthracycline, degradation products could exist in a culture environment that needs to be kept at physiological pH for cell survival.

Effectively, the results have shown that it is possible to have degradation products in the cellular system very early on in culture as well as drug loss through protein binding and adsorption. Although no daunorubicin degradation products have yet been reported to exert toxic effects, some anthracycline metabolites, *e.g.* daunorubicinol and idarubicinol, have been shown to be toxic with idarubicinol exhibiting as much toxicity as the parent drug (Limonta *et al*. 1990).

Previous literature reports have not attempted to dissect the processes of adsorption, degradation or protein binding, and as such it remains unclear whether previously reported alterations in resistance are due solely to interactions of the P-gp molecule with the parent drug or to interactions of the metabolites, degradation products or drug/protein complexes with P-gp.

2.7   CONCLUSION

Daunorubicin is an unstable drug under physiological culture conditions and, as conditions chosen for cell work must try to balance the stability of the drug with the probability of cell survival, it is possible for drug loss to occur via adsorption, degradation and protein binding. To aid in the isolation and identification of the molecules and systems responsible

for drug resistance it is therefore important to identify closely the likely constituents of the experimental model.

The results presented in this chapter led to the use of a modified RPMI solution, *i.e.* buffered to pH 7.2 with HEPES buffer, supplemented with 2mM L-glutamine and 10% (v/v) FBS. In addition the incubation times for future experiments would be limited to between 0.5-1.5h and daunorubicin would be maintained at μg/ml concentrations rather than lower ng/ml concentrations, depending on experimental requirements.

3. P-GLYCOPROTEIN EXPRESSION

## 3.1	INTRODUCTION

In B-CLL, the results of studies investigating the expression of P-gp have been conflicting. While several authors have described a large majority of patients expressing an increased amount of P-gp compared to healthy controls (Michieli *et al.* 1991; Shustik *et al.* 1991; Webb *et al.* 1998), others have detected low levels (Grulois *et al.* 1995).

These conflicting results may arise because different techniques have been used for P-gp analysis, all ranging in their sensitivities, *e.g.* studies on MDR1 gene expression have involved *in situ* mRNA hybridisation and reverse transcriptase-polymerase chain reaction (RT-PCR) to detect MDR1 mRNA (Mechetner & Roninson, 1992; Zhou *et al.* 1995), while flow cytometry & immunohistochemistry have been utilised to locate the P-gp protein in the cell (Schinkel *et al.* 1993a; Webb *et al.* 1998).

### 3.1.1	FLOW CYTOMETRY

Flow cytometry is a technique enabling rapid measurements of physical and chemical characteristics of cells or particles as they travel in suspension, one by one through a sensing point.

3.1.1.1 Hardware

The modern flow cytometer consists of a light source, fluidics system, collection optics, electronics and a computer to digitally process the data. In most modern cytometers the light source is a laser (light amplification by stimulated emission of radiation) which emits a beam of light at specific, selectable wavelengths. The fluidics system of the flow

cytometer then delivers particles of a random three-dimensional suspension one by one to a specific point in space intersected by the illuminating laser beam. Scattered and emitted fluorescent light is collected by two lenses (one set in front of the light source, *i.e.* forward scatter, and one set at right angles, *i.e.* side scatter) and by using a series of optics, beam splitters and filters, specific bands of fluorescence can be measured (Fig. 3.1).

Fig. 3.1 Generalised flow cytometer system
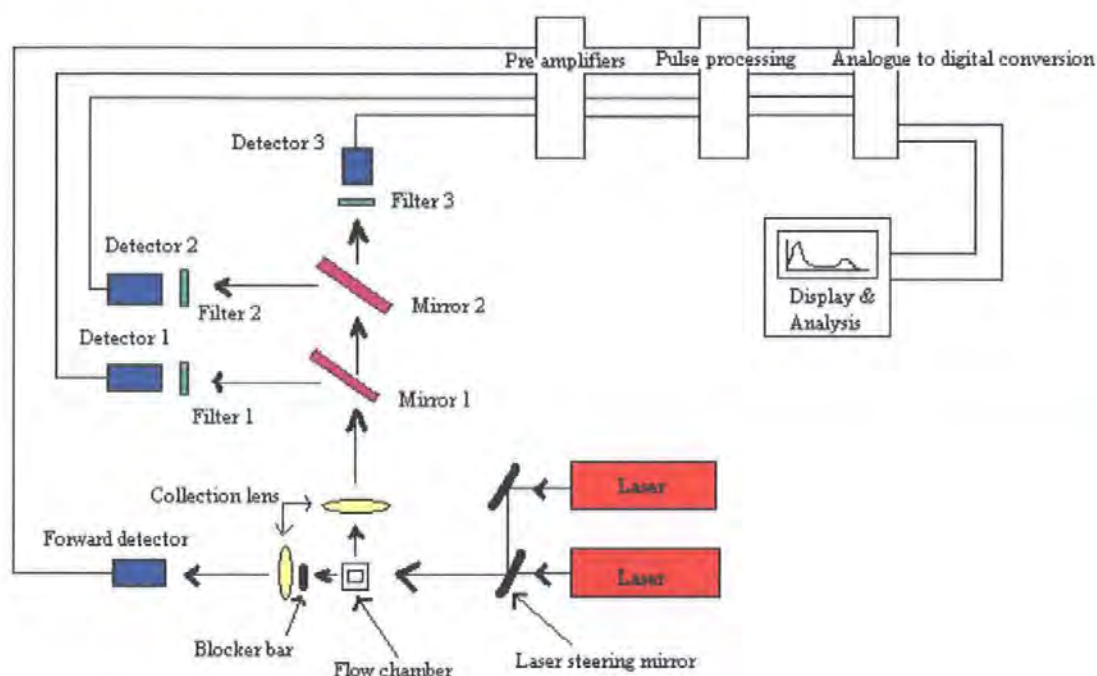
Fig. 3.1 From Flow Cytometry; A Practical Approach. M. G. Ormerod. Second Edition.: Oxford University Press (1996).

3.1.1.2 Analysis and Display

Flow cytometry has many different applications that usually involve using a fluorescent probe covalently bound to the cell or structure of interest, *e.g.* antibody conjugated to a fluorochrome. The fluorochrome is excited by the laser light and, as it returns to the unexcited (ground) state, it emits light of a longer wavelength detected by the flow

cytometer. Table 3.1 lists the major fluorochromes, their excitation and emission wavelengths together with their common applications.

Fluorochrome	Excitation (nm)	Emission (nm)	Laser type	Applications
Fluorescein	495	520	Argon	Phenotypic analysis
Phycoerythrin	495	575	Argon	Phenotypic analysis
PerCP	488	670	Argon	Phenotypic analysis
Allophycocyanin (APC)	630	660	Helium-Neon	Phenotypic analysis
APC-Cy7	630	760	Helium-Neon	Phenotypic analysis
Hoechst 33342	350	470	Argon	DNA analysis/Apoptosis
Hoechst 33258	350	470	Argon	DNA analysis/Apoptosis
DAPI	372	456	Argon	DNA analysis
Propidium Iodide	495	637	Argon	DNA analysis
Ethidium Bromide	493	620	Argon	DNA analysis
Acridine Orange	503	530/640	Argon	DNA, RNA
Fluo-3	488	530	Argon	Calcium flux measurement
Rhodamine 123	515	525	Argon	Mitochondria

Table 3.1 Some common fluorochromes From Imperial Cancer Research Fund webpage - http://www.icnet.uk/axp/facs/davies/flow.html

Light emitted from these fluorochromes is measured by a detector – usually a photomultiplier tube (PMT) – so generating a current that is transformed into analogue voltage pulses which are digitised. Computer systems allow sophisticated analysis of this data, the most common and useful ways of displaying the resulting values being the frequency histogram and the dual parameter correlated plot, often known as a cytogram or dot plot (Fig. 3.2).

Fig. 3.2 Two forms of flow cytometer display
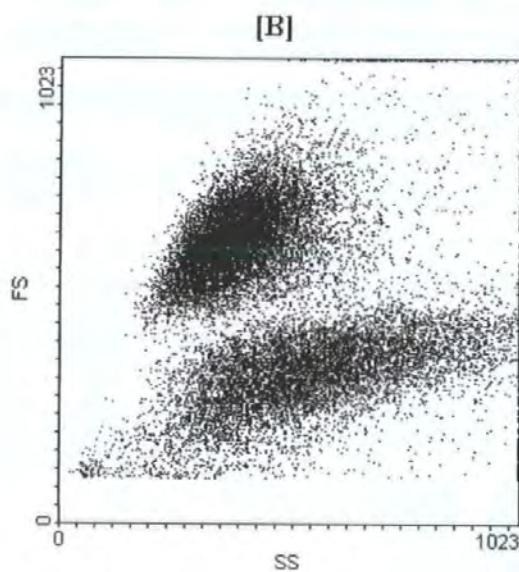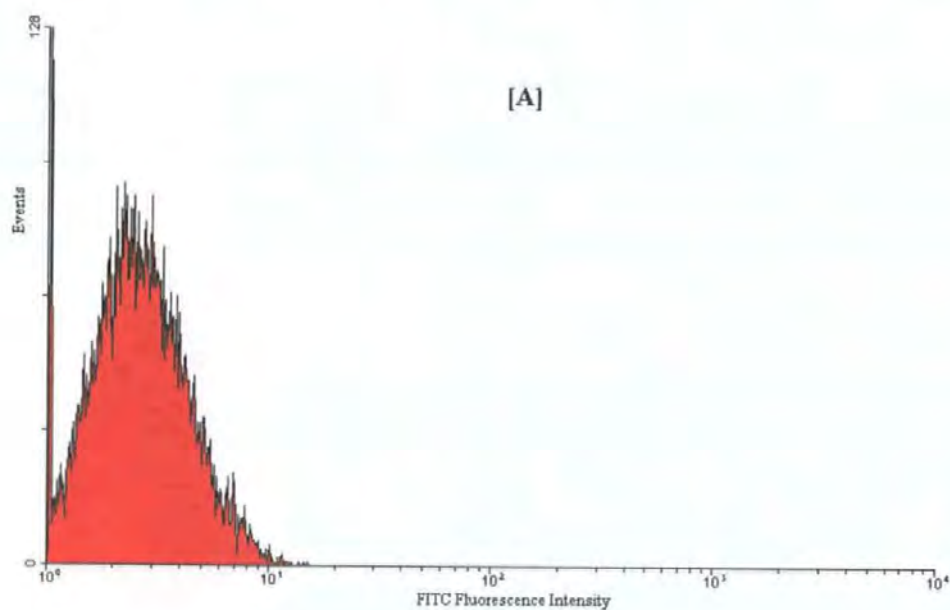
Fig. 3.2 [A]: typical frequency histogram of isolated B-lymphocytes displaying fluorescence intensity against number of events. [B]: dot plot of isolated B-lymphocytes displaying side scatter (SS) against forward scatter (FS), where each cell is represented as a dot at the co-ordinates appropriate to the measured values.

Statistical analysis of these displays is a straightforward task for the computer and markers can be set at specific areas of a histogram (fluorescence intensity) allowing percentage of total, mean fluorescence value and other statistics to be generated for these selected events (Fig. 3.3[B]). More complex analyses use the ability of the program to set gates or windows on areas of interest so that only cells that fall within the gates are analysed further (Fig. 3.3[A]).

3.1.1.2.1   Controls

When using monoclonal antibodies as fluorescent probes, isotype controls are often used to define the position of the negative cells and set the fluorescence markers. The use of isotype controls as a reference population assumes that the same non-specific staining and biochemical properties are present as in the test reagents.

An additional step that permits a measurement of non-specific staining is to analyse unstained cells in parallel with cells stained with a recommended panel of reagents. The position of the unstained cells (autofluorescence) can be compared to the isotype control populations observed in other samples. The separation between the populations (observed by overlaying the plots) provides a measure of the relative degree of non-specific binding of the antibodies to the cell population (Figs. 3.9 & 3.10, p153 & 154, respectively).

Fig. 3.3 Analysis of histogram and dot plot displays
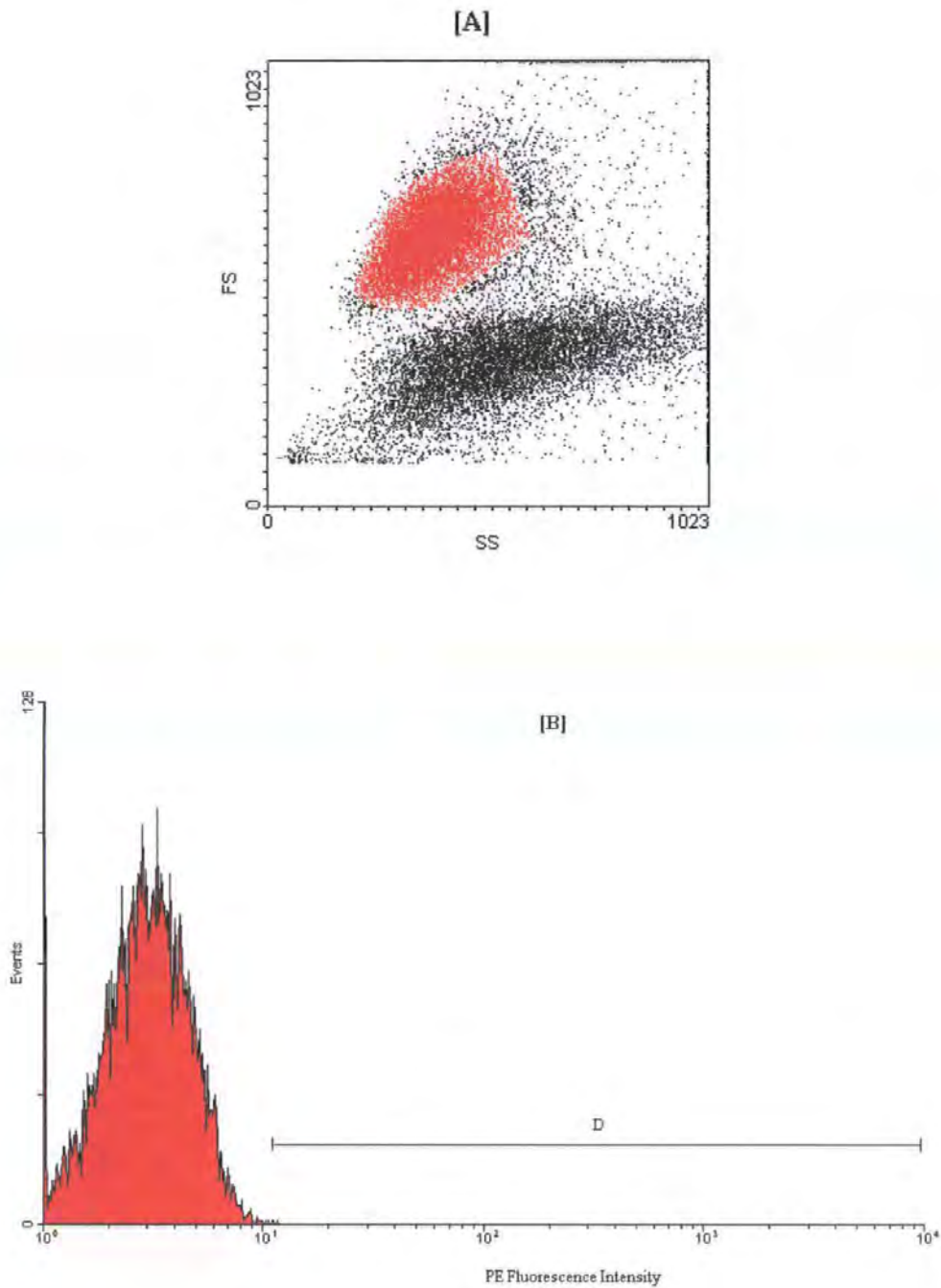
Fig. 3.3 [A]: gated dot plot of isolated B-lymphocytes. Gated region is denoted in red. [B]: histogram of gated B-lymphocytes in [A] - marker, D, denotes the region of analysis (positivity).

3.1.1.2.2   Compensation

Although filters select separate wavebands from fluorochromes, the emissions from some fluorochromes can overlap such that some fluorescence from one fluorochrome will pass to the detector intended to measure the fluorescence from the other and vice-versa. This spectral overlap can be corrected during signal processing by subtraction electronically of the proportion of the fluorescence due to spectral overlap and is termed compensation.

3.1.1.3 Detection of P-gp in B-CLL

The major problem in detecting P-gp-associated MDR in B-CLL has been the absence of a standardised method for detection and quantification of the MDR1 gene and P-gp. Herzog *et al.* (1992), using several cell lines with different levels of resistance, concluded that most techniques could detect low levels of MDR1 gene expression, but RT-PCR was the most reliable technique. For chronic lymphocytic leukaemia (CLL), however, Wall *et al.* (1993) observed an important discrepancy between a low percentage of patients positive for MDR1 mRNA and a higher percentage of positivity when assayed by a functional test for P-glycoprotein using flow cytometry analysis.

This emphasises the effectiveness of flow cytometry as a technique for P-gp-associated MDR investigations, as both P-gp protein expression as well as function can be determined quickly and effectively.

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3.1.1.3.1   Monoclonal antibodies (MAbs)

Flow cytometric detection of P-gp has led to the development of several MAbs targeting different epitopes on the P-gp protein (Table 3.2). These MAbs can be conjugated to different fluorochromes for flow cytometric use (Georges *et al.* 1990; Weinstein *et al.* 1990).

P-gp monoclonal antibody (MAb)	Location of P-gp Recognition site (epitope)
MRK16	extracellular
C219	intracellular
UIC2	extracellular
JSB-1	intracellular
4E3	extracellular

Table 3.2 Recognition sites for some P-gp monoclonal antibodies (MAbs)

Problems of non-specific hybridisation for different MAbs can lead to confusing results. For example, C219 recognises highly conserved amino acid sequences found in all P-gp isoforms characterised to date, including MDR3, but it also cross-hybridises to a small amount of skeletal muscle fibres. Similarly, the MRK16 MAb recognises external regions of the molecule but occasionally stains smooth muscle fibres. The cross reactivity exhibited by these MAbs does not normally present problems when using isolated B-lymphocytes, nevertheless, it was considered appropriate to follow consensus recommendations published in 1996 regarding the various P-gp detection methods used when analysing patients' tumours (Beck *et al.* 1996). A large variation between interlaboratory results was found and the recommendations suggested the use of more than one technique when

analysing P-gp, *e.g.* flow cytometry P-gp detection and MDR1 mRNA detection as well as P-gp functionality experiments.

3.1.1.3.2   Fluorochromes

Different fluorochromes possess different quantum efficiencies affecting their ability to fluoresce, *i.e.* a greater quantum efficiency is related to greater fluorescence intensity. A sample with a low amount of P-gp would therefore benefit from the use of a fluorochrome with high fluorescence intensity. Selection of the fluorochrome is therefore an important consideration when determining the presence of P-gp in a sample.

Consequently, in B-CLL, the use of phycoerythrin (PE) as the conjugated fluorochrome has been advised not only due to its higher fluorescence intensity (quantum efficiency) compared to other commonly used fluorochromes (such as fluorescein isothiocyanate (FITC)) resulting in an increased ability to detect low amounts of P-gp in clinical samples (Beck *et al.* 1996).

3.1.1.3.3   Use of cell lines

The preferred method of P-gp-associated MDR analysis is often the detection of the P-gp protein as the level of detectable mRNA does not always correlate with its protein counterpart. Accordingly, the development of a flow cytometric assay using P-gp specific MAbs requires P-gp positive and negative continuous cell lines to determine the specificity of the MAb before analysing patient samples.

Continuous cell lines are immortal cells which are ideal for use *in vitro*. These cells are derived from primary cells by a series of spontaneous or virus induced mutational events which result in rapidly proliferating transformed cells. Advantages of continuous cell lines include the ability to investigate cellular functions at different stages of the cell cycle and the relatively cheap costs of obtaining and maintaining cell lines in comparison to primary culture. Cell lines also provide a stable and reproducible cell culture for investigations within a defined set of parameters. Care must be taken however not to extend the passage of the cells within a single set of experiments to a number greater than 10. This is due to the potential for instability of cell phenotype as a result of genetic rearrangement which occurs following an extended number of passages.

It is also common for cell lines employed in validating methodology for future clinical investigations to resemble the cells in the clinical sample to be analysed. A leukaemic cell line is preferable when validating an assay for the study of leukaemic cells isolated from patients (section 3.3.1.1.1, p151).

3.1.2 REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION (RT-PCR) AND MDR1 mRNA DETECTION

3.1.2.1 Background

3.1.2.1.1   Deoxyribonucleic acid (DNA)

In eukaroytic cells the genetic information of the cell is coded within DNA which is localised to the nucleus of the cell. DNA is composed of 4 different nitrogenous bases; adenine, guanine, cytosine and thymine. These structures are referred to as nucleotides

when carrying the phosphate group and nucleosides without the phosphate group, consisting of only the base and sugar. The sequence of these 4 bases within a DNA molecule contains the genetic information. The DNA molecule is double stranded (Fig. 3.4) with each strand being complementary to each other, so that adenine binds thymine and guanine binds cytosine. Hydrogen bonding between the complementary bases holds the two stands together within a double helix structure. The two strands that form the double helix are also antiparallel to each other, with their 5', 3'-phosphodiester bonds running in opposite directions.

Fig. 3.4 Complementary arrangement of bases in double helix structure of DNA
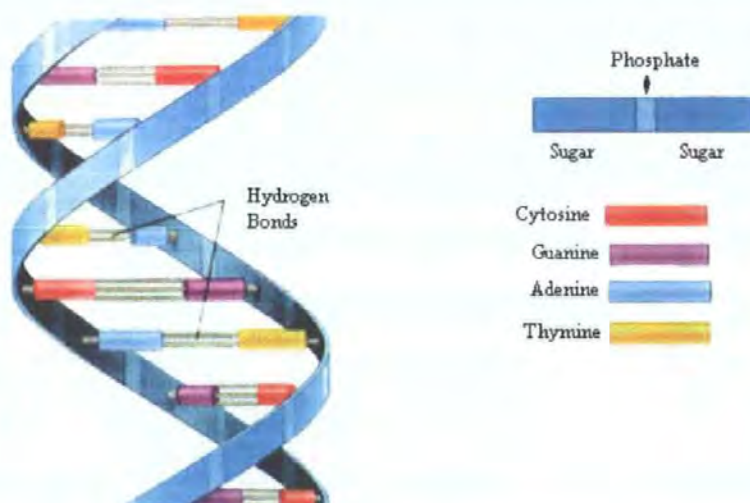
Fig. 3.4 From Mosby's Medical Encyclopedia for Windows 95, User's Guide. The Learning Company Inc. 1997 Edition.

3.1.2.1.2   Ribonucleic acid (RNA)

The expression of the genetic code in the form of protein synthesis takes place in the cytoplasm necessitating the transfer of the appropriate coded section outwith the nucleus. This is achieved by the use of an intermediate nucleic acid named ribonucleic acid (RNA). RNA forms a complementary strand to a single strand of DNA. This process is exactly the

same as for complementary DNA strand formation except for one exception. In RNA a thymine base is substituted by a fifth base named uracil. The RNA strand produced as a result of this process contains coding and non-coding regions, the latter of which regulate rather than code protein synthesis. The RNA which carries the genetic information from DNA to the protein synthesising ribosome is named messenger RNA (mRNA) and the process of mRNA formation from the DNA template is known as transcription.

3.1.2.1.3   Reverse transcription

Transcription, as explained in section 3.1.2.1.2, is the conversion of a DNA sequence into a complementary mRNA sequence. In the process of reverse transcription the opposite occurs and mRNA is reverse transcribed to produce complementary DNA (cDNA). The enzymes that carry out this function are termed reverse transcriptases.

3.1.2.1.4   Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR is a modification of the basic technique of PCR. RT-PCR allows the study of gene expression at the RNA level and is extremely sensitive, in theory RT-PCR being capable of detecting a single RNA molecule. The technique relies upon the extraction and then reverse transcription of cellular RNA to produce cDNA. This reverse transcription is carried out by naturally occurring reverse transcriptases.

Following reverse transcription the cDNA product is probed for the presence of a sequence of bases specific to the protein of interest during the process of PCR. This is achieved by the use of a set of primers which are short, single-stranded DNA molecules complementary to the ends of a defined sequence predicted to be present in the cDNA. In this study the

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defined sequence was specific to the P-gp protein. Consequently, production of the specific product indicates that RNA coding for P-gp was present in the cells suggesting P-gp gene expression *i.e.* MDR1 expression.

PCR begins with denaturation, by heating, of the double stranded cDNA into single strands (Fig. 3.5). Following cooling, primers anneal to complementary sequences on the cDNA and begin to extend along the cDNA strand by the action of DNA polymerase at a temperature suitable for the enzyme (stage ①). This primer extension phase occurs in the presence of excess free deoxynucleoside triphosphates (dNTPs). This cycle produces two new double-stranded DNA molecules (stage ②). Strand synthesis is then repeated again by the process of heat denaturation, primer annealing and primer extension (stage ③). As each new primer region produced acts as a template for further cycles of amplification the target DNA sequence is selectively amplified cycle after cycle (stage ④) and its ends are determined by the 5' ends of the primers. The quantity of the amplified product thus increases exponentially.

To allow detection and analysis, the PCR product may then be subjected to electrophoresis on an agarose or polyacrylamide gel and visualised by staining with ethidium bromide - a fluorescent dye that intercalates with DNA. Accurate size determination of the product may be achieved by incorporation of a base-pair size marker into the gel.

Fig. 3.5 Schematic representation of the PCR reaction
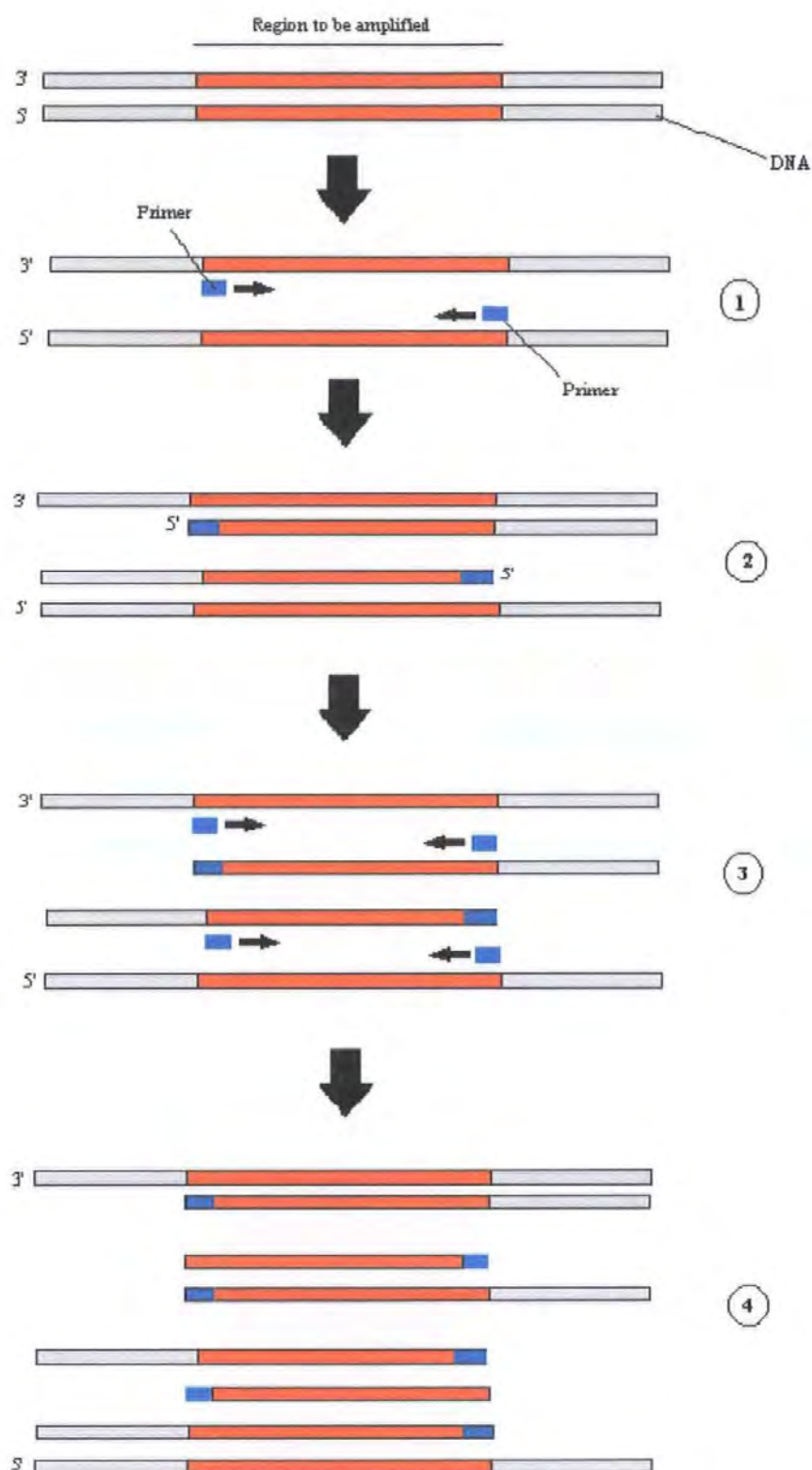
Fig. 3.5 Stage 1; primer annealing & extension: Stage 2; DNA double strand production: Stage 3; repetition of stages 1 & 2: Stage 4; selective amplification. Adapted from O'Driscoll *et al*. 1993

3.1.2.1.5 Splicing and RT-PCR

A primary transcript for a eukaryotic mRNA typically contains sequences encompassing one gene. However, the sequences encoding the polypeptide are usually not contiguous. Instead, in the majority of cases, the coding sequence is interrupted by noncoding tracts called introns; the coding segments are called exons. In a process called splicing, the introns are removed from the primary transcript and the exons joined to form a contiguous sequence specifying a functional polypeptide. Therefore, when designing or deciding on which primers to use for RT-PCR it is important to consider the process of splicing such that the primers used are designed to anneal to an exon sequence rather than an intron sequence. In addition, the primers should be selected so that they reside in separate exons to enable detection, by looking at the size of product, of any contaminating DNA which has been amplified.

3.1.2.1.6 Housekeeping genes

The levels of specific gene products rise and fall within a cell in response to molecular signals. Such signals may be associated with environmental stimuli such as the requirement for specific metabolic enzymes as food sources change or are depleted, or the differentiation of a cell which may require the expression of a certain protein for only a brief period of time. Some gene products are however required continually. Such genes are constitutively expressed and are termed 'housekeeping genes'. They may be used to indicate consistent sample preparation or as a constant arbitrary level of expression against which relative changes in expression of other genes may be illustrated. One such enzyme, hypoxanthine-guanine phosphoribosyl transferase (HGPRT), is essential in the synthesis of nucleotides by the cell via *de novo* or salvage pathways and is used as a housekeeping gene

product in this section of work to monitor the quality of sample preparation.

3.1.2.2 RT-PCR detection of MDR1 mRNA

As mentioned in section 1.5.3.6.1, detection of MDR1 RNA has involved the use of RT-PCR using primers specific to a known sequence in the MDR1 gene.

The primers chosen in this study to enable the detection of MDR1 mRNA (Fig 3.6 and section 3.2.4) were first published by Noonan *et al.* (1990) and result in a region of 157bp being amplified (Fig. 3.7A & Fig. 3.7B). The primers flank a sequence that crosses an intron so that MDR1 DNA contamination can be detected. If the relevant area of DNA was amplified, a band of 1257bp would result in comparison to a band of 157bp from cDNA. Therefore, by virtue of the fact that the selected region has an intron present in the DNA, if contaminating DNA is amplified, it should be possible to identify it by its greater length in comparison to the amplified region of cDNA. Another strategy to detect contaminating DNA would be to eliminate the reverse transcription of samples such that amplification would be due to contaminating DNA only. A pure sample of RNA would not be reverse transcribed to cDNA in the absence of reverse transcriptase and there would be no amplification.

These MDR1 primers are both 100% homologous and specific to the MDR1 mRNA so avoiding amplification of the MDR3 product (Fig. 3.8) which closely resembles that of MDR1. However, even with this specificity in primer design, the possibility of non-specific amplification prompted the inclusion of an additional diagnostic step. This study used a diagnostic restriction enzyme site, *Mae I* (O'Driscoll *et al.* 1993), so that digestion of the

MDR1 RT-PCR product with this enzyme results in fragments 84bp and 73bp in size (Fig. 3.7A and Fig. 3.7B).

Fig. 3.6 Complete MDR1 mRNA sequence and associated primer binding sites and *Mae I* restriction site

```
atggatcttgaagggggaccgcaatggaggagcaaagaagaagaacttttttaaactgaacaataaaagtgaaa
aagataagaaggaaaagaaaccaactgtcagtgtatttttcaatgtttcgctattcaaattggcttgacaagtt
gtatatggtggtgggaactttggctgccatcatccatggggctggacttcctctcatgatgctggtgtttgga
gaaatgacagatatctttgcaaatgcaggaaatttagaagatctgatgtcaaacatcactaatagaagtgata
tcaatgatacagggttcttcatgaatctggaggaagacatgaccaggtatgcctattattacagtggaattgg
tgctggggtgctggttgctgcttacattcaggtttcattttggtgcctggcagctggaagacaaatacacaaa
attagaaaacagttttttcatgctataatgcgacaggagataggctggtttgatgtgcacgatgttggggagc
ttaacacccgacttacagatgatgtctccaagattaatgaaggaattggtgacaaaattggaatgttctttca
gtcaatggcaacatttttcactgggtttatagtaggatttacacgtggttggaagctaacccttgtgattttg
gccatcagtcctgttcttggactgtcagctgctgtctgggcaaagatactatcttcatttactgataaagaac
tcttagcgtatgcaaaagctggagcagtagctgaagaggtcttggcagcaattagaactgtgattgcatttgg
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ttttagtgttggacaggcatctccaagcattgaagcatttgcaaatgcaagaggagcagcttatgaaatcttc
aagataattgataataagccaagtattgacagctattcgaagagtgggcacaaaccagataatattaagggaa
atttggaattcagaaatgttcacttcagttacccatctcgaaaagaagttaagatcttgaagggtctgaacct
gaaggtgcagagtgggcagacggtggccctggttggaaacagtggctgtgggaagagcacaacagtccagctg
atgcagaggctctatgacccacagaggggatggtcagtgttgatggacaggatattaggaccataaatgtaa
ggtttctacgggaaatcattggtgtggtgagtcaggaacctgtattgtttgccaccacgatagctgaaaacat
tcgctatggccgtgaaaatgtcaccatggatgagattgagaaagctgtcaaggaagccaatgcctatgacttt
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agaggatcgccattgcacgtgccctggttcgcaacccaagatcctcctgctggatgaggccacgtcagcctt
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gctcatcgtttgtctacagttcgtaatgctgacgtcatcgctggtttcgatgatggagtcattgtggagaaag
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```

agatccagtctaataagaaaaagatcaactcgtaggagtgtccgtggatcacaagcccaagacagaaagctta

gtaccaaagaggctctggatgaaagtatacctccagtttccttttggaggattatgaagctaaatttaactga

atggccttattttgttgttggtgtattttgtgccattataaatggaggcctgcaaccagcatttgcaataata

ttttcaaagattatagggtttttacaagaattgatgatcctgaaacaaaacgacagaatagtaacttgtttt

cactattgtttctagcccttggaattatttcttttattacatttttccttcagggtttcacatttggcaaagc

tggagagatcctcaccaagcggctccgatacatggttttccgatccatgctcagacaggatgtgagttggttt

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tggttggcaactaacactgttactcttagcaattgtacccatcattgcaatagcaggagttgttgaaatgaaa

atgttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgctactgaagcaatag

aaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaacatatgtatgctcagagtttgcaggt

accatacagaaactctttgaggaaagcacacatctttggaattacattttccttcacccaggcaatgatgtat

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gaaggcctaatgccgaacacattggaaggaaatgtcacatttggtgaagttgtattcaactatcccacccgac

cggacatcccagtgcttcagggactgagcctggaggtgaagaagggccagacgctggctctggtgggcagcag

tggctgtgggaagagcacagtggtccagctcctggagcggttctacgaccccttggcagggaaagtgctgctt

gatggcaaagaaataaagcgactgaatgttcagtggctccgagcacacctgggcatcgtgtcccaggagccca

tcctgtttgactgcagcattgctgagaacattgcctatggagacaacagccgggtggtgtcacaggaagagat

tgtgagggcagcaaaggaggccaacatacatgccttcatcgagtcactgcctaataaatatagcactaaagta

ggagacaaaggaactcagctctctggtggccagaaacaacgcattgccatagctcgtgcccttgttagacagc

ctcatattttgcttttggatgaagccacgtcagctctggatacagaaagtgaaaaggttgtccaagaagccct

ggacaaagccagagaaggccgcacctgcattgtgattgctcaccgcctgtccaccatccagaatgcagactta

atagtggtgtttcagaatggcagagtcaaggagcatggcacgcatcagcagctgctggcacagaaaggcatct

atttttcaatggtcagtgtccaggctggaacaaagcgccagtga

Fig. 3.6 Sense and antisense primer regions are shown in red while the sequence highlighted in pink represents the recognition sequence of the *Mae I* restriction enzyme.

Mae I restriction sites

sense primer

Mae I restriction site

exon 21

PCR product

exon 22

antisense primer

(A)

Mae I restriction site

Sense primer

Antisense primer

157bp PCR product

Exon 21   Exon 22

(B)

Fig. 3.8 Complete MDR3 mRNA sequence and *Mae I* restriction sites

(A)

← — Mae I Restriction sites —→

(B)
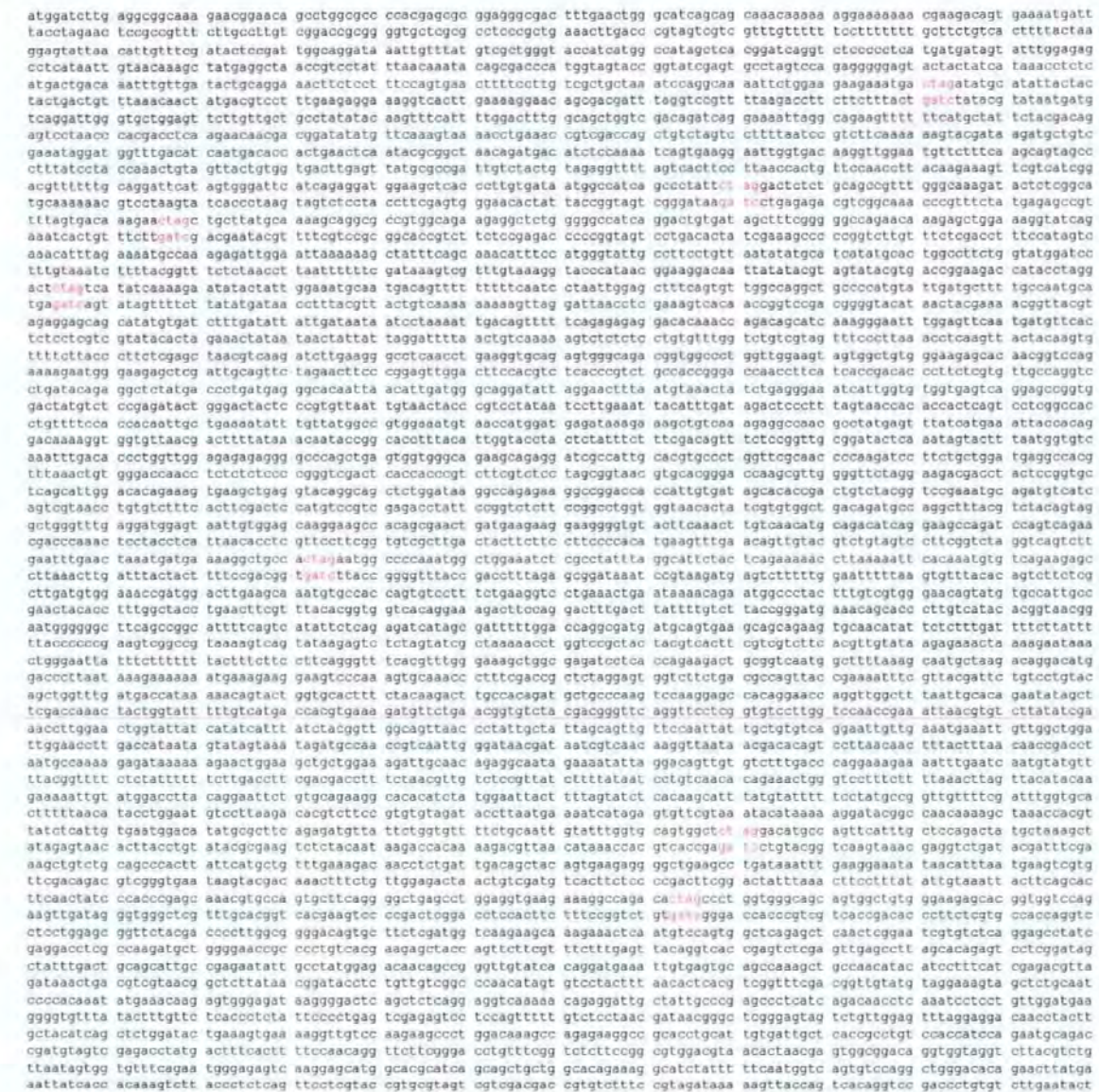
Fig. 3.8 (A) complete MDR3 mRNA sequence with *Mae I* restriction recognition sites highlighted in pink.

(B) schematic of MDR3 mRNA sequence and associated *Mae I* restriction sites. Note absence of MDR1

primer binding.

Although methods for P-gp detection do vary between laboratories, analysis using flow cytometry has been well documented (Beck *et al.* 1996; Wall *et al.* 1993; Webb *et al.* 1998) and as this technique provides a rapid method of detecting the P-gp protein it is a preferred technique when investigating P-gp-associated MDR. However, the inconsistency of previous investigations has meant that the role of P-gp in B-CLL drug resistance remains unclear, therefore, this study further investigated the issue of P-gp expression in B-lymphocytes isolated from B-CLL patients utilising both the techniques of flow cytometry, to analyse P-gp expression and function, and RT-PCR, to determine MDR1 mRNA expression.

3.2   MATERIALS

Unless otherwise stated, materials used in this study were of at least analytical grade and obtained from Sigma-Aldrich Company Ltd., Poole, UK.

All water used was $3M\Omega$ water, filtered and deionised to $18M\Omega$ by an Elga UHQ II water purifier (Elga Ltd., Buckinghamshire, UK).

3.2.1   GENERAL MATERIALS

Equipment

- Heraeus Labofuge 400 Centrifuge (Jencons Scientific Ltd., Bedfordshire, UK).

Reagents

- Absolute ethanol (Hayman Ltd., Essex, UK).

3.2.2   TISSUE CULTURE MATERIALS

Equipment

- 9ml vacuette Sodium Heparin blood tubes (Greiner Labortechnik Ltd., Gloucestershire, UK).

- Sterile Universal tubes [25ml] (Greiner Labortechnik Ltd., Gloucestershire, UK)

Reagents

- Detachabead CD19 (Dynal (UK) Ltd., Merseyside, UK)

- Dynabeads M-450 CD19 (Dynal (UK) Ltd., Merseyside, UK)

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- Fetal Bovine Serum (Sigma-Aldrich Company Ltd., Poole, UK).

- Lymphoprep (Nycomed (UK) Ltd., Birmingham, UK)

- MESSA human uterine sarcoma cell line (ECACC, Wiltshire, UK)

- MESSA/Dx-5 human uterine sarcoma cell line (ECACC, Wiltshire, UK)

- McCoys 5A buffered medium (with sodium bicarbonate & 25mM HEPES) (Sigma-Aldrich Company Ltd., Poole, UK).

- RPMI 1640 buffered medium (with sodium bicarbonate & 25mM HEPES) (Sigma-Aldrich Company Ltd., Poole, UK)..

3.2.3 FLOW CYTOMETRY MATERIALS

Equipment

- Epics Elite Flow cytometer (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

- 7mL Falcon tubes (Greiner Labortechnik Ltd., Gloucestershire, UK)

- Epics Elite Flow software, version 4.02 (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

Reagents

- MAb CD19-FITC (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

- MAb CD45-FITC/CD14-PE (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

- MAb CD5-PE (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

- MAb IgG1-FITC mouse isotype control (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

- MAb IgG1-FITC/IgG2a-PE isotype control (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

- MAb IgG2a-PE mouse isotype control (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

- MAb, UIC2-PE (Immunotech Ltd., Bedfordshire, UK)

3.2.4 RT-PCR MATERIALS

Equipment

- Cetus DNA thermal cycler 480 (Perkin Elmer Ltd., Buckinghamshire, UK)

- DR1 Block DB-2A Heat Block (Techne, R & D Systems Europe Ltd., Oxon, UK)

- Genius EQ25 thermal cycler (Techne, R & D Systems Europe Ltd., Oxon, UK)

- Software; Microsoft windows 98 & Kodak digital science 1D (Kodak scientific imaging systems, New Haven, USA).

Reagents

- 123 base-pair ladder (Life Technologies, Paisley, UK)

- 25 base-pair ladder (Life Technologies, Paisley, UK)

- Ficoll 400 (Life Technologies, Paisley, UK)

- *MaeI* restriction endonuclease (Boehringer Mannheim, East Sussex, UK).

- Primer – MDR1 Antisense - 5', GTTCAAACTTCTGCTCCTGA, 3' (Life Technologies, Paisley, UK)

- Primer – MDR1 Sense - 5', CCCATCATTGCAATAGCAGG, 3' (Life Technologies, Paisley, UK)

- Primer – HGPRT Antisense - 5', GTCAAGGGCACATCCTACAA, 3' (Life Technologies, Paisley, UK)

- Primer – HGPRT Sense - 5', CTTGCTGGTGAAAAGGACCC, 3' (Life Technologies, Paisley, UK)

- Propan-2-ol HPLC Grade (Fisher Scientific, Leicestershire, UK)

- Trizol (Life Technologies, Paisley, UK)

- Ultra pure Agarose – electrophoresis grade (Life Technologies, Paisley, UK)

3.3   GENERAL METHODS

3.3.1   METHOD DEVELOPMENT

3.3.1.1 Flow cytometry

Flow cytometric assessment of P-gp presence in B-CLL patients required P-gp-positive and -negative cell lines with which to validate and standardise a flow cytometry assay.

3.3.1.1.1   Choice of cell line

For analytical accuracy, it would have been preferable to use an immortalised B-CLL cell line exhibiting similar characteristics to the patients' malignant B-lymphocytes. However, this type of cell line could not be obtained from interlaboratory contacts or the 'European Collection of Animal Cell Culture' (ECACC). Immortalisation of isolated B-lymphocytes could have been attempted in the laboratory but this option was thought to be impractical. Therefore, in the absence of a readily available immortalised B-CLL cell line, ECACC provided a doxorubicin sensitive human uterine sarcoma cell line - MESSA - and its P-gp overexpressing, doxorubicin resistant subline, MESSA/Dx-5. Although not ideal, these were the only commercially available cell lines that provided both a P-gp negative(-ve) and a P-gp positive(+ve) control for method validation and were used in this study.

3.3.1.1.2   Choice of MAb and fluorochrome

UIC2 was the anti-P-gp antibody selected. Its use and specificity for P-gp has been well documented (Beck *et al.* 1996; Schinkel *et al.* 1993) and its ability to recognise an external P-gp epitope potentially allowed the correlation of P-gp expression with other cell surface antigens in multicolour flow cytometry as well as functional measurements of dye efflux.

To increase the sensitivity of detection, the UIC2 antibody was conjugated to phycoerythrin (PE) which has fluorescence emission in the red region. PE has a much greater quantum efficiency than FITC permitting more reliable detection of low levels of P-gp expression in haematological samples (section 3.1.1.3.2, p134).

The P-gp results using the +ve and −ve cell line analysis (section 3.3.8, p165) were compared and it was found that, under identical conditions, the fluorescence shift of the resistant cell line into the positive region compared to the sensitive cell line gave a good indication that the assay was able to identify the presence of the P-gp protein (Fig 3.9 and Fig 3.10). An ability to detect different amounts of P-gp expression was demonstrated by the range of fluorescence observed (Fig. 3.10).

= P-gp UIC2 antibody - 0.7%

= Isotype control - 0.2%

= Control - 0.2%

<u>Fig 3.9</u> A region of analysis (positivity) was set using a marker on the control sample (section 3.1.1.2.1). When compared with the isotype control the increase in % positivity using the UIC2 monoclonal antibody is very low with only a 0.5% increase in positivity, falling well within the acceptable margin of error for a Flow cytometer. ANOVA and post-hoc analysis with the Bonferroni test showed that this increase in fluorescence intensity was not significant ($P > 0.05$).

Fig 3.10 UIC2 P-gp detection in anthracycline resistant cells (MESSA/Dx-5) v IgG2a isotype control.
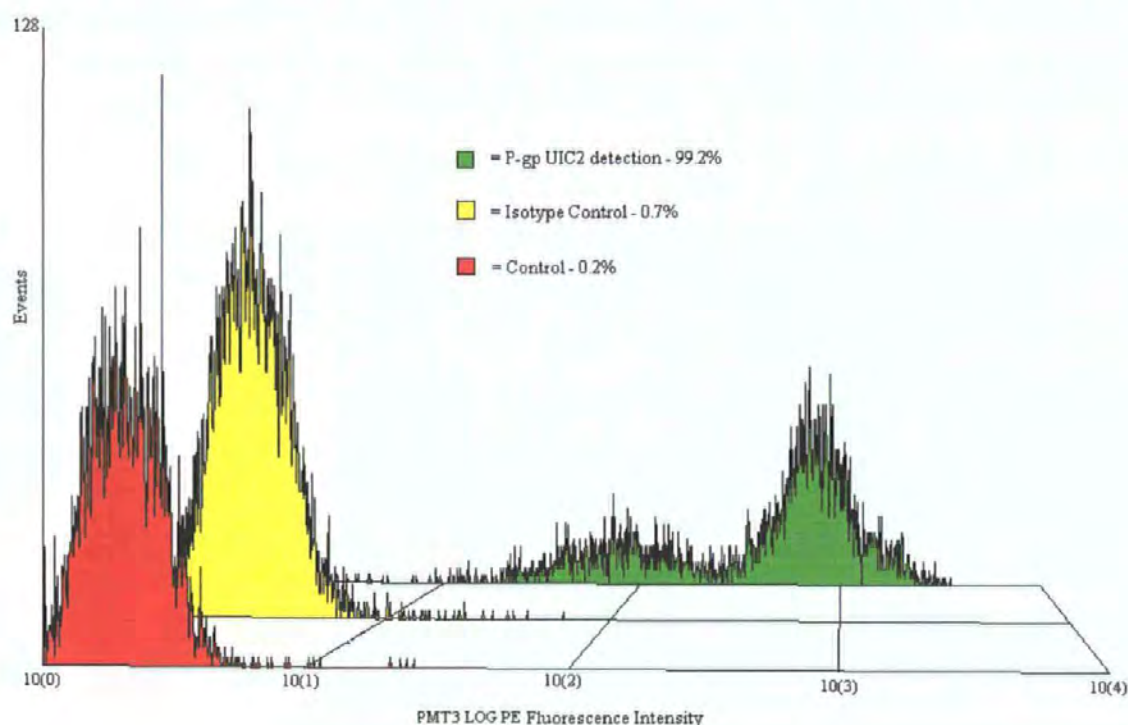
Fig 3.10 A region of analysis (positivity) was set using a marker on the control sample (section 3.1.1.2.1). Under identical conditions the increased 99.2% positivity shows a substantial ability to detect P-gp presence. ANOVA and post-hoc analysis with the Bonferroni test showed that this increase in fluorescence intensity was significant ($P > 0.05$).

Successful P-gp detection led to the use of this methodology for patient P-gp analysis.

3.3.1.2 RT-PCR

To help corroborate the flow cytometry results, the 'Access' RT-PCR kit (Promega, Southampton, UK) was used to detect the presence of MDR1 mRNA (section 3.4.4, p169).

Due to time constraints, however, substantial method development was not possible.

Instead, the integrity (as determined by the presence of 18S and 28S ribosomal subunits) of extracted cellular RNA was verified by running an aliquot of the extracted samples on a 1.4% agarose gel (section 3.4.2, p168 and Fig. 3.11 below) and also by performing a RT-PCR reaction on the HGPRT housekeeping genes endogenous to many mammalian cells (section 3.4.3, p169 and Fig. 3.12).

Fig. 3.11 Integrity of RNA in patient samples.

Fig. 3.11 Lanes 1-5; healthy volunteers: Lanes 6, 29 & 49; Blank: Remaining numbered lanes; patients: Control and •; control RNA from isolated mononuclear cells: MESSA; P-gp negative cell line: MESSADx-5; P-gp positive cell line. A solid line, |, indicates the faint presence of RNA bands comparable to the control RNA samples.

The RNA extracted from some patient samples could be identified (Fig. 3.11). It was theorised that any inability to visualise RNA in the rest of the samples was due to variability in extraction efficiency. HGPRT RT-PCR was continued on these samples as it was thought that low RNA levels would not substantially hinder the PCR due to the amplification procedure.

Fig. 3.12 HGPRT housekeeping gene RT-PCR product.
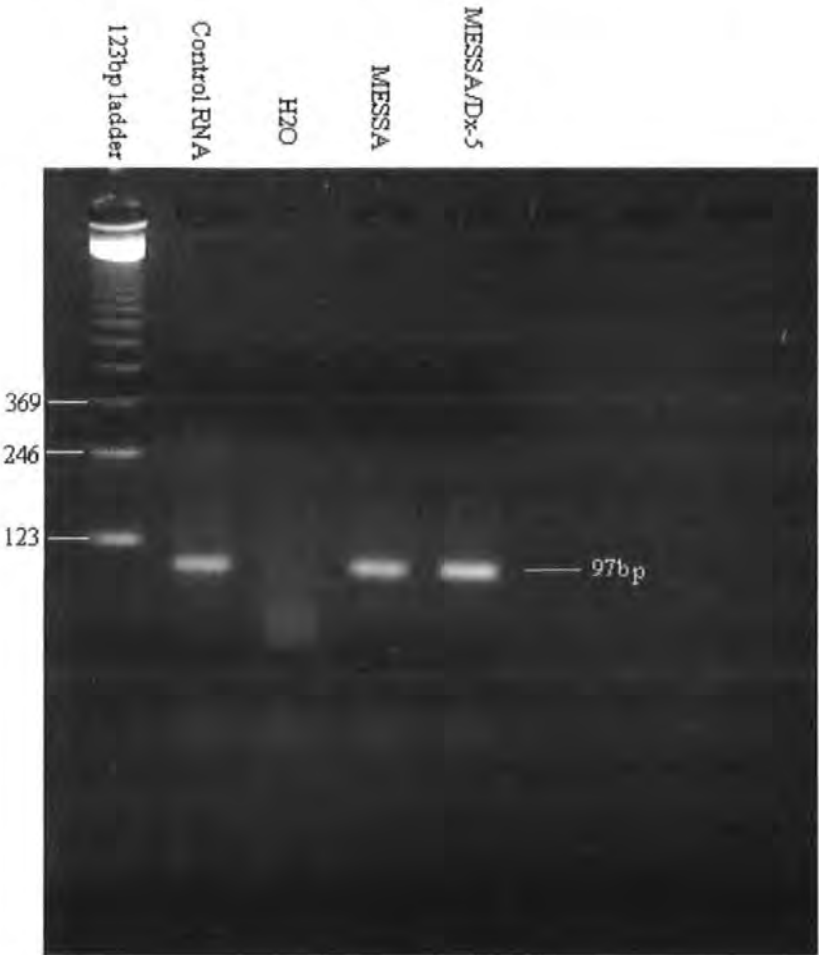
Fig. 3.12 Control RNA; RNA isolated from mononuclear cells: H2O; nuclease-free water: MESSA; P-gp -ve cell line: MESSA/Dx-5; P-gp +ve cell line. A 97bp HGPRT RT-PCR product was detected in the control RNA sample as well as the positive and negative control cell lines, demonstrating the integrity of the RNA.

The RT-PCR for HGPRT was only performed with the cell lines due to time constraints and it was found that the RNA was able to be amplified by RT-PCR. Upon verification of RNA integrity, optimisation of the magnesium sulphate ($MgSO_4$) concentration in the 'Access' RT-PCR kit was completed. To determine optimum $MgSO_4$ concentration for analysis, PCR was conducted on an H_2O control and the two cell lines MESSA & MESSA/Dx-5 (P-gp -ve & P-gp +ve) with varying concentrations of magnesium sulphate (Fig 3.13).

The results demonstrated the absence of a 157bp product with the -ve and H_2O controls, while the P-gp +ve sample showed a clear band at a position approximately 157bp in size. Higher concentrations of magnesium sulphate also gave the same result but there were also extra bands, possibly from non-specific primer binding or DNA contamination during the RNA extraction procedure (section 3.3.10.2, p167). A decision was therefore made to employ a magnesium sulphate concentration of 1mM throughout the remaining PCR analysis .

After optimisation of the $MgSO_4$ concentration, RT-PCR analysis of MDR1 mRNA was performed on previously isolated patient and healthy volunteer samples using published sense and anti-sense primers for the MDR1 gene (Noonan *et al.* 1990) (sections 3.4.4 & 3.5.1.2, p169 & 172).

Fig. 3.13 Optimisation of Magnesium Sulphate (MgSO₄)concentration
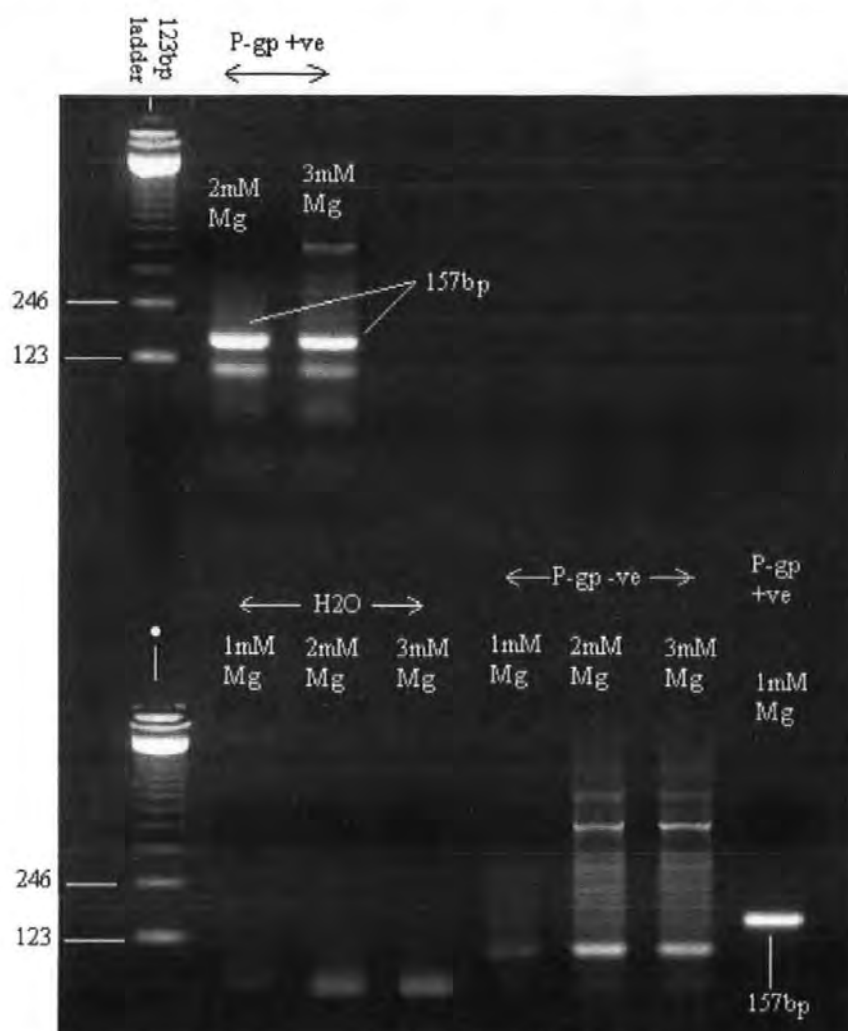
Fig. 3.13 Samples of water, P-gp -ve cells & P-gp +ve cells were run in triplicate with the following varying concentrations of Mg - 1mM, 2mM & 3mM. Each Mg concentration allowed the detection of the 157bp MDR1 RT-PCR product in the P-gp positive cell line compared to water and P-gp negative control. At concentrations higher than 1mM MgSO₄, however, separation became contaminated by other bands/products showing optimum Mg concentration to be 1mM.

3.3.2 PREPARATION OF GENERAL SOLUTIONS/MATERIALS

3.3.2.1 Culture Media

Both buffered RPMI & McCoys 5A media were purchased in 500ml quantities. Frozen aliquots of L-glutamine and FBS were defrosted at 37°C; 55ml of medium was then removed from the 500ml bottles of media and replaced with 5ml of 200mM L-glutamine & 50ml of FBS. The final media therefore had 10% FBS (v/v) and a final concentration of 2mM L-glutamine.

3.3.2.2 Ficoll Loading Buffer

This loading buffer was made up as follows: -

15% (w/v) Ficoll 400

0.25% (w/v) Bromophenol Blue

0.25% (w/v) Xylene Cyanol Blue

3.3.2.3 Agarose gel preparation

The appropriate amount of agarose was weighed out (Table 3.3) and transferred to a conical flask. 50ml of 1 x TBE buffer was added and the mixture was then placed in the microwave and heated on medium power until all the agarose had melted and no solid particles remained. 2.5µl of ethidium bromide solution (10mg/mL) was added and gently agitated to ensure thorough mixing, avoiding the creation of air bubbles. The agarose was then cooled to 50-60°C and poured into a casting tray. The gel was allowed to set for a minimum of 20min.

	% agarose gel	
	1.4%	2%
grams of agarose for 50mL gel	0.7	1.0

Table 3.3 Quantity of agarose for different gel strengths

3.3.2.4 Mini polyacrylamide gel electrophoresis (PAGE) gel preparation

10ml of the PAGE gel mix was made up according to Table 3.4.

Stock solutions	10% gel
5 x TBE	2ml
H2O	5.5ml
40% (w/v) polyacrylamide	2.5ml
10% (w/v) ammonium persulphate	50μl
TEMED	11μl

Table 3.4 Constituents for PAGE gel mix

Approximately 9ml of the PAGE mix was added to the gel casting assembly and a 12-tooth comb inserted, ensuring that no air bubbles were present around the comb. The gel was allowed to set for at least 30min.

3.3.3 CELL LINE HARVEST/PASSAGE & B-LYMPHOCYTE ISOLATION

Harvest/passage and isolation techniques were used to maintain stocks of cell lines, isolate peripheral blood mononuclear cells (PBMCs) from normal volunteers, and isolate patient B-lymphocytes. Both P-gp -ve and P-gp +ve cell lines (MESSA & MESSA/Dx-5, respectively) were passaged when they reached confluence. All procedures were carried out under aseptic conditions.

3.3.3.1 Cell Line harvest/passage

Tissue culture medium (M^{c}Coys 5A) was prepared and warmed to 37^{o}C, prior to use. The medium in the culture flask (10ml) was carefully decanted and a small amount of sterile ethylenediaminetetraacetic acid (EDTA) 0.02% (w/v) solution added. The flask was then incubated (37^{o}C) for 10-15min. During this incubation the cells detached from the bottom of the flask. After the incubation period the flask was removed from the incubator and gently tapped to dislodge any remaining cells. This cell mixture was then transferred to a sterile universal flask and an equal amount of warmed medium added to the EDTA/cell mixture. The cells were dispersed using a sterile pipette and flasks were re-seeded into 4 T25cm^2 adherent culture flasks, *i.e.* 1:4 split. Approximately 10ml of warmed medium were added to each new flask and adequate cell dispersal was checked microscopically before returning the flasks to the incubator.

3.3.3.2 B-lymphocyte isolation

Peripheral blood was collected from B-CLL patients into 9ml sodium heparin tubes. The B-lymphocytes from the patient sample were isolated using magnetic bead separation,

targeting the CD19 antigen on the cell surface & so effecting positive cell isolation (Appendix C). Purity of sample from magnetic bead separation was checked on the flow cytometer using CD19 and CD5 as the antigenic targets with corresponding isotype controls (section 3.1.1.2.1, p130).

3.3.3.3 Peripheral Blood Mononuclear Cells (PBMCs)

Due to difficulties in isolating sufficient numbers of B-lymphocytes from the healthy volunteers using magnetic beads (due to their low B-lymphocyte count) peripheral blood mononuclear cells were isolated instead (Appendix D). As a mixed population of cells was obtained by the isolation of mononuclear cells, dual staining was carried out on these samples allowing the gating of CD19 +ve cells and analysis of P-gp presence in the gated population (section 3.1.1.2, p127).

3.3.4   CELL CULTURE

All procedures were carried out under aseptic conditions.

3.3.4.1 Cell Lines

The doxorubicin sensitive (MESSA) and doxorubicin-resistant (MESSA/Dx-5) cell lines were grown as a monolayer in T25cm^2 adherent flasks (Sarstedt Ltd., Leicestershire, UK) in M^{c}Coys 5A medium supplemented with 10% (v/v) FBS and 2mM L-glutamine. Cells were harvested with EDTA 0.02% (w/v) prior to experimentation (section 3.3.3.1, p161).

3.3.4.2 Patient B-lymphocytes

The isolated B-lymphocytes from patients were maintained as a suspension population in T25cm^2 suspension flasks (Sarstedt Ltd., Leicestershire, UK) in RPMI medium supplemented with 10% FBS (w/v) and 2mM L-glutamine. The cells were harvested by centrifuging at 280g for 5 min and pelleting the cells at the bottom of a sterile universal flask, before resuspension in 5 ml RPMI culture medium. Aliquots of the resuspended cells were taken for experimentation as described in section 3.3.8 (p165).

3.3.5 DETERMINATION OF CELL NUMBER AND VIABILITY

Cells were harvested as described in sections 3.3.3.1 & 3.3.3.2 (p161) and pelleted by centrifugation at 280g for 5min. The cells were resuspended in 10.1ml of warmed culture medium and thoroughly dispersed. A 100µl aliquot of the cell suspension was mixed with 900µl of Trypan Blue (dilution factor (df) of 10). A portion of this mixture was taken for determination of cell count using a haemocytometer. Non-viable cells took up the dye and appeared blue while the viable cells appeared translucent. The four outer corner squares and middle square of the haemocytometer grid were counted for viable and non-viable cells. An average cell count per haemocytometer square was taken and the cell number calculated as shown below:

To calculate cell number:

Average cells/ml $\quad\quad\quad\quad$ = average cell count per square x df x 10^4

$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$ = no. cells x 10^4/ml

Divide by 100 to convert to $\quad$ = no. cells x 10^6/ml

df = Dilution factor.

To calculate cell viability:

Total viable cells/ Total no. of cells x 100 = % viability

3.3.6 CRYOPRESERVATION OF CELLS

Previous investigations within the laboratory had shown that patients' B-lymphocytes were too fragile for cryopreservation, therefore, flow cytometry experimentation was always conducted on freshly isolated B-lymphocytes avoiding the need for cryopreservation. By contrast, the cell lines needed to be cryopreserved to maintain future cell stocks and were found to tolerate the procedure well.

Both cell lines were harvested using EDTA 0.02% (w/v) as described in section 3.3.3.1 (p161). The harvested cell suspension was centrifuged for 5min at 280g and the cell pellet resuspended in 10.1ml of RPMI medium. Cell number and viability was then determined (section 3.3.5, p163). The cell suspension was again centrifuged to pellet the cells which were then resuspended in cold cryosolution (95% (v/v) FBS, 5% (v/v) DMSO) to give a final concentration of 1 x 10^6 viable cells/ml. 1ml aliquots were transferred to cryovials (Fisher Scientific, Leicestershire, UK) and placed into a Cryo1°C freezing container (Fisher Scientific, Leicestershire, UK) with isopropanol allowing controlled rate freezing with a

stepwise decrease in temp of -1°C/min, when incubated at lower temperatures like - 80°C. The cells were left for 48h, at -80°C, in the isopropanol-filled Cryo1°C container and the cryovials were then transferred to -196°C (liquid nitrogen) and stored until required.

3.3.7 CELL RECUPERATION

M^{c}Coys 5A medium was warmed to 37°C in preparation for the extraction of the required frozen cell aliquots. When the medium had been warmed, the cryovials were removed from the liquid nitrogen and heated quickly at 37°C until completely defrosted. As DMSO is toxic to cells, the defrosted cell suspension was transferred to a 25cm^2 culture flask as quickly as possible and 10ml of warmed medium added to de-activate the DMSO. The culture flask was incubated at 37°C for 24h and the medium changed. The cells were then cultured as described previously (section 3.3.4.1, p162).

3.3.8 P-GP DETECTION IN CELL LINES BY FLOW CYTOMETRY

P-glycoprotein was assayed by incubating 1μg/ml UIC2, conjugated to PE, per 5×10^5 cells, for 15 min at room temperature. The antibody controls used were all isotypically matched and used at the same concentration as the primary anti-P-gp antibody, *i.e.* an equivalent amount of IgG2a-PE antibody was added to another aliquot of cells as an isotype-matched negative control. After two additional washes in PBS the cells were resuspended in 0.5ml PBS and analysed by flow cytometry using the following procedure. A control cell sample (unstained cells) was run on the flow cytometer for 2-3min to determine the position of the cell population using forward and side scatter. A gate was established around the unstained control cell population (Fig. 3.3[A], p131) and a region of positivity marked (Fig. 3.3[B], p131). The experimental samples (including the isotype

control) were analysed by counting 10000 gated cells/sample and determining the percent positivity using the previously marked region. Cellular fluorescence associated with UIC2 labelling was compared to that of the IgG2a control and the difference in percentage positivity noted. A sample was considered to be expressing P-gp if the percent positivity was at least 10% greater than the isotypic control value.

3.3.9   PREPARATION OF CELLS FOR PROTEIN AND RNA EXTRACTION

Cells (cell lines & patients' B-lymphocytes) were pelleted in a sterile universal flask by centrifugation at 280g and resuspended in warmed medium for cell counting. The cells were pelleted once more and resuspended in FBS-free medium. The cells were washed 2 more times with FBS-free medium to remove all traces of proteins and a sample containing 5×10^5 cells removed to a cryovial. The cryovial was centrifuged at 280g to pellet the cells and the medium discarded. The cell pellet was snap frozen to -80°C and then transferred to liquid nitrogen for storage.

3.3.10 RNA TECHNIQUES

3.3.10.1 General considerations for RNA work

RNA manipulation is very sensitive to contaminant RNases and therefore a clean area was set aside for RNA work. Any pipettes used were regularly cleaned (3% H_2O_2) and designated for use with RNA-grade solutions only. Sterile tips and microfuge tubes were designated for RNA work only and gloves were changed frequently. RNA-grade reagents were used at all times with sterile nuclease-free water.

3.3.10.2 Extraction of RNA using TRIzol reagent

In accordance with the Trizol reagent protocol (Appendix E) RNA was extracted from the frozen +ve and -ve cell line pellets and the frozen patient samples. The resulting RNA pellet was resuspended in nuclease-free water and stored at -20°C until required (Chomczynski, 1993). Aliquots of these samples as well as portions of a control RNA sample (from isolated mononuclear cells) were run on an agarose gel (1.4%) to determine the integrity of the extracted RNA and therefore the quality of the RNA extraction procedure (section 3.4.2, p168), *i.e.* a gel was run to determine the integrity of the 18S and 28S ribosomal subunits.

3.4   EXPERIMENTAL METHODS

3.4.1   P-GP DETECTION IN PATIENTS BY FLOW CYTOMETRY

55 patients with B-CLL and 10 healthy adult volunteers were analysed in this study. The patient sample population was split into 4 analytical groups to attempt to distinguish any relationship between P-gp expression and treatment modalities: normal volunteers (10 samples); untreated B-CLL patients (33 samples); B-CLL patients treated with drugs not transported by P-gp (20 samples); B-CLL patients being treated with drugs transported by P-gp (CHOP regimen, 2 samples).

The entire patient sample (55 patients) could not be subsequently analysed for P-gp functionality. Therefore 12 of these patients were investigated for P-gp and MDR1 mRNA expression as well as P-gp functionality.

In each case P-gp analysis was carried out on freshly isolated B-lymphocytes (section 3.3.3.2, p161). A cell count was then performed (section 3.3.5, p163) and P-gp expression determined using the procedure described in section 3.3.8 (p165). Previous investigations have used a threshold limit to distinguish between P-gp positive and negative samples. An analytical cut-off point commonly used is that of a greater than 10% increase in P-gp MAb staining compared to an isotype control to denote P-gp positivity and this threshold was initially used in this study.

3.4.2 AGAROSE GEL ELECTROPHORESIS OF RNA SAMPLES FROM ORIGINAL PATIENT SAMPLE

Successful RNA extraction (section 3.3.10.2, p167) was determined by running a portion of the extracted sample on a 1.4% agarose gel using the following procedure. The previously extracted RNA samples were defrosted on ice while 1μl of Ficoll loading buffer was added to labelled 0.5ml microfuge tubes. 5μl of each defrosted RNA sample was then added to the appropriate microfuge tube and the remaining RNA sample was refrozen for PCR analysis the next day. The microfuge tubes were then heated at 65°C for 5 min in a DR1 Block DB-2A Heat Block (Techne, R & D Systems Europe Ltd., Oxon, UK) and placed immediately on ice. The samples (6μl) were loaded into the wells of a 1.4% agarose gel (containing 10mg/mL ethidium bromide) and the gel was run at 100V for 20-25min on GNA-100 gel electrophoresis apparatus (Pharmacia). The RNA was visualised with a 3 U.V™ Transilluminator UVP U.V. box. Pictures were taken and stored using the "Electrophoresis documentation and analysis system 120" from Kodak Digital Science.

3.4.3 DETECTION OF HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE (HGPRT) BY PCR

RNA integrity was also investigated by performing a RT-PCR reaction on the HGPRT housekeeping genes endogenous to many mammalian cells. This PCR amplifies a specific region of the gene encoding HGPRT from cellular RNA as a method to confirm template quality. This test was carried out to confirm that the extracted RNA was of amplifiable quality.

The HGPRT sense (5', CTTGCTGGTGAAAAGGACCC, 3') and antisense (5', GTCAAGGGCACATCCTACAA, 3') primers were used with the 'Access' RT-PCR kit following the manufacturers protocol (Appendix F). The template was replaced by nuclease-free water for the negative control to check for contamination of the reaction and a previously tested sample of RNA was used as the positive control. The PCR products were run on a 2% agarose gel (containing 10mg/mL ethidium bromide) at 100V for 20-25min on GNA-100 gel electrophoresis apparatus (Pharmacia). The PCR products were visualised with a 3 U.V^{TM} Transilluminator UVP U.V box at 302nm. Pictures were taken and stored using the 'Electrophoresis documentation and analysis system 120' from Kodak Digital Science.

3.4.4 ANALYSIS OF MDR1 GENE EXPRESSION BY REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

The cell samples used for RT-PCR were isolated simultaneously with cells isolated for flow cytometry analysis and stored in liquid nitrogen until required (section 3.3.9, p166). The samples were stored in liquid nitrogen as quickly as possible to delay any RNA

degradation. However, the primers specific for amplification of the MDR1 gene yield small cDNA PCR products (Fig. 3.7A & 3.7B) so that even partially degraded mRNA could have been used as a template (Noonan *et al.*, 1990). The stored samples were analysed in a RT-PCR reaction using the 'Access' RT-PCR system from Promega (Appendix F) with MDR1 sense (5', CCCATCATTGCAATAGCAGG, 3') and anti-sense primers (5', GTTCAAACTTCTGCTCCTGA, 3'). Optimisation of this reaction was carried out with regards to magnesium sulphate ($MgSO_4$) concentration by comparing the PCR product for the water control and the P-gp −ve & P-gp +ve cell lines using concentrations of $MgSO_4$ at 1mM, 2mM or 3mM. Following optimisation of the $MgSO_4$ concentration to 1mM, the final MDR1 mRNA RT-PCR product was separated on a 2% agarose gel (containing 10mg/mL ethidium bromide).

A diagnostic enzyme restriction was included as an extra test in order to differentiate between the MDR1 & MDR3 product in the event of non-specific primer annealing and amplification of MDR3 (O'Driscoll *et al.* 1993). If the amplified product was MDR1 then it would be digested by the restriction endonuclease *Mae 1* (Appendix G) such that a 84bp and 73bp fragment would be distinguishable on a 10% polyacrylamide gel after separation.

Additionally, a gel-purified RT-PCR sample from the P-gp positive cell line control (MESSA/Dx-5, p148) as well as 5 random patient RT-PCR samples (also gel-purified) were sent to MWG Biotech (UK) Ltd. (Milton Keynes) for sequencing analysis. The gel purification was performed by Dr. J Farrugia who subsequently sent 10µL of each DNA sample as well as the MDR1 sense and antisense primers (120 µL of each at 10pmol/µL) shown above.

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3.4.5 STATISTICAL ANALYSIS

Where possible chi square analysis was performed on the results in the patient contingency tables comparing one sample group of patients to another with regards to P-gp presence or absence. All other statistics in this chapter were calculated using a one-way ANOVA followed by post-hoc analysis at the 95% confidence interval ($P < 0.05$) using the Bonferroni test.

3.5   RESULTS

This study was designed to determine the expression of MDR1 mRNA and P-gp in B-CLL patients.

3.5.1 ANALYSIS OF P-GP AND MDR1 mRNA EXPRESSION IN B-CLL PATIENTS

3.5.1.1 Flow Cytometry detection of P-gp in isolated samples

	Normals	B-CLL - Untreated	B-CLL - single agent therapy	B-CLL – combination therapy
Pgp positive	2	10	6	0
Pgp negative	8	23	14	2
Total sample No.	10	33	20	2

Table 3.5 P-gp positivity in four patient sample groups using flow cytometry analysis

B-lymphocytes were isolated from patients with B-CLL and analysed for the presence of P-gp using the MAb UIC2. The PBMCs from normal volunteers were isolated and dual stained with CD19 -FITC and UIC2-PE for the analysis of P-gp on B-lymphocytes. Normals; healthy volunteers: B-CLL untreated; patients not treated with chemotherapy: B-CLL single agent therapy; patients treated with single agent regime, *e.g.* chlorambucil, fludarabine: B-CLL combination therapy; patients being treated with CHOP combination regime. There was no significant association between P-gp positivity and B-CLL or single agent treatment of B-CLL by chi square analysis. No statistical analysis was possible for the patients on combination therapy as the sample population was too small.

Fig 3.14 Typical P-gp positivity profile in patient sample
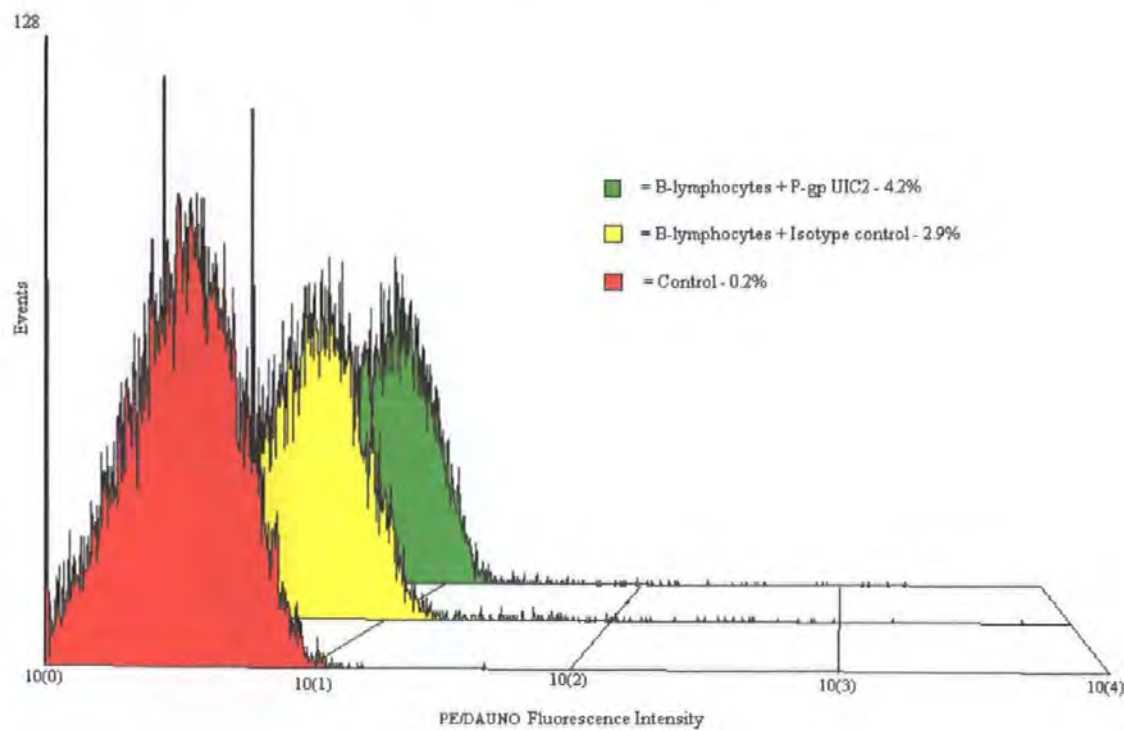
Fig 3.14 The patient B-lymphocytes were gated and analysed for P-gp expression. The samples were compared with each other using ANOVA followed by post-hoc analysis using the Bonferroni test. No significant difference was found between UIC2 P-gp staining v isotype control.

3.5.1.2 Reverse transcriptase-polymerase chain reaction (RT-PCR) of isolated samples

RT-PCR analysis on 50 patient samples and cell line controls suggests a much higher P-gp positivity than flow cytometry (Tables 3.5, p171 & 3.6 and Fig. 3.15 & 3.16, p175 & 176).

	Normals	B-CLL - Untreated	B-CLL - single agent therapy	B-CLL – combination therapy
Pgp positive	5	26	15	2
Pgp negative	0	7	0	0
Sample No.	5	33	15	2

Table 3.6 P-gp positivity in four sample groups using RT-PCR analysis Cells from healthy volunteers and B-CLL patients were isolated and analysed for the presence of MDR1 mRNA using RT-PCR. Normals; healthy volunteers: B-CLL untreated; patients not treated with chemotherapy: B-CLL single agent therapy; patients treated with single agent regime, *e.g.* chlorambucil, fludarabine: B-CLL combination therapy; patient treated with CHOP combination regime. There was no significant association between P-gp positivity and B-CLL single agent therapy by chi square analysis. No statistical analysis was possible for the patients on combination therapy as the sample population was too small.

The comparative increase in P-gp detection using RT-PCR versus flow cytometry had to be confirmed however as amplification of the MDR1 product also had the potential to result in the amplification of the MDR3 product and both are very similar in size (O'Driscoll *et al.* 1993).

The *Mae1* restriction enzyme recognises sites specific to the MDR1 gene product. Therefore, the amplification of the MDR1 product was confirmed by digesting the RT-PCR product with *Mae1*. The ability of this enzyme to digest the samples for P-gp +ve cell line

and four B-CLL patients suggested that the amplified gene product in all these cases is that of the MDR1 gene and not MDR3 (Fig. 3.17, p177).

However, sequencing of the amplified gene products was the only way to absolutely confirm that the RT-PCR products were definitely a product of MDR1 gene amplification. Unfortunately, of the 6 gel-purified RT-PCR samples sent for sequencing, only 4 were able to be analysed. These four samples consisted of; Pgp +ve control cell line MESSA/Dx-5, patient 3, patient 4 and patient 8 (Table 3.7).

Sample	Sequence
Pgp +ve MESSA/Dx-5	tgttgtctggacagcayygaaagataagaaagaactagaaggttctgcgaagatcgctactgaagcaatagaa aacttccgaaccgttgtttctttgactcaggagcagaagtttgaaca
Patient 3	gttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgctactgaagcaatagaa aacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
Patient 4	ttgtctggacagcactgaaagataagaaagaactagaaggtgctgggaagatcgctactgaagcaatagaaaa cttccgaaccgttgtttctttgactcaggagcagaagtttgaac
Patient 8	aatgttgtctggaca(a)gcactgaaagataagaaagaactagaaggtgctgggaagatcgctactgaagcaat agaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac

Table 3.7 Sense sequences for the Pgp+ve control cell line, MESSA/Dx-5, and some random patient samples Gel purified RT-PCR samples were sent to MWG Biotech (UK) Ltd. for sequencing using the sense primer for MDR1 (p170).

The homology of the above sense sequences, compared to the known MDR1 sequence, was assessed by performing a BLAST search at the NCBI internet site (www.ncbi.nlm.nih.gov/blast/Blast.cgi). The Blast search in each case returned a hit of ≥ 95% homology compared to homo sapiens P-glycoprotein (PGY1) mRNA (Appendix H) confirming the RT-PCR product to be that of the amplified MDR1 gene and not the MDR3 gene.

Fig 3.15 MDR1 RT-PCR product from healthy volunteers and patient samples.

[A]

[B]

[C]
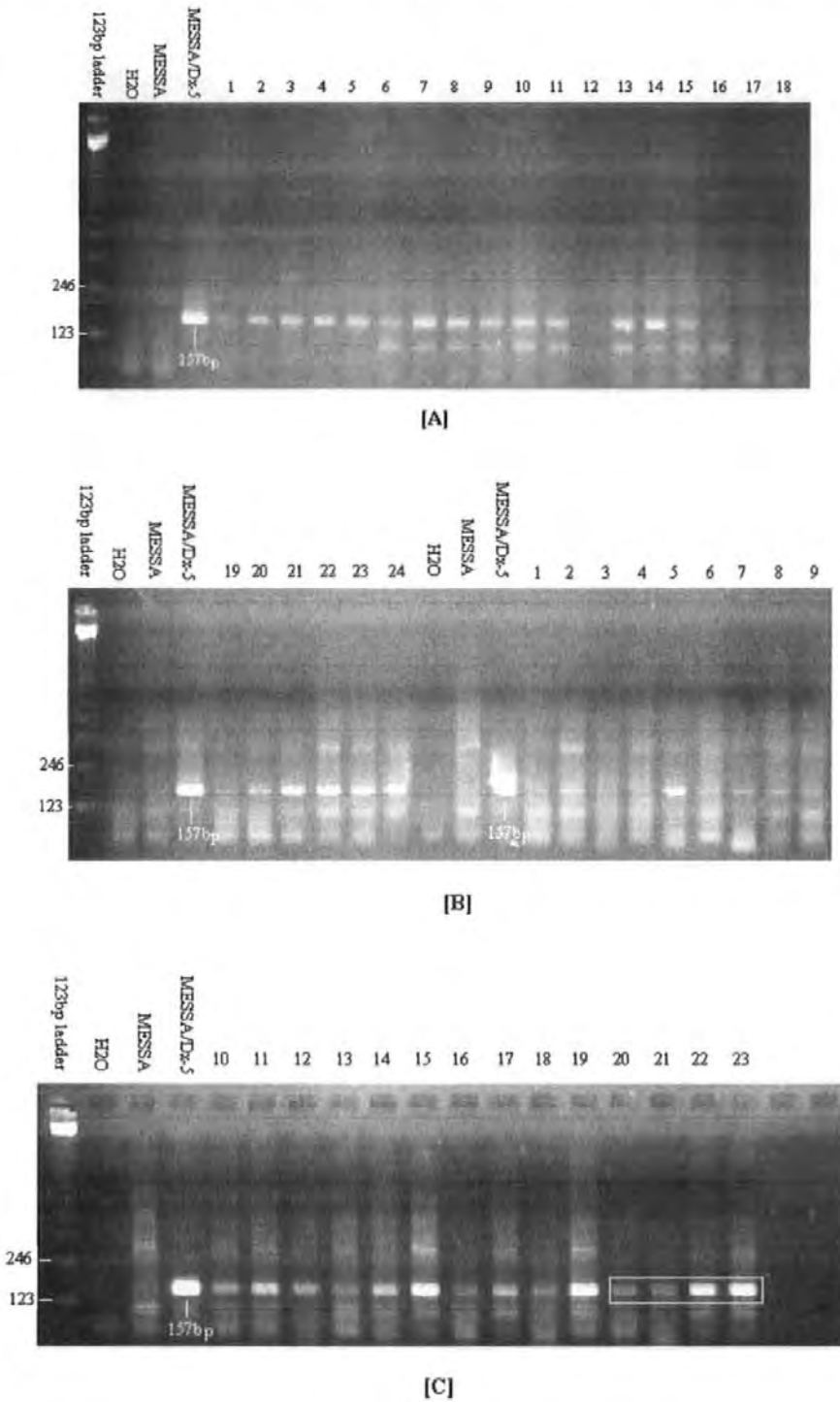
Fig 3.15 One single agarose gel with 3 separate combs, [A], [B] and [C]. H2O; nuclease free water: MESSA; P-gp -ve cell line: MESSA/Dx-5; P-gp +ve cell line. [A] Lanes 1-5, healthy volunteers; lanes 6-18, untreated patients. [B] Lanes 19-23, patients on single agent therapy; lane 24, patient on combination therapy; lanes 1-9, untreated patients. [C] Lanes 10-14, untreated patients; lanes 15-19, patients on single agent therapy; and lanes 20-23, patient on combination therapy over time *i.e.* rectangle; same patient over time.
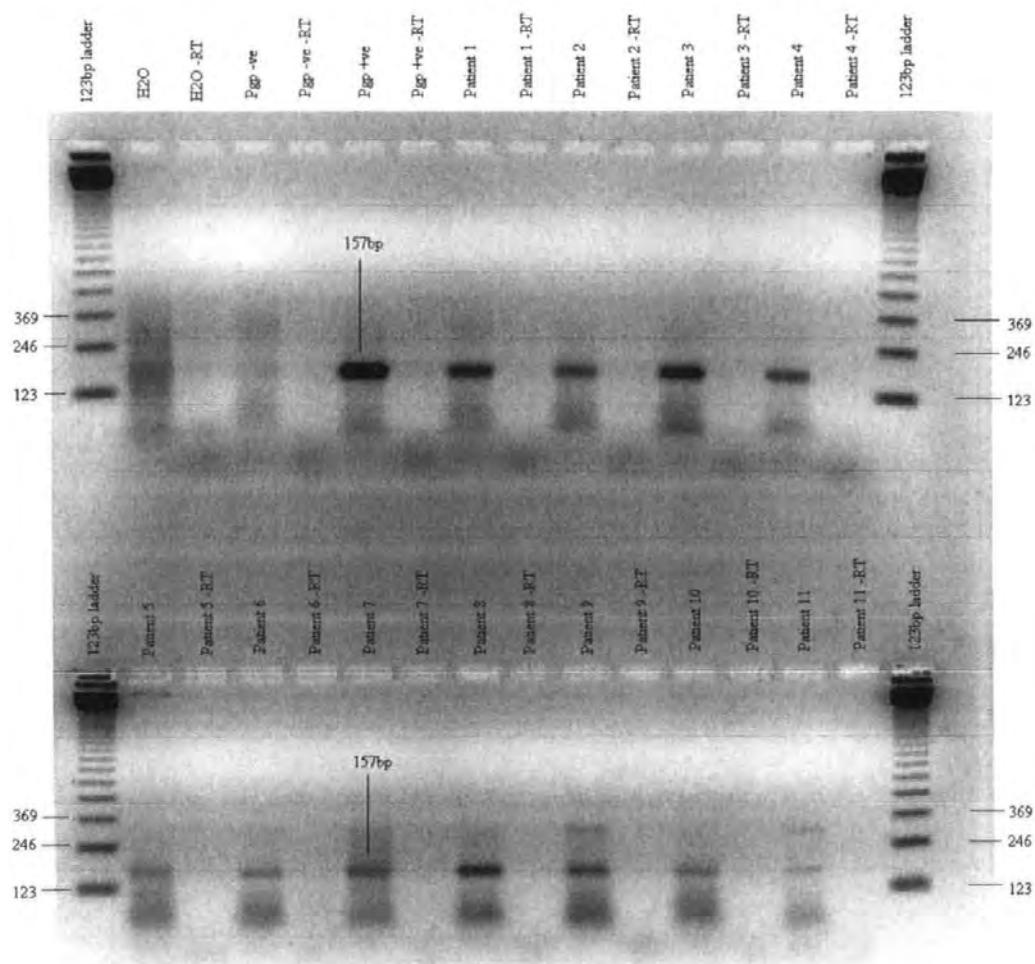
Fig 3.16 H2O; nuclease free water: P-gp -ve; MESSA cell line: P-gp +ve; MESSA/Dx-5 cell line: 'N' -RT; PCR analysis without the reverse transcription, where 'N' = H2O, P-gp -ve, P-gp +ve or patient sample.

Fig 3.17 PAGE of digestion of patient PCR product using *MaeI* restriction enzyme

1 = 25bp ladder
2 = Pgp +ve MESSA/Dx-5
3 = Pgp +ve MESSA/Dx-5 Digest
4 = Patient 8
5 = Patient 8 Digest
6 = Patient 9
7 = Patient 9 Digest
8 = Patient 10
9 = Patient 10 Digest
10 = Patient 11
11 = Patient 11 Digest
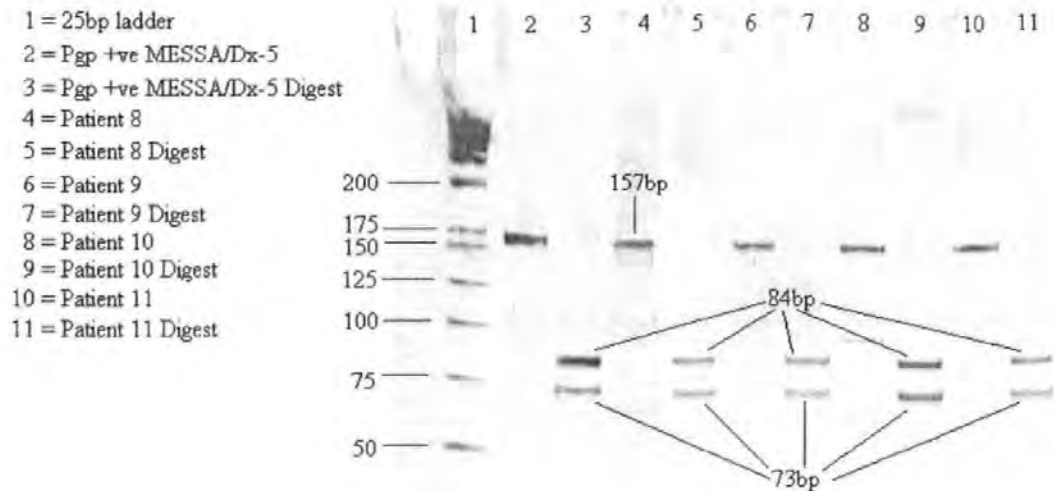
Fig 3.17 Digestion of the 157bp P-gp product results in 2 fragments, 84 & 73bp in size.

3.6   DISCUSSION

With flow cytometry, the initial inability to detect P-gp in the majority of the patient sample (16/55 were positive) and healthy adult volunteer samples (2/10 were positive) (Table 3.5, p171 & Fig. 3.14, p172) suggested that, in general, this protein was absent. In addition, it was only possible to compare the effect of single agent therapy on P-gp expression as other treatment group sizes were too small for statistical analysis. In this case, with chi square analysis, no association was found between single agent therapy and P-gp expression.

The low P-gp incidence by flow analysis could perhaps be explained by a lack of sensitivity of the assay. Although the P-gp-positive and -negative control cell lines demonstrated that the assay was able to distinguish between presence and absence of the P-gp molecule (section 3.3.1.1, p151), the resistant cell line has been engineered to overexpress P-gp and may not reflect the true clinical levels of this protein.

Uncertainty of the level at which MDR expression becomes significant within the clinical context means that although P-gp may be detected in only small quantities or not at all, its limited presence may still render a patient prone to drug resistance. In view of this, the investigation was directed towards analysis of the presence of P-gp at a molecular level.

The detection of mRNA in the patient group and volunteer samples throws into question the sensitivity of the flow cytometry results. The presence of MDR1 mRNA (Fig. 3.15, p175; Fig. 3.16, p176; Table 3.6, p173) in 43/50 samples suggests that flow cytometry analysis may not be sensitive enough to detect very low quantities of P-gp which may still be significant in the clinical setting.

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These seemingly inconsistent results have been found by others. In 1993, Wall *et al.* found that some of their patients were positive for MDR1 mRNA detection but negative when analysed with the P-gp monoclonal antibody, MRK 16. It is unlikely that such a similar result occurred due to coincidence and some explanations for this are proposed.

1] The flow cytometry analysis may not have been sensitive enough to detect P-gp. Either the levels of P-gp were extremely low or the intensity from the fluorescent conjugate was not sufficient for detection (Beck *et al.* 1996). However, this investigation used UIC2/PE as the chosen monoclonal antibody/fluorescent conjugate marker, for ease of acquisition and its use and reliability have been well documented (Beck *et al.* 1996; Schinkel *et al.* 1993).

2] It may be that the mRNA is constitutively expressed in circulating peripheral B-lymphocytes from patients and volunteers and translation into the P-gp protein requires another stimulus, perhaps in the form of an intense chemotherapeutic insult. This would sustain the recent findings by Webb *et al.* (1998) who reported low amounts of P-gp in B-CLL patients in general, but a higher level in B-CLL patients treated with P-gp transportable chemotherapy agents. Only 2/55 patients in this study were treated with a chemotherapy regime containing P-gp transportable agents (CHOP regime containing, doxorubicin and vincristine). It is possible that without this specific toxic insult mRNA translation into the P-gp protein may not occur and detection of P-gp would therefore not be possible.

3] It has been reported that point mutations in the MDR1 gene are related to phenotypic diversity and that alternative splice variants of P-gp can exist (Greenberger *et al* 1994). These could presumably lead to variations in structure of the resultant P-gp protein and,

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depending on which variant is expressed in the disease, the use of certain monoclonal antibodies may have negative results due to their incapacity to bind to P-gp.

Although the above are all acceptable explanations, there is another interesting possibility which may offer a better explanation for the varying results.

4] Aberrant sialylation of lymphocytes in CLL, leading to epitope masking, has been well characterised (Brown *et al.* 1985) and cells have a reported increase in levels of sialyl transferase. Treatment with neuraminidase, thus affecting sialylation patterns, was reported to greatly increase the amount of detectable fluorescence by the antibody MRK16 in samples of lymphocytes from CLL patients (Cumber *et al.* 1990). This epitope masking might explain, in part, the discordance in P-gp staining of clinical samples and it is possible that the low P-gp flow cytometry results are due, at least in part, to this phenomenon.

Of the two patients on a combination regime containing P-gp transportable drugs (CHOP regime containing doxorubicin and vincristine), the MDR1 mRNA PCR signal varied with each subsequent analysis over an extended period of time (Fig. 3.15[C], p175). It would have been interesting to compare the PCR product over time by performing quantitative PCR, however, this PCR technique was not utilised during this investigation. Additionally, there were few patients available being treated with anthracycline-containing combination regimes thus limiting the analysis of MDR1 mRNA expression and P-gp presence versus anthracycline treatment. Nevertheless, Webb *et al.* (1998) showed a low incidence of MDR1 mRNA (and P-gp protein) in most B-CLL patients and volunteers analysed, while patients treated with prolonged combination regimes showed a correlation between time and amounts of MDR1 mRNA.

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3.7   CONCLUSION

In general, a smaller proportion of patients (16/55) express P-gp when analysed by flow cytometry compared to RT-PCR analysis (43/50) for MDR1 mRNA presence (29% and 86%, respectively). These conflicting results make it difficult to determine the role of P-gp in these clinical samples.

However, while this chapter alone does not determine the role of P-gp-associated MDR in B-CLL it does help to show that P-gp and MDR1 mRNA can be expressed in B-CLL patients. Many investigators have previously assumed that the presence of the MDR1 mRNA indicates P-gp expression even though transcription of a gene into its correspondent RNA is not necessarily an indication of translation. As suggested by the results, P-gp may be present in amounts not detectable by a flow cytometer, therefore, functionality studies are needed to either help support or refute the question of P-glycoprotein presence and activity in B-CLL drug resistance.

4. P-GLYCOPROTEIN FUNCTION AND MODULATION

4.1   INTRODUCTION

The investigations which were performed to determine the expression of MDR1 mRNA and P-gp in B-CLL patients are described in Chapter 3. Flow cytometric studies showed that a small proportion of patients (16/55) expressed P-gp while RT-PCR studies indicated the majority (43/50) were positive for MDR1 mRNA. This is not unusual and, in B-CLL, one explanation given for conflicting literature data on P-gp expression is the use of different analytical techniques. The limitations of these techniques have now been recognised and the recommendation for the analysis of P-gp-associated MDR is that detection of MDR1 mRNA and P-gp should be combined with P-gp functionality studies (Beck *et al*. 1996).

4.1.1   P-GP FUNCTIONALITY STUDIES

4.1.1.1 Drug accumulation/efflux

Functionality experiments were conducted firstly to evaluate the presence of low levels of P-gp, not detected using flow cytometric methods, and secondly to assess the potential of IFN-α to modulate P-gp function.

Functionality studies examining drug accumulation and efflux apply the principle that P-gp function can be followed by monitoring the intracellular levels of P-gp transportable agents, with inherent colour or fluorescent properties.

The theory that resistant cells expressing the P-gp molecule will exhibit a lower fluorescent intensity than sensitive cells has been demonstrated by many groups *in vitro* (Green *et al*.

2001; Guerci *et al.* 1995; Lizard *et al.* 1995; Merlin *et al.* 1994; Merlin *et al* 2000; Quesada *et al.* 1996; Scala *et al* 1991)

The agents chosen to investigate P-gp functionality in this study were rhodamine 123 (rho123, a commonly used P-gp transportable agent fluorescing in the green spectrum) and daunorubicin (the anthracycline drug of interest fluorescing in the red spectrum).

4.1.1.2 Drug toxicity

Successful anticancer treatment relies on the intracellular accumulation of anticancer agents to therapeutic concentrations. The resultant toxicity is generally cell death, and this concept has been used when investigating P-gp function; since fewer cells expressing functional P-gp will die compared to non-P-gp expressing cells, due to lower intracellular drug accumulation.

Various analytical methods have been employed to monitor the relationship between drug toxicity and cell viability including colourimetric cytotoxicity assays such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and detection of cell death by flow cytometry analysis (Table 4.1).

Basis of viability/toxicity assay	Detection method
Detection of esterase enzyme activity thus monitoring enzyme activity and cell permeability	Use of fluorogenic esterase substrates *e.g.* calcein
Determination of cell membrane changes with nucleic acid stains	Cell-impermeable nucleic acid stains *e.g.* propidium iodide
Determination of cell metabolic activity by analysis of cell oxidation/reduction	Use of water-soluble, colourless probes prone to oxidation or reduction resulting in precipitation of coloured product *e.g.* reduction of tetrazolium salts to coloured formazan crystals *i.e.* MTT assay
Detection of apoptotic cell death markers	Detection of apoptosis-related cell surface markers and apoptosis-associated enzymes *e.g.* detection of phosphatidyl serine externalisation or caspase activity.
Monitoring of changes in cellular ion gradients set by ion pumps and ion channels	Measure specific ion levels or transmembrane potentials as well as changes in cell energy *e.g.* chemiluminescent detection of ATP levels

Table 4.1. Analysis of drug toxicity and cell viability

4.1.1.2.1 Detection of cell death by flow cytometry

It has been reported that the B-lymphocytes in B-CLL die by apoptosis (Mainou-Fowler *et al.* 1994; Panayiotidis *et al* 1994). Successful anticancer treatment is generally manifest as cell death, usually by apoptosis and, as this event can be influenced by the presence or absence of P-gp, it was considered a good physiological end-point with which to study the functionality and modulation of P-gp in B-CLL. It was postulated that a lower proportion of P-gp +ve (resistant) cells would undergo apoptosis compared to P-gp -ve (sensitive) cells due to lower intracellular drug accumulation.

The widespread involvement of apoptosis in various disease states is becoming more apparent and its increasing importance has led to the development of a variety of

techniques for detection of this type of cell death. Many techniques use flow cytometry and can measure a variety of parameters such as changes in light scatter, cell organelles or plasma membrane. Some common methods for apoptosis detection are listed in Table 4.2.

Analytical parameter	Method
Light scatter	Forward scatter and dead cell exclusion
Membrane changes	Hoechst and Propidium Iodide staining
	Annexin V staining
	Merocyanine 540 staining
Changes to cell organelles	CMXRos staining
	Rhodamine 123 staining

Table 4.2 Techniques for detection of apoptosis by flow cytometry From Imperial Cancer Research webpage - http://www.icnet.uk/axp/facs/davies/apop.html

Annexin V Staining

One of the membrane changes in the early to intermediate stages of apoptosis is the translocation of phosphatidylserine (PS) from the inner surface of the cell membrane to the outside. It is possible to detect PS by using FITC-labelled Annexin V, which is a Ca^{++} dependent phospholipid-binding protein. By combining staining of Annexin-FITC with propidium iodide (PI), a cell profile can be obtained where live cells are negative for both dyes, dead (necrotic) and late apoptotic cells are positive for both and early and intermediate apoptotic cells are positive only for Annexin-FITC.

As Annexin V was supplied conjugated to the green fluorochrome, FITC, it was postulated that multiparameter analysis would also be possible, *e.g.* the detection of apoptotic B-

lymphocytes (using Annexin-FITC/PI and a CD19 MAb conjugated to PE) could be attempted simultaneously to monitoring changes in intracellular daunorubicin concentrations. This would have meant the use of more than one red fluorochrome, leading to a strong degree of overlap in the red spectrum. Although compensation techniques can be done to limit the overlap on the flow cytometer (section 3.1.1.2.2, p132), this was considered to be an inaccurate method of analysis. Another option was time lapse analysis, where the detection of each red fluorescence is delayed and analysed sequentially. This would have meant reconfiguring the flow cytometer and proved impractical .

Single parameter analysis of apoptotic cells was a logical alternative, but using daunorubicin as the toxic agent for inducing apoptosis meant that the cells already contained a red fluorescing agent, the drug itself, in the intracellular matrix. In addition, the two resulting red fluorescent wavelengths are identical, making even the most intricate colour compensation impossible.

Due to these difficulties, another method of apoptosis detection was investigated where there was no necessity for fluorescent analysis. Instead the MTT cytotoxicity assay was employed (below and Appendix I). The intracellular concentration of P-gp transportable agents in resistant cells should be lower than sensitive cells, therefore resulting in a decreased amount of cell death and a more intense spectrophotometric reading. The spectrophotometric reading is converted into '% survival fraction' using the calculation shown below:

% survival fraction   =   experimental reading x 100%
                            control reading

4.1.1.2.2   MTT assay

This assay is commonly used for the detection of damage caused by cytotoxic or cytostatic agents (Carmichael *et al.* 1987; Mosmann, 1983). When exposed to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), the dehydrogenases localised in viable mitochondria reduce this salt to a purple formazan crystal which can then be solubilised in acidified isopropanol. The intensity of the purple colour is then determined using a spectrophotometer where the intensity of the purple colour is proportional to cell viability. Cells expressing functional P-gp demonstrate greater colour intensity and thus greater cell viability, than P-gp -ve cells.

The advantage of the MTT assay is that it is rapid and easy to perform, and gives a good indication of the effect of P-gp modulators. In the presence of modulators the percentage of resistant cells killed should approach that of sensitive cells. However, this technique is not without disadvantages. Glutathione-S-transferase enzymes (GSTs) are present to protect cells from toxic compounds (section 1.5.1, p44) and it has been reported that GSTs have the ability to reduce MTT *in vitro* (York *et al.* 1998). As GSTs, especially GST-π, are known to be overexpressed in a variety of tumour cells (Tew, 1994), it is possible that these enzymes could interfere with an MTT assay in cancer cell lines and this has to be considered when interpreting results as a measure of cell viability.

Alternative assays for monitoring cell viability and cell death are becoming available and more recent developments include colourimetric apoptotic assays detecting mitochondrial enzyme activity such as caspase 3 (Choi *et al.* 2000; Finucane *et al.* 1999; Komoriya *et al.* 2000). Caspase analysis also provides a fast and effective tool and seems to be a more robust technique avoiding interference from endogenous, intracellular compounds such as

GSTs. However, this type of assay also has limitations, the major disadvantage being the incorrect use of this assay when toxic insult causes cell death by a caspase-independent mechanism. Unless the mechanism of cell death triggered by a specific toxic agent is known, it would be inaccurate to use a technique limited to the detection of only one specific mechanism of cell death. In addition, recent reports have highlighted the ability of P-gp to protect cells from caspase-dependent apoptosis. However, P-gp was not shown to protect from caspase-independent cell death (Johnstone *et al.* 1999). The potential of P-gp to interfere with a caspase-dependent assay was considered, in addition to the above outlined advantages and limitations, and it was decided to analyse cell viability with the MTT assay.

4.1.2   P-GP MODULATION

4.1.2.1 Modulation of P-gp by Drugs

The presence of P-gp can also be demonstrated by the use of specific inhibitors of P-gp function.. This group of inhibitors/modulators is chemically diverse and includes calcium channel blockers such as verapamil and nifedipine.

These modulators have a varying degree of potency with regards to their ability to inhibit P-gp function but the well characterised modulators such as verapamil allow detection of the presence and function of P-gp. In the presence of a modulator the efflux of the fluorescent compound is impeded and therefore the fluorescent intensity of resistant cells should return towards the level seen in the sensitive cells.

Although these modulators have a significant role *in vitro*, use in the clinical setting has been limited by their toxicity *in vivo* (Fisher and Sikic, 1995; Sikic 1993). New compounds are being continuously designed and tested in an attempt to combat the serious problem of drug resistance, but, although novel compounds such as PSC 833 (currently in clinical trials) have potential, there are no P-gp modulators currently available for effective use in the clinic.

4.1.2.2 Cytokine modulation of P-gp

The general aim upon discovering a resistance mechanism is to find methods to overcome this resistance. The problem of *in vivo* toxicity experienced with many P-gp modulators means that cytokines present attractive candidates for use as reversing agents and publications regarding their modulatory abilities are of great interest (Fogler *et al.* 1995; Scala *et al.* 1991; Tambur *et al.* 1998).

4.1.2.2.1   Cytokine modulation of P-gp function in B-CLL

Although the role of P-gp-associated MDR in B-CLL has yet to be clarified, some groups have detected the presence and function of P-gp in a number of patient samples (Arai *et al.* 1997; Webb *et al.* 1998). Continued investigation into the role of P-gp-associated MDR could confirm the presence and function of P-gp in a large percentage of the patient population. Consequently, if P-gp transportable drugs are included in the therapeutic regime, these patients may benefit from treatment with a P-gp modulator that does not possess the *in vivo* toxicity associated with the majority of agents studied so far. The publication by Scala *et al.* (1991) concerning the reversal of doxorubicin resistance by recombinant interferon-alpha (IFN-α) was therefore of particular interest as IFN-α is

already used for disease treatment and, although it has its own inherent toxicity *in vivo*, the effective IFN-α concentration reported by Scala *et al* would give acceptable toxicity *in vivo* (Guttermann *et al.* 1982).

The aims of this study were, subsequently, to investigate the function and modulation of P-gp in both sensitive (MESSA) and resistant (MESSA/Dx-5) cell lines as well as isolated patient B-lymphocytes and to determine the effect of IFN-α as a potential P-gp modulator.

The effects of two concentrations of verapamil (2μM & 20μM) were compared (Quesada *et al.* 1996) while the IFN-α - in the cell line investigations - was used at what was determined experimentally, in this study & others, to be a non-toxic concentration *i.e.* 500 I.U/ml (Guttermann *et al.* 1982). For analysis of IFN-α modulation in patient B-lymphocytes, three concentrations of IFN-α were investigated; 500 I.U/ml; 1000 I.U/ml; 5000 I.U/ml. The rationale behind the use of higher IFN-α concentrations was the suggestion that higher concentrations of IFN-α were tolerated in the clinic (personal communication).

4.2   MATERIALS

Unless otherwise stated, materials used in this study were of at least analytical grade and obtained from Sigma-Aldrich Company Ltd., Poole, UK.

All water used was 3MΩ water, filtered and deionised to 18MΩ by an Elga UHQ II water purifier (Elga Ltd., Buckinghamshire, UK).

4.2.1   GENERAL MATERIALS

Equipment

• Heraeus Labofuge 400 Centrifuge (Jencons Scientific Ltd., Bedfordshire, UK).

Reagents

• Absolute ethanol (Hayman Ltd., Essex, UK).

4.2.2   TISSUE CULTURE MATERIALS

Equipment

• 24-well culture plates (Sarstedt Ltd., Leicestershire, UK)

• 9ml vacuette Sodium Heparin blood tubes (Greiner Labortechnik Ltd., Gloucestershire, UK)

• 96-well culture plates (Sarstedt Ltd., Leicestershire, UK)

• Epson LX-300 Plate reader (Dynex Technologies, Middlesex, UK)

• Sterile Universal tubes [25ml] (Greiner Labortechnik Ltd., Gloucestershire, UK)

Reagents

- Detachabead CD19 (Dynal (UK) Ltd., Merseyside, UK)

- Dynabeads M-450 CD19 (Dynal (UK) Ltd., Merseyside, UK)

- Fetal Bovine Serum (Sigma-Aldrich Company Ltd., Poole, UK).

- Lymphoprep (Nycomed (UK) Ltd., Birmingham, UK)

- MESSA human uterine sarcoma cell line (ECACC, Wiltshire, UK)

- MESSA/Dx-5 human uterine sarcoma cell line (ECACC, Wiltshire, UK)

- McCoys 5A buffered medium (with sodium bicarbonate & 25mM HEPES) (Sigma-Aldrich Company Ltd., Poole, UK).

- RPMI 1640 buffered medium (with sodium bicarbonate & 25mM HEPES) (Sigma-Aldrich Company Ltd., Poole, UK).

4.2.3 FLOW CYTOMETRY MATERIALS

Equipment

- Epics Elite Flow cytometer (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

- 7mL Falcon tubes (Greiner Labortechnik Ltd., Gloucestershire, UK)

- Epics Elite Flow software, version 4.02 (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

Reagents

- Annexin-V binding buffer (PharMingen, Oxford, UK)

- Annexin-V-FITC (PharMingen, Oxford, UK)

- Annexin-V-FITC Apoptosis Kit (Calbiochem-Novabiochem, CN Biosciences, Nottingham, UK)

- MAb CD19-FITC (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

- MAb CD5-PE (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

- MAb IgG1-FITC mouse isotype control (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

- MAb IgG2a-PE mouse isotype control (Beckman/Coulter (UK) Ltd., Buckinghamshire, UK)

- MAb UIC2-PE (Immunotech Ltd., Bedfordshire, UK)

4.3   GENERAL METHODS

4.3.1 PREPARATION OF GENERAL SOLUTIONS/MATERIALS

4.3.1.1 Culture Media

This was prepared as described in section 3.3.2.1, p159

4.3.2   CELL LINE HARVEST/PASSAGE & B-LYMPHOCYTE ISOLATION

This was used to maintain stocks of cell lines, and isolate patient B-lymphocytes. Isolation of PBMCs from normals was not performed as functionality experiments required a pure cell type to enable final experimental analysis. Both P-gp -ve and P-gp +ve cell lines (MESSA & MESSA/Dx-5, respectively) were passaged when they reached confluence. All procedures were carried out under aseptic conditions, using methods described in section 3.3 (p151).

4.4   EXPERIMENTAL METHODS

4.4.1   CELL LINE GROWTH CURVES

21, 25cm^2 culture flasks of MESSA (sensitive) and MESSA/Dx-5 (resistant) cells were plated at 30000cells/cm^2 and grown in buffered McCoys 5A medium (section 3.3.2.1, p159) for 144h. 3 flasks were harvested in triplicate at each of the following time points 0, 24, 48, 72, 96, 120, and 144h, and a viable cell count performed using Trypan Blue. Average number of viable cells/ml was plotted against time to determine the growth curve

profile for each cell line. For each cell line N = 3, where N is the number of separate experiments performed.

4.4.2   CYTOTOXICITY INVESTIGATIONS

4.4.2.1 Interferon-alpha toxicity in sensitive and resistant cell lines

MESSA (sensitive) and MESSA/Dx-5 (resistant) cells were plated at 30000cells/cm^2/well in 96-well plates (180µl cell solution/well) and grown for 48h, *i.e.* until the start of the exponential phase of growth. After 48h the medium was changed and the cells were further cultured in the presence or absence of IFN-α at 500, 1000, 10,000, 50,000, and 100,000 I.U./ml for 72h using the following procedure. A sterile, freshly made stock solution of 1 x 10^6 I.U./ml IFN-α (3ml) was diluted to 5000, 10,000, 100,000, 500,000 I.U./ml in sterile, plastic Bijou bottles using sterile, buffered McCoys 5A medium (section 3.3.2.1, p159). 20µl of each solution, including the stock, was added to the appropriate wells (6 replicates per concentration), giving a 10 fold dilution of each. After 72h of further cell growth the surviving cell fraction was evaluated by MTT assay (Appendix I) compared to the control containing no IFN-α.

4.4.2.2 Daunorubicin toxicity in sensitive and resistant cell lines

This method followed that described in section 4.4.2.1, but after 48h the medium was changed and the cells were further cultured for 72h in the presence or absence of daunorubicin at 0.2, 0.8, 1.2, 2, and 6 µg/ml using the following procedure. A sterile, freshly made stock solution of 368µg/ml daunorubicin in 18MΩ water was diluted to 2, 8, 12, 20, and 60µg/ml in sterile, plastic Bijou bottles using sterile, buffered McCoys 5A

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medium (section 3.3.2.1, p159). 20μl of each daunorubicin solution was added to the appropriate wells (6 replicates per concentration) giving a 10 fold dilution of each. After 72h of further cell growth the surviving cell fraction was evaluated by MTT assay (Appendix I) compared to the control without daunorubicin.

4.4.2.3 Effect of P-gp modulators on daunorubicin toxicity in sensitive and resistant cell lines

MESSA and MESSA/Dx-5 cells were plated at 30000cells/cm^2/well in 96-well plates (160μl cell solution/well) and grown for 48huntil the start of the exponential phase of growth. After 48h the medium was changed and the cells were further cultured for 72h in the presence or absence of daunorubicin at 0.2, 0.8, 1.2, 2, and 6 μg/ml with or without 2μM verapamil, 20μM verapamil or 500 I.U./ml IFN-α using the following procedure. A sterile, freshly made stock solution of 368μg/ml daunorubicin in 18MΩ water was diluted to 2, 8, 12, 20, and 60μg/ml in sterile, plastic Bijou bottles using sterile, buffered McCoys 5A medium (section 3.3.2.1, p159). Similarly, sterile, freshly made stock solutions of 600μM verapamil and 1 x 10^6 I.U./ml IFN-α in 18MΩ water were diluted to 20μM, 200μM and 5000 I.U./ml, respectively. 20μl of each daunorubicin solution was added to the appropriate wells in triplicate with or without 20μl of 20μM verapamil, 200μM verapamil or 5000 I.U./ml IFN-α, giving a 10 fold dilution of each daunorubicin solution as well as each verapamil and IFN-α solution. After 72h of further cell growth the surviving cell fraction was evaluated by MTT assay (Appendix I) compared to the control without daunorubicin.

To determine the effect of modulators on daunorubicin toxicity a comparison was made between the IC_{50} values (concentration of daunorubicin resulting in 50% growth inhibition) for daunorubicin toxicity alone and daunorubicin toxicity in the presence of modulator. The IC_{50} value was determined from the survival curve generated for each cell line (Figs 4.4 & 4.5, p209 & 212) by reading the concentration (X-axis) equating to 50% survival (Y-axis).

4.4.2.4 Interferon-alpha toxicity in isolated patient B-lymphocytes

Isolated patient B-lymphocytes were plated at $180000cells/cm^2$/well in 96-well plates ($180\mu l$ cell solution/well) and cultured in the presence or absence of IFN-α at 500, 1000, 10,000, 50,000, and 100,000 I.U./ml for 72h using the following procedure. A sterile, freshly made stock solution of 1×10^6 I.U./ml IFN-α (3ml) was diluted to 5000, 10,000, 100,000, 500,000 I.U./ml in sterile, plastic Bijou bottles using sterile, buffered RPMI medium (section 3.3.2.1, p159). $20\mu l$ of each solution, including the stock, was added to the appropriate wells (6 replicates/concentration), giving a 10 fold dilution of each. After 72h of cell culture the surviving cell fraction was evaluated by MTT assay (Appendix I) compared to the control containing no IFN-α.

4.4.2.5 Daunorubicin toxicity in isolated patient B-lymphocytes

This method followed that described in section 4.4.2.4, but cells were cultured for 72h in the presence or absence of daunorubicin at 0.2, 0.8, 1.2, 2, and 6 $\mu g/ml$ using the following procedure. A sterile, freshly made stock solution of $368\mu g/ml$ daunorubicin in $18M\Omega$ water was diluted to 2, 8, 12, 20, and $60\mu g/ml$ in sterile, plastic Bijou bottles using sterile, buffered RPMI cell culture medium. $20\mu l$ of each daunorubicin solution was added to the appropriate wells (6 replicates per concentration) giving a 10 fold dilution of each. After

72h of cell culture the surviving cell fraction was evaluated by MTT assay (Appendix I) compared to the control without daunorubicin.

4.4.3   DRUG ACCUMULATION/EFFLUX STUDIES

4.4.3.1 Cell lines

MESSA and MESSA/Dx-5 cells were plated at 30000 cells/cm^2/well in 24-well plates (1.8ml cell solution/well) and grown for 48huntil the start of the exponential phase of growth (Fig 4.1, p204). Fresh medium was then added to 6 control wells, as well as medium containing 2μM rho123 (6 wells) and 2μg/ml daunorubicin (6 wells). The cells were immediately returned to incubate at 37^{o}C for 30min for drug accumulation (Quesada *et al.* 1996). After the 30min incubation, 3 wells of each treatment were harvested (section 3.3.3.1, p161) and the contents of each well placed into a separate tube on ice until flow cytometry analysis. In the remaining wells the medium was replaced with drug-free medium and the cells incubated for a further 90min to allow for drug efflux (Quesada *et al.* 1996). At the end of the 90min period the remaining wells were harvested, as above, and the cells washed and then placed on ice for flow cytometry analysis (section 3.1.1, p126).

Analysis was carried out according to the following procedure. A control cell sample (unstained cells) was run on the flow cytometer for 2-3min to determine the position of the cell population. A gate was established around the unstained control cell population and a region of positivity marked *i.e.* from the end of the unstained cell peak to the end of the X-axis (Fig. 3.3, p131). The triplicate experimental samples were analysed by counting 10000 gated cells per sample and determining the percent positivity using the previously marked region. The mean percent positivity for each triplicate was calculated and accumulation of

2μM rho123 and 2μg/ml daunorubicin after 30min incubation was compared to the harvested control cells (no addition of either rho123 or daunorubicin), while efflux of rho123 and daunorubicin was compared to the rho123 and daunorubicin samples harvested after the 30min accumulation period.

4.4.3.2 Isolated patient B-lymphocytes

Isolated B-lymphocytes (section 3.3.3.2, p161) were plated out at 1 x 10^6 cells/well in 24-well plates. Each well contained a final volume of 1.8ml and as patient B-lymphocytes have been reported to spontaneously apoptose *in vitro* (Mainou-Fowler *et al.* 1994), the following procedure was performed as quickly as possible. 200μl of a sterile, freshly made 20μM rhodamine 123 stock solution or 20μg/mL daunorubicin stock solution was added to each of 6 wells, giving a final concentration of 2μM and 2μg/mL, respectively, and the cells incubated at 37°C for 30min for drug accumulation (Quesada *et al.* 1996). At the end of the 30min incubation period the cells in 3 rho123-containing wells, 3 daunorubicin-containing wells and 3 control wells (no rho123 or daunorubicin addition) were harvested (section 3.3.3.2, p161), the contents of each well placed into a separate tube on ice until flow cytometry analysis. In the remaining wells the medium was replaced with drug-free medium and the cells incubated for a further 90min, to allow drug efflux. At the end of the 90min period the remaining wells were harvested, as above, the cells washed and then placed on ice for flow cytometry analysis (section 3.1.1, p126). Analysis was carried out as in section 4.4.3.1.

4.4.4 MODULATION OF DRUG ACCUMULATION/EFFLUX

For cell lines and patient B-lymphocytes , flow cytometric studies of P-gp modulation were conducted over a 2h time period rather than the 72h time period used for MTT studies. As these flow cytometry experiments were looking at drug accumulation and efflux, the lack of a significant difference in cell death between the two cell lines at 2μg/ml daunorubicin was not thought to pose a problem with this shorter type of experiment. In addition, a clear difference in drug accumulation between the sensitive (MESSA) cells and resistant (MESSA/Dx-5) cells could be seen at this concentration and, for this reason, 2μg/ml daunorubicin was used for flow cytometry P-gp modulation analysis.

4.4.4.1 Cell lines

MESSA and MESSA/Dx-5 cells were plated at 30000 cells/cm^2/well in 24-well plates (1.8ml cell solution/well) and grown for 48h until the start of the exponential phase of growth (Fig 4.1, p204). Fresh medium was then added to 15 control wells, medium containing 2μM rho123 to 15 wells and medium containing 2μg/ml daunorubicin to a further 15 wells. The cells were immediately incubated at 37°C for 30min for drug accumulation (Quesada *et al.* 1996). After the 30min incubation, 3 wells of each treatment were harvested (section 3.3.3.1, p161), the contents of each well placed into a separate tube on ice until flow cytometry analysis. In the remaining wells the medium was replaced (in triplicate) with medium with or without P-gp modulator, either verapamil (2μM or 20μM) or IFN-α (500 I.U./ml), and the cells incubated for a further 90min to allow drug efflux with or without modulation (Quesada *et al.* 1996). At the end of the 90min period the remaining wells were harvested, as above, and the cells washed and then placed on ice for flow cytometry analysis (section 3.1.1, p126). Analysis was carried out as in section 4.4.3.1

where modulation of rho123 and daunorubicin efflux was assessed by comparing intracellular fluorescence intensities to those of cells having effluxed 2μM rhodamine123 in the absence of modulators.

4.4.4.2 Isolated patient B-lymphocytes

Isolated B-lymphocytes were obtained from 6 untreated and 6 treated B-CLL patients.

Isolated B-lymphocytes (section 3.3.3.2, p161) were plated out as described in section 4.4.3.2. 200μl of sterile RPMI medium (section 3.3.2.1, p159) was added to 21 control wells and 200μl of a sterile, freshly made 20μM rhodamine 123 stock solution in addition to a 20μg/mL daunorubicin stock solution was each added to 21 wells, giving a final concentration of 2μM and 2μg/mL, respectively. The cells were then incubated at 37°C for 30min for drug accumulation (Quesada *et al.* 1996). At the end of the 30min incubation period the cells in 3 rho123-containing wells, 3 daunorubicin-containing wells and 3 control wells (RPMI only) were harvested (section 3.3.3.2, p161), the contents of each well placed into a separate tube on ice until flow cytometry analysis. In the remaining wells the medium was replaced (in triplicate) with medium with or without P-gp modulator (verapamil (2μM or 20μM) or IFN-α (500 I.U./ml, 1000 I.U./ml or 5000 I.U./ml) and the cells incubated for a further 90min to allow for drug efflux with or without modulation. At the end of the 90min period the remaining wells were harvested, as above, the cells washed and then placed on ice for flow cytometry analysis (section 3.1.1, p126). Analysis was carried out as in section 4.4.3.1 where modulation of rho123 and daunorubicin efflux was assessed by comparing intracellular fluorescence intensities to those of B-lymphocytes having effluxed 2μM rhodamine123 in the absence of modulators.

4.4.5 STATISTICAL ANALYSIS

All statistics in this chapter were calculated using a one-way ANOVA followed by either a student t-test or post-hoc analysis at the 95% confidence interval ($P < 0.05$) using a Dunnett's test.

4.5 RESULTS

4.5.1 CELL LINES

4.5.1.1 Growth curves

Analysis of MESSA (sensitive cell line) and MESSA/Dx-5 (resistant cell line) growth curves showed the exponential phase of growth, in both cell lines, commencing at 48h (Fig. 4.1). As deleterious effects on cell growth or viability are more noticeable during the exponential phase of growth, drug additions and incubations during cell line experimentation were started at 48h.

Fig. 4.1 Rate of cell growth for MESSA (sensitive) and MESSA/Dx-5 (resistant) cell lines
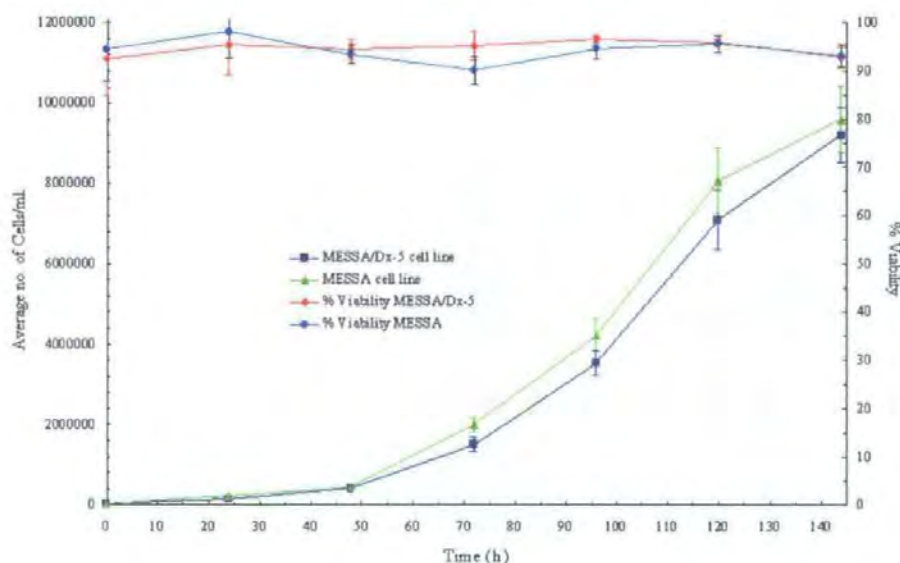
Fig. 4.1 21, 25cm^2 flasks of MESSA and MESSA/Dx-5 cells were grown for 144h. 3 flasks were harvested in triplicate at each of the following time points 0, 24, 48, 72, 96, 120, and 144h, and a viable cell count performed using Trypan Blue. For each cell line N = 3, where N is the number of separate experiments performed.

4.5.1.2 Cytotoxicity investigations

4.5.1.2.1   Interferon-alpha toxicity in sensitive and resistant cell lines

Initial investigation into IFN-α toxicity had dissimilar results in the sensitive and resistant cells (Fig. 4.2).

Fig. 4.2 IFN-α toxicity in sensitive and resistant cell lines
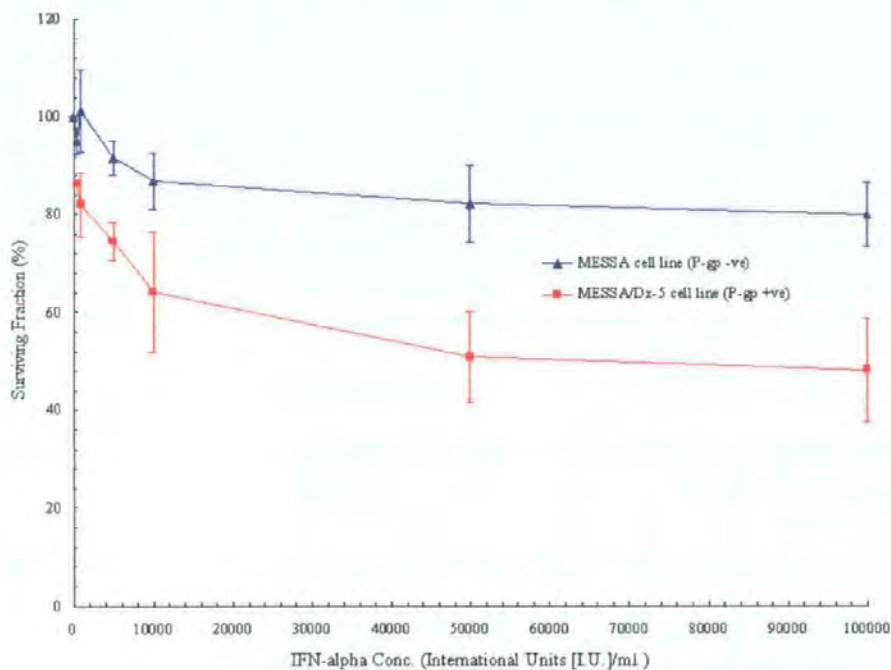
Fig. 4.2 MESSA and MESSA/Dx-5 cells were grown for 48h until exponential phase of growth and then incubated for 72h in the presence of varying concentrations of IFN-α. Cell viability was determined using the MTT assay. IFN-α was significantly more toxicity to MESSA/Dx-5 cells than the MESSA cells (P < 0.05), using ANOVA. For each cell line N = 3, where N is the number of separate experiments performed.

Minimum cell death was observed at a concentration of 500 I.U. in both cell lines with greater growth inhibition occurring on increasing IFN-α concentrations, however, the

resistant cell line showed a greater susceptibility to IFN-α toxicity than the sensitive one (section 4.6, p236).

As the data presented in Fig 4.2 shows least cell death in both cell lines to occur at 500 I.U./ml and both Gutterman *et al.* (1982) and Scala *et al.* (1991) suggested that an IFN-α concentration of 500I.U./ml could be well tolerated *in vivo*, 500I.U./ml was the concentration of IFN-α used for consequent P-gp modulation analysis in cell lines.

4.5.1.2.2   Daunorubicin toxicity in sensitive and resistant cell lines

Prior to P-gp modulation analysis in both MESSA and MESSA/Dx-5 cell lines, the effect of differing concentrations of daunorubicin was established using the MTT assay (Appendix I). Fig. 4.3 demonstrates the effectiveness of the P-gp molecule in the resistant cell line MESSA/Dx-5. Increased cell survival was seen in this cell line at daunorubicin concentrations of 0.2 – 1.2µg/ml compared to the sensitive MESSA cell line, indicating the presence of a lower intracellular toxic drug concentration due to the function of the P-gp efflux pump. At higher concentrations of 2-6µg/ml, however, no significant difference in percent cell kill was seen.

Fig. 4.3 Degree of daunorubicin toxicity in sensitive and resistant cell lines
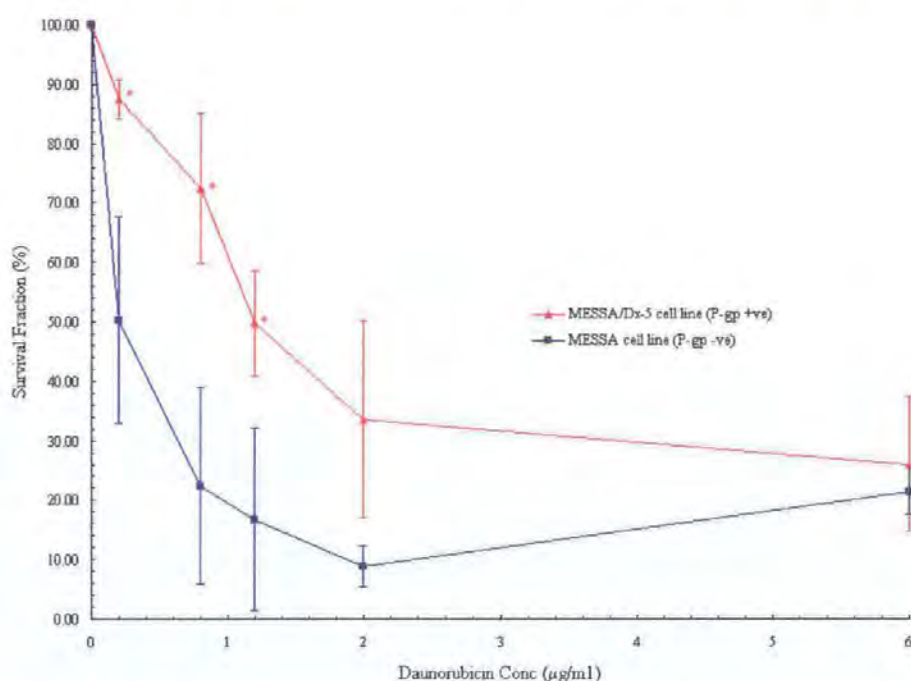
Fig. 4.3 MESSA and MESSA/Dx-5 cells were grown for 48h until exponential phase of growth and then incubated for 72h in the presence of varying concentrations of daunorubicin. Cell viability was determined using the MTT assay. * : statistically significant ($P < 0.05$), using ANOVA followed by student t-test. For each cell line $N = 3$, where N is the number of separate experiments performed.

It was speculated that at higher concentrations of daunorubicin, the P-gp molecules had become saturated with daunorubicin over the 72h period and, although functioning, were not able to expel sufficient amounts of intracellular drug to prevent a similar degree of cell death as seen in the sensitive cell line. Even though the difference between cell death in sensitive and resistant cell lines was not significantly different at concentrations of 2 and 6 µg/ml, for completeness of analysis these higher concentrations were still included in P-gp modulation investigations using MTT assay.

The flow cytometry investigations into P-gp modulation, using both cell lines and patient B-lymphocytes (sections 4.4.3.1, p199 & 4.4.3.2, p200), were conducted over a shorter

time period than that using MTT analysis with total experiment times of 2h v 72h, respectively. As these flow cytometry experiments were looking at drug accumulation and efflux, the lack of a significant difference in cell death between the two cell lines at 2μg/ml daunorubicin was not thought to pose a problem with this shorter type of experiment. In addition, a clear difference in drug accumulation between the sensitive (MESSA) cells and resistant (MESSA/Dx-5) cells could be seen at this concentration and, for this reason, 2μg/ml daunorubicin was used for flow cytometry P-gp modulation analysis.

4.5.1.2.3   Effect of P-gp modulators on daunorubicin toxicity in sensitive and resistant cell lines

The investigation continued with the examination of the effect of P-gp modulators on the sensitivity of cell lines to daunorubicin. These effects were first determined with the sensitive cell line using 2μM & 20μM verapamil and IFN-α at a concentration of 500 I.U./ml.

As MESSA does not express P-gp it was speculated that these modulators would have no effect on the sensitivity to daunorubicin.
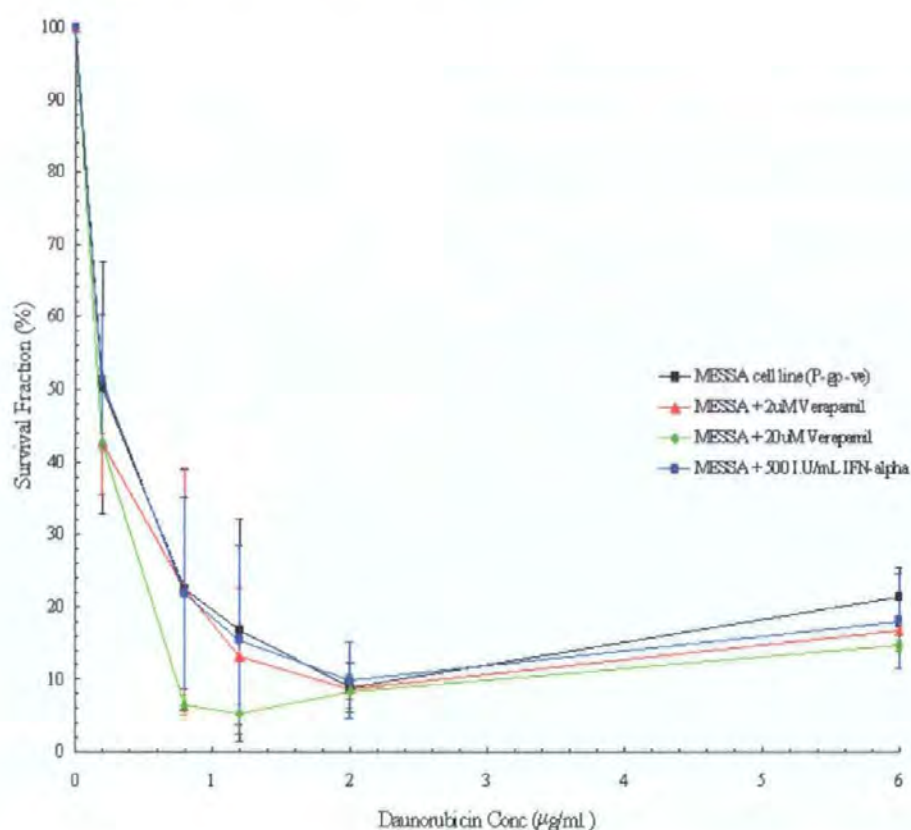
Fig. 4.4 MESSA cells were grown for 48h until exponential phase of growth and then incubated for 72h in the presence of varying concentrations of daunorubicin +/- modulator (2μM verapamil, 20μM verapamil or 500I.U./ml IFN-α). Cell viability was determined using the MTT assay. No significant difference was found between control cells and those exposed to the different modulators.

Fig. 4.4 confirms this hypothesis, showing that treatment with 2μM & 20μM verapamil, as well as 500I.U./ml of IFN-α, did not significantly modulate the daunorubicin sensitivity of MESSA cells *i.e.* at the daunorubicin concentrations used, cells were inhibited to 50-90% of control (no daunorubicin) and both verapamil and IFN-α did not change the sensitivity to daunorubicin (Tables 4.3-4.5 & Fig. 4.4).

In contrast, treatment with 2μM & 20μM of verapamil decreased the resistance of MESSA/Dx-5 cells while IFN-α did not significantly change the sensitivity to daunorubicin, *i.e.* MESSA/Dx-5 cells alone were resistant to the daunorubicin concentrations, but after 72h treatment with verapamil (2μM & 20μM) there was growth inhibition to 50% of control with 200ng/ml of daunorubicin (Tables 4.3-4.5 & Fig. 4.5, p212).

Although not investigated, it is speculated that higher doses of IFN-α would not result in significantly greater daunorubicin toxicity and would instead result in toxicity due to IFN-α alone (Fig. 4.2, p205). In addition, the concentration of IFN-α was being kept to that reported to be tolerated *in vivo* rather than increasing the concentration to more toxic levels (Gutterman *et al.* 1982).

	IC_{50} to MESSA cells (μg/ml)			IC_{50} to MESSA/Dx-5 cells (μg/ml)		
VER. TIME	IC_{50} +	IC_{50} -	Gain	IC_{50}+	IC_{50}-	Gain
72h	0.17	0.20	1.18	0.20	1.20	6.00

Table 4.3 Effect of 2μM verapamil on daunorubicin toxicity in sensitive and resistant cell lines MESSA and MESSA/Dx-5 cells were incubated for 72h with different concentrations of daunorubicin +/- 2μM verapamil. Cell survival was measured using the MTT assay. The IC_{50} was measured in the presence (IC_{50} +) and absence (IC_{50} -) of verapamil. The gain in sensitivity was defined by the IC_{50} - /IC_{50} + ratio. The gain for the MESSA/Dx-5 cells was statistically significant ($P < 0.001$) but not for the MESSA cells, using student t-test.

	IC$_{50}$ to MESSA cells (µg/ml)			IC$_{50}$ to MESSA/Dx-5 cells (µg/ml)		
VER. TIME	IC$_{50}$ +	IC$_{50}$ -	Gain	IC$_{50}$+	IC$_{50}$-	Gain
72h	0.17	0.20	1.18	0.20	1.20	6.00

Table 4.4 Effect of 20µM verapamil on daunorubicin toxicity in sensitive and resistant cell lines MESSA and MESSA/Dx-5 cells were incubated for 72h with different concentrations of daunorubicin +/- 20µM verapamil. Cell survival was measured using the MTT assay. The IC$_{50}$ was measured in the presence (IC$_{50}$ +) and absence (IC$_{50}$ -) of verapamil. The gain in sensitivity was defined by the IC$_{50}$ -/IC$_{50}$ + ratio. The gain for the MESSA/Dx-5 cells was statistically significant (P < 0.001) but not for the MESSA cells, using student t-test.

	IC$_{50}$ to MESSA cells (µg/ml)			IC$_{50}$ to MESSA/Dx-5 cells (µg/ml)		
IFN TIME	IC$_{50}$ +	IC$_{50}$ -	Gain	IC$_{50}$+	IC$_{50}$-	Gain
72h	0.20	0.20	1.00	1.06	1.20	1.13

Table 4.5 Effect of 500I.U./ml IFN-α on daunorubicin toxicity in sensitive and resistant cell lines MESSA and MESSA/Dx-5 cells were incubated for 72h with different concentrations of daunorubicin +/- 500I.U./ml IFN-α. Cell survival was measured using the MTT assay. The IC$_{50}$ was measured in the presence (IC$_{50}$ +) and absence (IC$_{50}$ -) of IFN-α. The gain in sensitivity was defined by the IC$_{50}$ -/IC$_{50}$ + ratio. The gains for the MESSA/Dx-5 and MESSA cells were not statistically significant.

Fig. 4.5 Effect of 2μM & 20μM verapamil and 500I.U./ml IFN-α on daunorubicin toxicity in resistant cell line, MESSA/Dx-5
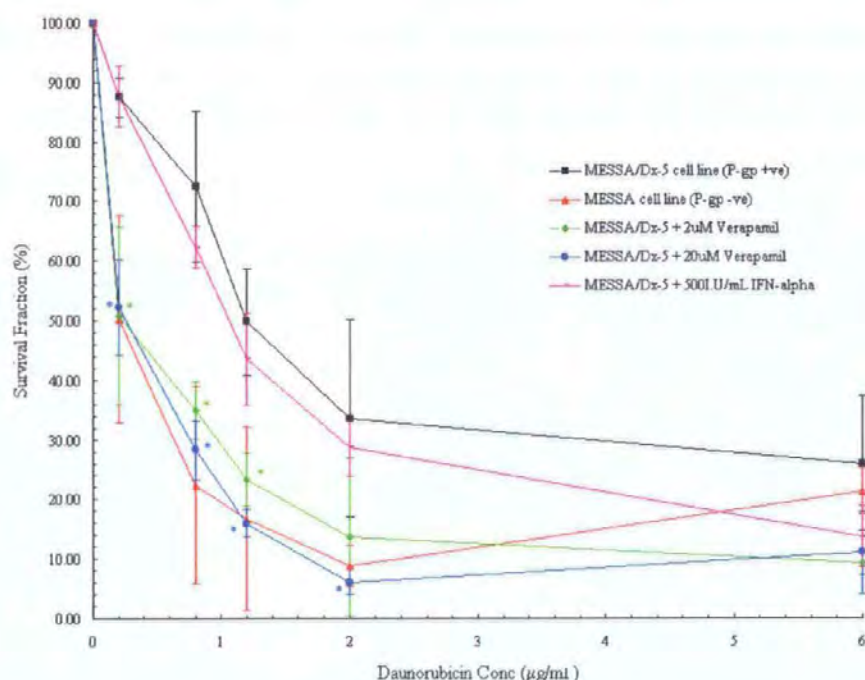
Fig. 4.5 MESSA/Dx-5 cells were grown for 48h until exponential phase of growth and then incubated for 72h in the presence of varying concentrations of daunorubicin +/- modulator (2μM verapamil, 20μM verapamil or 500I.U./ml IFN-α). Cell viability was determined using the MTT assay. * = statistically significant ($P < 0.05$), using ANOVA followed by student t-test.

4.5.1.3 Drug accumulation/efflux studies

To confirm that verapamil was decreasing resistance by increasing intracellular drug concentrations, rho123 and daunorubicin accumulation was measured by flow cytometry.

The two cell lines were characterised by determining total rhodamine 123 (rho123) accumulation in addition to total daunorubicin accumulation. As a well characterised P-gp transportable agent, rho123, was thought to be an ideal second agent for the investigation of P-gp-associated drug resistance. Intracellular accumulation was investigated by comparing the fluorescence intensities of each compound after loading an equal number of cells from each cell line with $2\mu M$ rho123 and $2\mu g/ml$ daunorubicin for 30min (Quesada *et al.* 1996).

Greater accumulation of both compounds was observed in the MESSA sensitive cell line compared to the MESSA/Dx-5 resistant cell line (Figs. 4.6 & 4.7). The resistant cells showed a large population of cells achieving a fluorescent intensity (x-axis) similar to the control cells (no drug loading) verifying the presence of an active efflux mechanism. Monoclonal antibody investigations showed this active efflux to be P-gp related (section 3.3.1.1, p151).

Fig. 4.6 Rhodamine 123 accumulation in sensitive and resistant cell lines
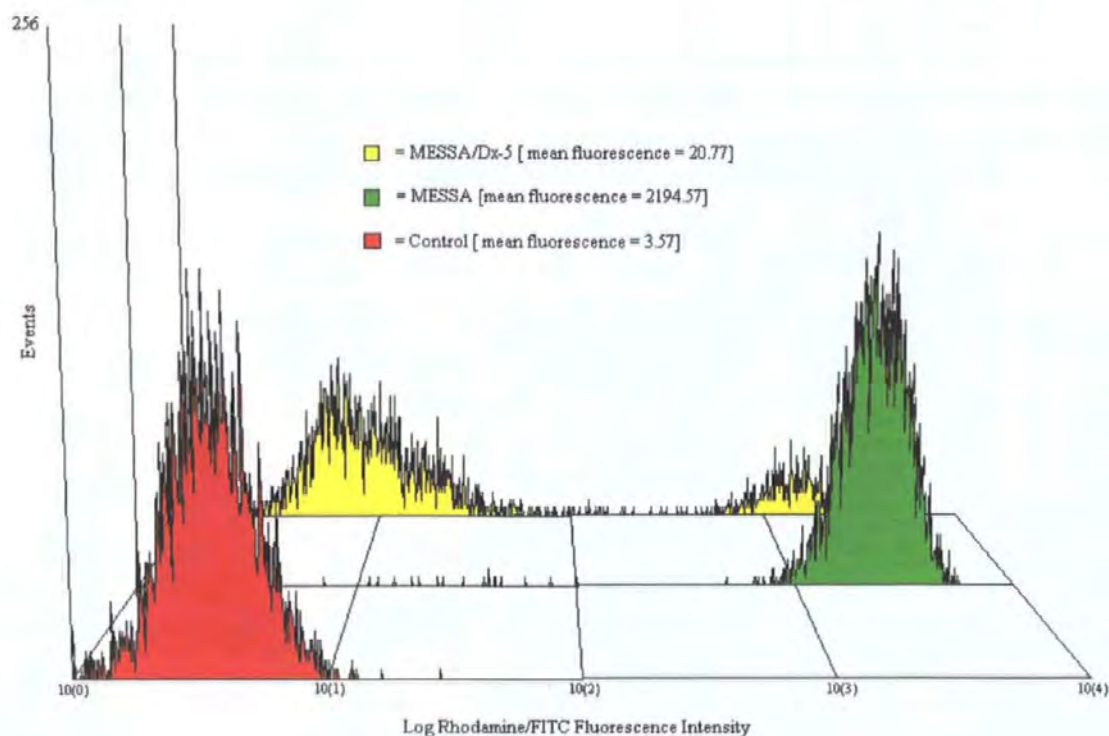
Fig. 4.6 After gating, a region of positivity was marked, using the control population (no drug loading) as the reference, *i.e.* 10^1-10^4 units of fluorescence intensity. The difference in sensitivity between each cell line was easily distinguishable on single fluorescence histograms: the MESSA cells not expressing P-gp showed a significantly stronger accumulation of rho123 and strong positivity ($P < 0.05$ using student t-test) than the MESSA/Dx-5 cells expressing P-gp which demonstrated a weaker accumulation of rho123.

Fig. 4.7 Daunorubicin accumulation in sensitive and resistant cell lines
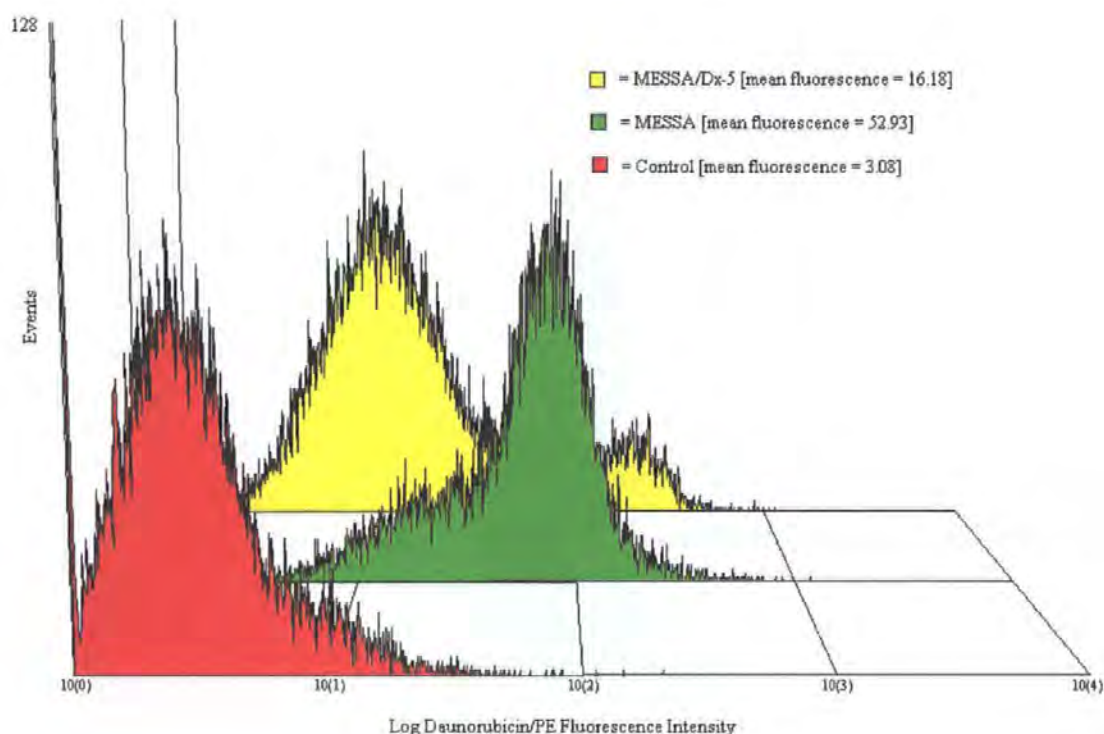
Fig. 4.7 After gating, a region of positivity was marked, using the control population (no drug loading) as the reference. The difference in sensitivity between each cell line was easily distinguishable on single fluorescence histograms: the MESSA cells not expressing P-gp showed a significantly greater accumulation of daunorubicin ($P < 0.05$ using student t-test) than the MESSA/Dx-5 cells expressing P-gp.

It was noted that for both rho123 and daunorubicin, the results revealed a small sub-population in the resistant cells demonstrating similar fluorescence intensity (x-axis) to the sensitive cell line. As the MESSA/Dx-5 cells were selected to overexpress P-gp, it was speculated that this sub-population represented a minority of MESSA/Dx-5 cells either devoid of the P-gp protein or a sub-population expressing non-functional P-gp, therefore

exhibiting characteristics identical to those of the sensitive cells. The presence of this sub-population was noted in the remainder of the cell line experiments.

4.5.1.4 Modulation of drug accumulation/efflux

The activities of the P-gp reversing agent, verapamil, as well as IFN-α, were evaluated from green (rhodamine) and red (daunorubicin) fluorescence histograms. Verapamil was used at concentrations of $2\mu M$ and $20\mu M$, while IFN-α was used at 500I.U./ml.

Comparisons were initially performed on the sensitive MESSA cells where little modulation was expected to occur due to the absence of the target P-gp protein (Figs. 4.8 & 4.9).

Fig. 4.8 Rhodamine 123 accumulation, efflux, and P-gp modulation in sensitive cell line
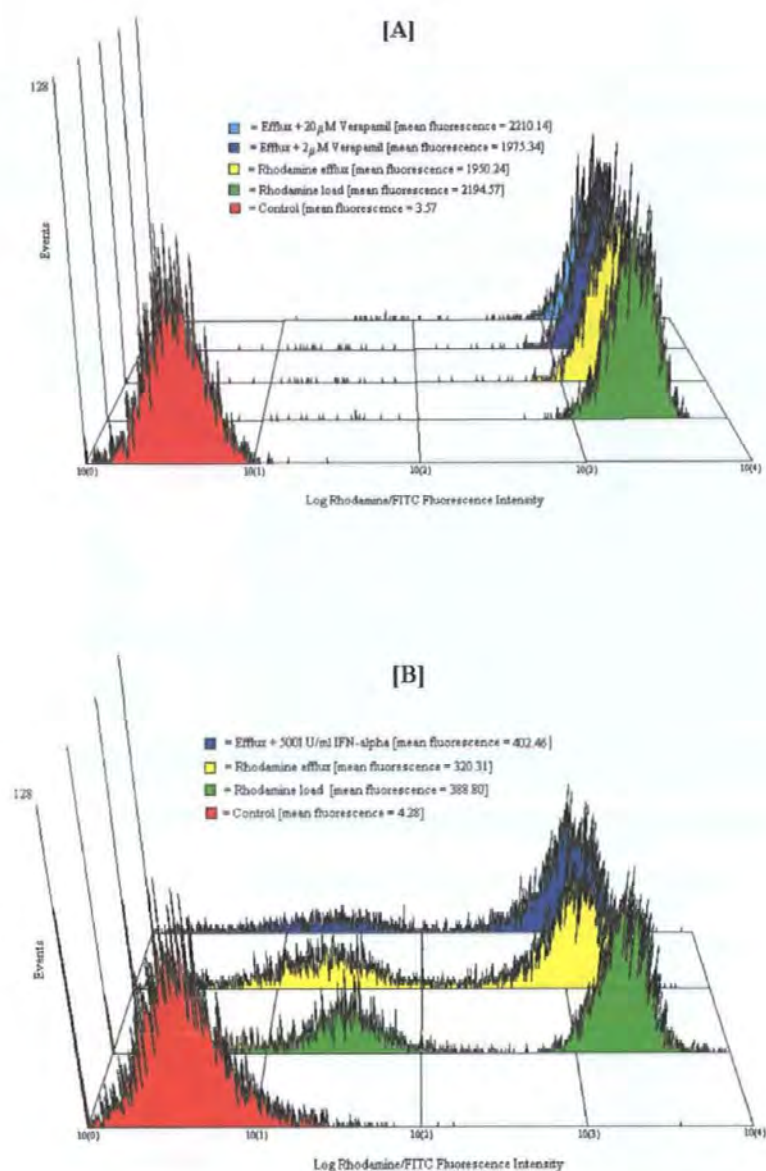
Fig. 4.8 [A]: After 30min drug loading MESSA cells were exposed to fresh medium +/- verapamil (2μM & 20μM) and left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of cells having effluxed 2μM rho123 in the absence of modulators. [B]: After 30min drug loading MESSA cells were exposed to medium +/- IFN-α (500I.U./ml) and left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of cells having effluxed 2μM rho123 in the absence of modulators. Control: MESSA cells not loaded with rho123. No significant modulation was detected with verapamil or IFN-α - $P > 0.05$, using ANOVA followed by Dunnett's post-hoc analysis.

Fig. 4.9 Daunorubicin accumulation, efflux, and P-gp modulation in sensitive cell line

[A]

Legend:
- = Efflux + 20μM Verapamil [mean fluorescence = 93.44]
- = Efflux + 2μM Verapamil [mean fluorescence = 79.25]
- = Daunorubicin efflux [mean fluorescence = 79.15]
- = Daunorubicin load [mean fluorescence = 84.85]
- = Control [mean fluorescence = 2.58]

Events

Log Daunorubicin/PE Fluorescence Intensity

[B]

Legend:
- = Efflux + 500I.U./ml IFN-alpha [mean fluorescence = 25.10]
- = Daunorubicin efflux [mean fluorescence = 24.62]
- = Daunorubicin load [mean fluorescence = 55.51]
- = Control [mean fluorescence = 2.91]

Events

Log Daunorubicin/PE Fluorescence Intensity

Fig. 4.9 [A]: After 30min drug loading MESSA cells were exposed to medium +/- verapamil (2μM & 20μM) and left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of cells having effluxed 2μg/ml daunorubicin in the absence of modulators. [B]: After 30min drug loading MESSA cells were exposed to medium +/- IFN-α (500I.U./ml) and left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of cells having effluxed 2μg/ml daunorubicin in the absence of modulators. Control: MESSA cells not loaded with daunorubicin. No significant modulation was detected with verapamil or IFN-α - $P > 0.05$, using ANOVA followed by Dunnett's post-hoc analysis.

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As expected, neither verapamil nor IFN-α had any significant (P > 0.05 using ANOVA and Dunnett's post-hoc analysis) modulatory effect on the amount of fluorescent agent lost from the cells after incubation. It was considered that in the absence of P-gp, any observed decrease in fluorescent intensity would be due to either diffusion or the activity of another membrane protein pump, however, the results did not show much decrease in intensity.

The reverse was seen in the resistant cell line when exposed to the two different concentrations of verapamil (Figs. 4.10[A] & 4.11[A]). In both cases 2μM verapamil restored the fluorescent intensity to that determined before efflux. When exposed to 20μM verapamil, the intensity exceeded that recorded before efflux. A possible explanation for this could be a slight toxicity on the cells from such a high concentration of verapamil (Sikic, 1997), effectively leading to membrane-altered cells and presenting an opportunity for greater drug accumulation. 500 I.U./ml IFN-α had no modulatory effect on the amount of fluorescent agent effluxed from the cells after incubation (Figs. 4.10[B] & 4.11[B]).

Fig 4.10 Rhodamine 123 accumulation, efflux, and P-gp modulation in resistant cell line

[A]

= Efflux + 20μM Verapamil [mean fluorescence = 31.58]
= Efflux + 2μM Verapamil [mean fluorescence = 16.24]
= Rhodamine efflux [mean fluorescence = 7.77]
= Rhodamine load [mean fluorescence = 16.18]
= Control [mean fluorescence = 3.67]

[B]

= Efflux + 500I.U /ml IFN-alpha [mean fluorescence= 18.20]
= Rhodamine efflux [mean fluorescence= 20.22]
= Rhodamine load [mean fluorescence = 41.18]
= Control [mean fluorescence = 3.85]
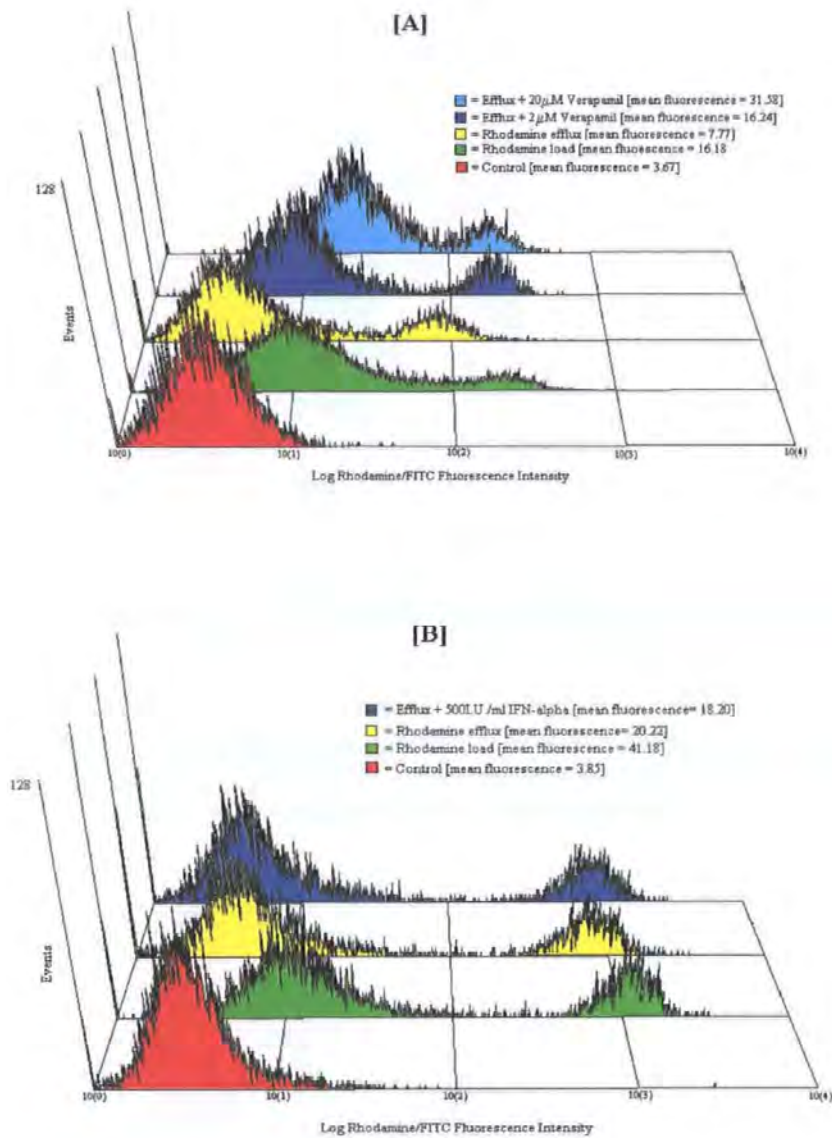
Fig 4.10 [A]: After 30min drug loading MESSA/Dx-5 cells were exposed to medium +/- verapamil (2μM & 20μM) and left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of cells having effluxed 2μM rho123 in the absence of modulators. [B]: After 30min drug loading MESSA/Dx-5 cells were exposed to medium +/- IFN-α (500I.U./ml) and left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of cells having effluxed 2μM rho123 in the absence of modulators. Control: MESSA/Dx-5 cells not loaded with rho123. Significant modulation was achieved with 2μM & 20μM verapamil (P < 0.05 using ANOVA followed by Dunnett's post-hoc analysis), while IFN-α had no significant effect (P > 0.05).

Fig. 4.11 Daunorubicin accumulation, efflux, and P-gp modulation in resistant cell line

[A]

■ = Efflux + 20μM Verapamil [mean fluorescence = 31.38]
■ = Efflux + 2μM Verapamil [mean fluorescence = 16.28]
□ = Daunorubicin efflux [mean fluorescence = 7.41]
■ = Daunorubicin load [mean fluorescence = 16.29]
■ = Control [mean fluorescence = 3.55]

Log Daunorubicin/PE Fluorescence Intensity

[B]

■ = Efflux + 500I.U./ml IFN-alpha [mean fluorescence = 5.46]
□ = Daunorubicin efflux [mean fluorescence = 4.88]
■ = Daunorubicin load [mean fluorescence = 17.75]
■ = Control [mean fluorescence = 3.12]

Log Daunorubicin/PE Fluorescence Intensity

Fig. 4.11 [A]: After 30min drug loading MESSA/Dx-5 cells were exposed to medium +/- verapamil (2μM & 20μM) and left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of cells having effluxed 2μg/ml daunorubicin in the absence of modulators. [B]: After 30min drug loading MESSA/Dx-5 cells were exposed to medium +/- IFN-α (500I.U./ml) and left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of cells having effluxed 2μg/ml daunorubicin in the absence of modulators. Control: MESSA/Dx-5 cells not loaded with daunorubicin. Significant modulation was achieved with 2μM & 20μM verapamil ($P < 0.05$ using ANOVA followed by Dunnett's post-hoc analysis), while IFN-α had no significant effect ($P > 0.05$).

4.5.2   ISOLATED PATIENT B-LYMPHOCYTES

To confirm the presence of P-gp in B-CLL, further analysis of P-gp function was required in patient cells.

4.5.2.1 Cytotoxicity Investigations

4.5.2.1.1   Interferon-alpha toxicity in isolated B-lymphocytes

Although giving a faster and more efficient analytical method than Annexin V/PI detection, the MTT assay, in this case, was not 'suitable' for use on patients' B-lymphocytes. It was found that on a 96-well spectrophotometric plate reader with the number of B-lymphocytes required in each well to maintain optimum cell density (approx. 180 000 - 200 000 cells/180µL/well *i.e.* 1 x 10^6 cells/ml), no purple colour could be detected. Neither the control cells (not exposed to cytotoxic drug or modulator) nor the cells exposed to drug and modulator resulted in a detectable purple colour suggesting either total cell death or insufficient cell density. As other groups have used the MTT assay to investigate drug toxicity over a period of 96h (Sargent *et al.* 1999), and flow cytometry analysis performed on the day of isolation demonstrated viable cells, it was theorised that the problems associated with MTT analysis were due to insufficient cell density - Sargent *et al* describe a cell concentration of 3 x 10^6 cells/ml. However, higher cell densities were not possible due to limitations in the number of B-lymphocytes able to be extracted from patient blood samples. Therefore, the MTT assay was not used for analysis of daunorubicin toxicity or P-gp modulation in patient B-lymphocytes.

4.5.2.1.2   Daunorubicin toxicity in isolated B-lymphocytes

As section 4.5.2.1.1 above

4.5.2.2 Drug accumulation and modulation

4.5.2.2.1   Rhodamine efflux and modulation

An investigation using B-lymphocytes isolated from 12 patients (11 patients full analysis, 1 patient restricted analysis) revealed an efflux of 2μM rhodamine from the gated cells in 11/12 patients after incubation at 37°C for 90min. In each case rhodamine efflux was modulated by verapamil and, in some cases, by IFN-α (Figs. 4.13, 4.15 & 4.16).

4.5.2.2.1.1   Untreated patients

These six patients had not been previously treated for B-CLL. Typical results from an untreated patient are presented in Fig 4.12 (for other patient data, see Appendix J), and a summary of data from untreated patients presented in Fig 4.13.

Fig. 4.12 Rhodamine accumulation, efflux, and P-gp modulation in patient # 4
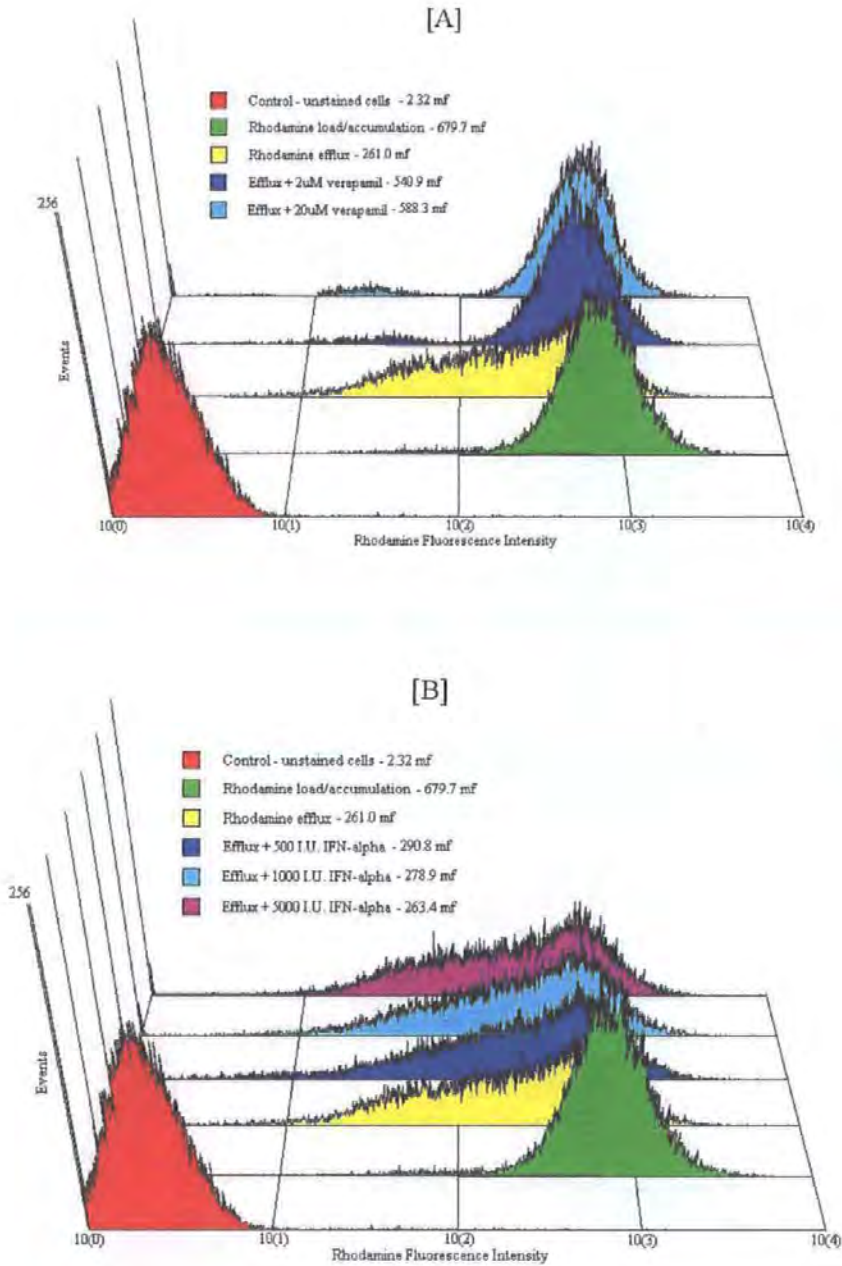
Fig. 4.12 Patient B-lymphocytes were simultaneously exposed to [A] 2μM rhodamine 123 +/- verapamil (2μM & 20μM) and [B] 2μM rhodamine 123 +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μM rhodamine123 in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil as well as 500I.U/ml IFN-α (P < 0.01 using ANOVA followed by Dunnett's post-hoc analysis). 1000I.U/ml & 5000I.U/ml IFN-α had no significant effect (P > 0.05).

Fig. 4.13 Summary of rhodamine accumulation, efflux, and P-gp modulation in untreated patients
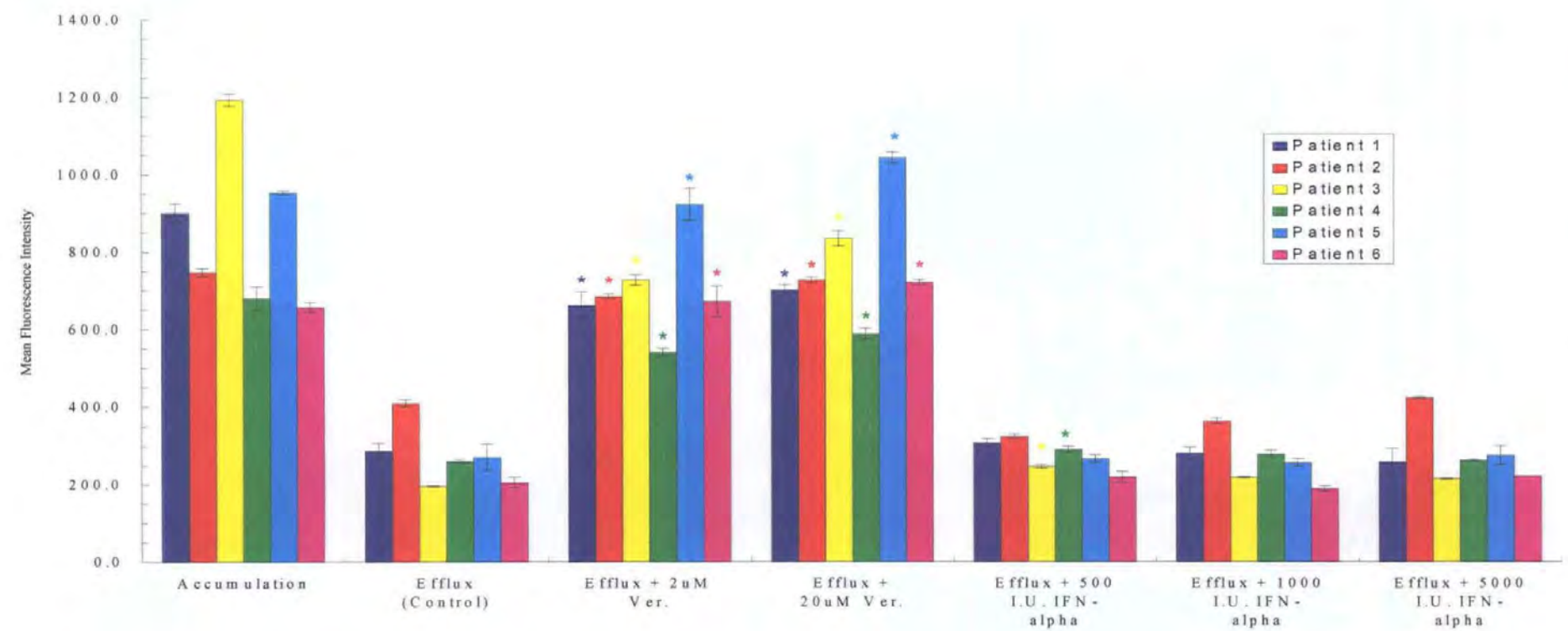
Fig. 4.13 *: statistically significant difference compared to efflux control. (P < 0.01 using ANOVA followed by Dunnett's post-hoc analysis).

4.5.2.2.1.2   Single agent treated patients

Five patients were treated for B-CLL with either chlorambucil or fludarabine. Typical results from a treated patient are presented in Fig 4.14 (for other patient data, see Appendix J), and a summary of data from treated patients presented in Fig 4.15.

Fig. 4.14 Rhodamine accumulation, efflux, and P-gp modulation in patient # 11
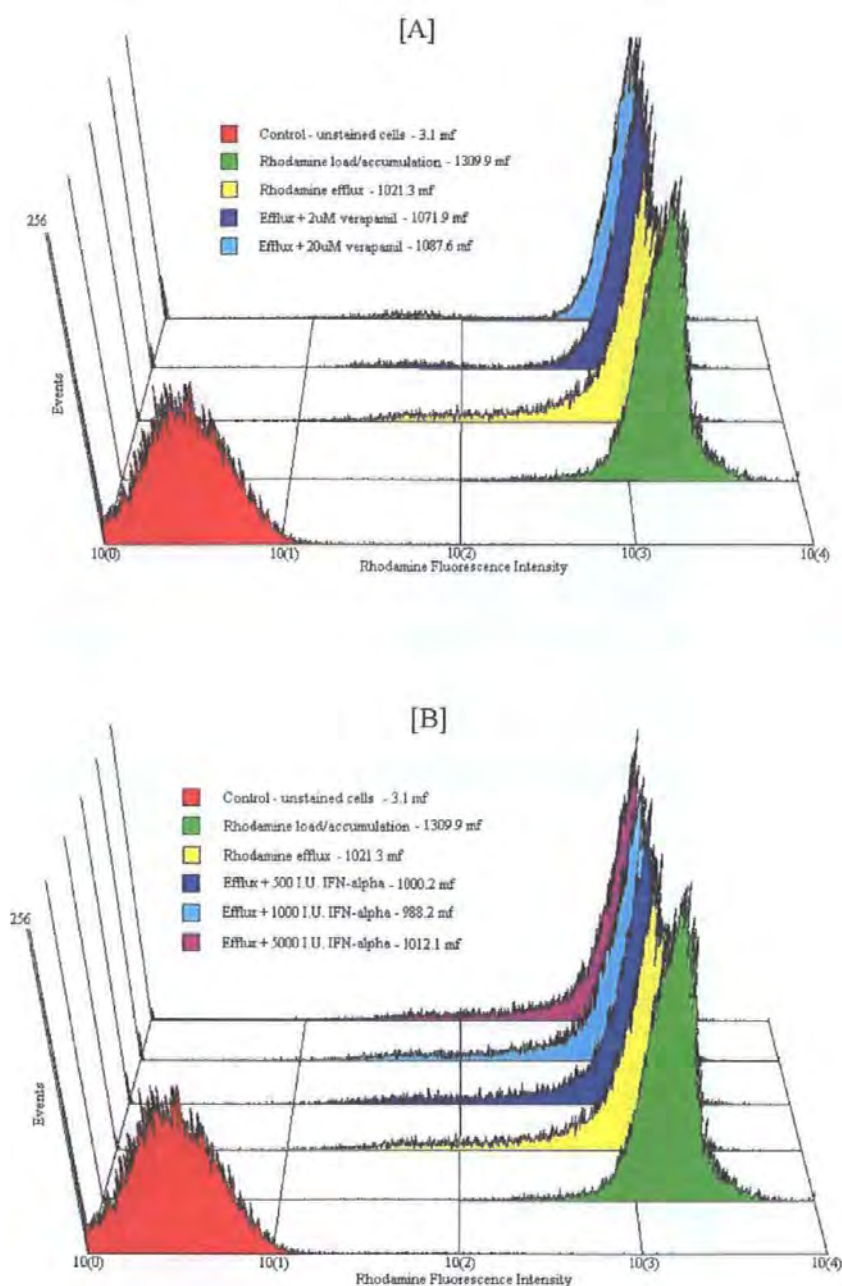
Fig. 4.14 Patient B-lymphocytes were simultaneously exposed to [A] 2μM rhodamine 123 +/- verapamil (2μM & 20μM) and [B] 2μM rhodamine 123 +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μM rhodamine123 in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil ($P < 0.01$ using ANOVA followed by Dunnett's post-hoc analysis). IFN-α had no significant effect ($P > 0.05$).

Fig. 4.15 Summary of rhodamine accumulation, efflux, and P-gp modulation in treated patients
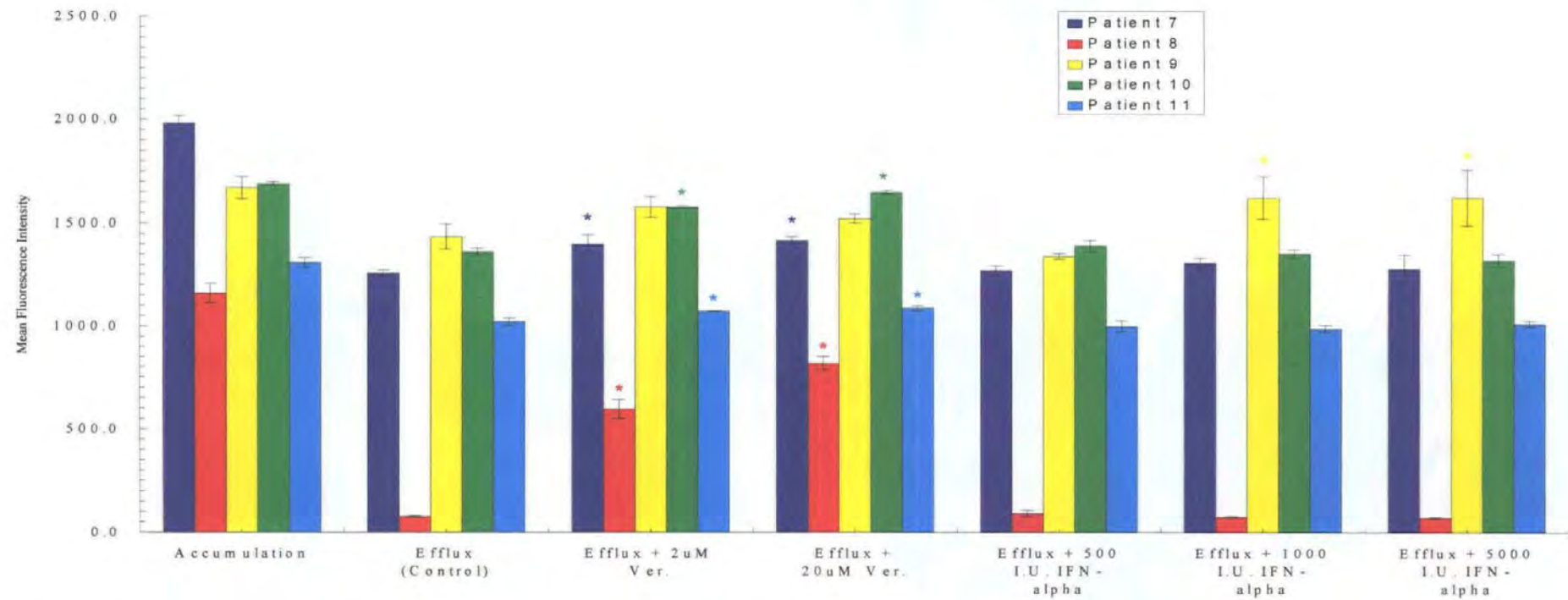
Fig. 4.15 *: statistically significant difference compared to efflux control. (P < 0.01 or 0.05 using ANOVA followed by Dunnett's post-hoc analysis).

Restricted analysis on one patient treated with CHOP was possible. Rhodamine 123 efflux

was examined along with modulation by 2μM, 20μM verapamil and 500 I.U./ml IFN-α

(Fig. 4.16)

Fig. 4.16 Rhodamine 123 accumulation, efflux, and P-gp modulation in B-lymphocytes isolated from a patient on combination therapy (patient # 12)
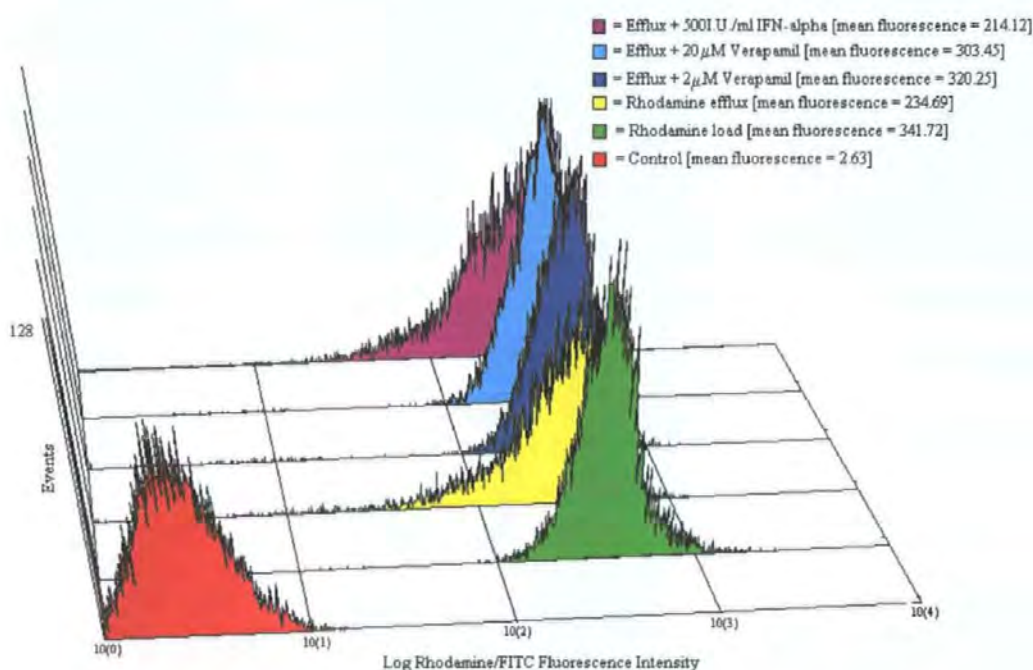
Fig. 4.16 Patient B-lymphocytes were simultaneously exposed to 2μM rhodamine 123 +/- verapamil (2μM & 20μM) and 2μM rhodamine +/- IFN-α (500I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μM rhodamine in the absence of modulators. Significant modulation was achieved with both verapamil concentrations ($P < 0.05$ for both 2μM & 20μM using ANOVA followed by Dunnett's post-hoc analysis) while IFN-α had no effect ($P > 0.05$ using ANOVA).

4.5.2.2.2   Daunorubicin efflux and modulation

An investigation using B-lymphocytes isolated from 10 patients (5 previously treated and 5 untreated) (insufficient cells were isolated from patient 3 to analyse daunorubicin efflux) revealed an efflux of $2\mu g/ml$ daunorubicin from the gated cells in 9/10 patients after incubation at $37^{o}C$ for 90min. In each case daunorubicin efflux was modulated by differing concentrations of verapamil and, in some cases, by IFN-α (Figs. 4.18 & 4.20).

4.5.2.2.2.1   Untreated patients

These five patients had not been previously treated for B-CLL. Typical results from an untreated patient are presented in Fig 4.17 (for other patient data, see Appendix J), and a summary of data from untreated patients presented in Fig 4.18.

Fig. 4.17 Daunorubicin accumulation, efflux, and P-gp modulation in patient # 2
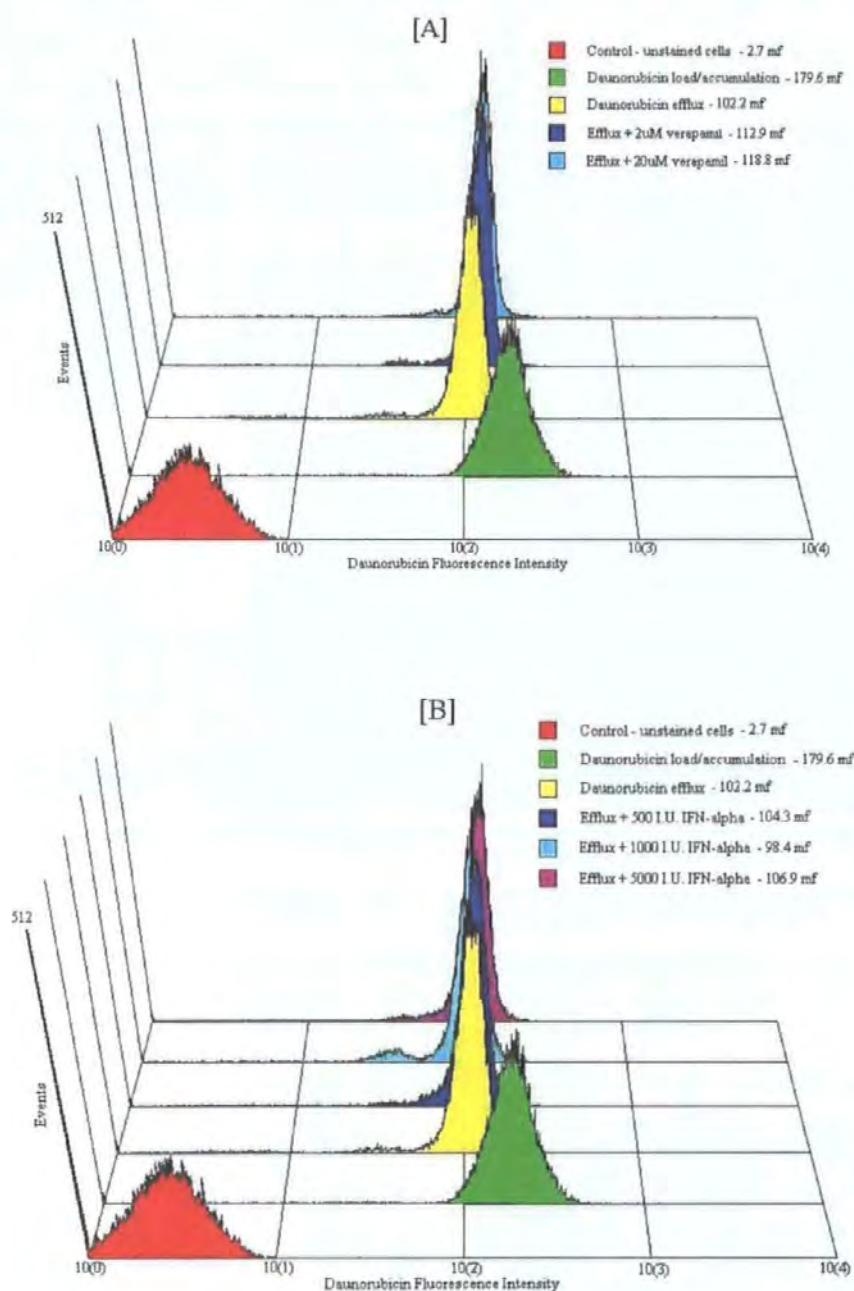
Fig. 4.17 Patient B-lymphocytes were simultaneously exposed to [A] 2μg/ml daunorubicin +/- verapamil (2μM & 20μM) and [B] 2μg/ml daunorubicin +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μg/ml daunorubicin in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil (P < 0.01 using ANOVA followed by Dunnett's post-hoc analysis). IFN-α had no significant effect (P > 0.05).

Fig. 4.18 Summary of daunorubicin accumulation, efflux, and P-gp modulation in untreated patients
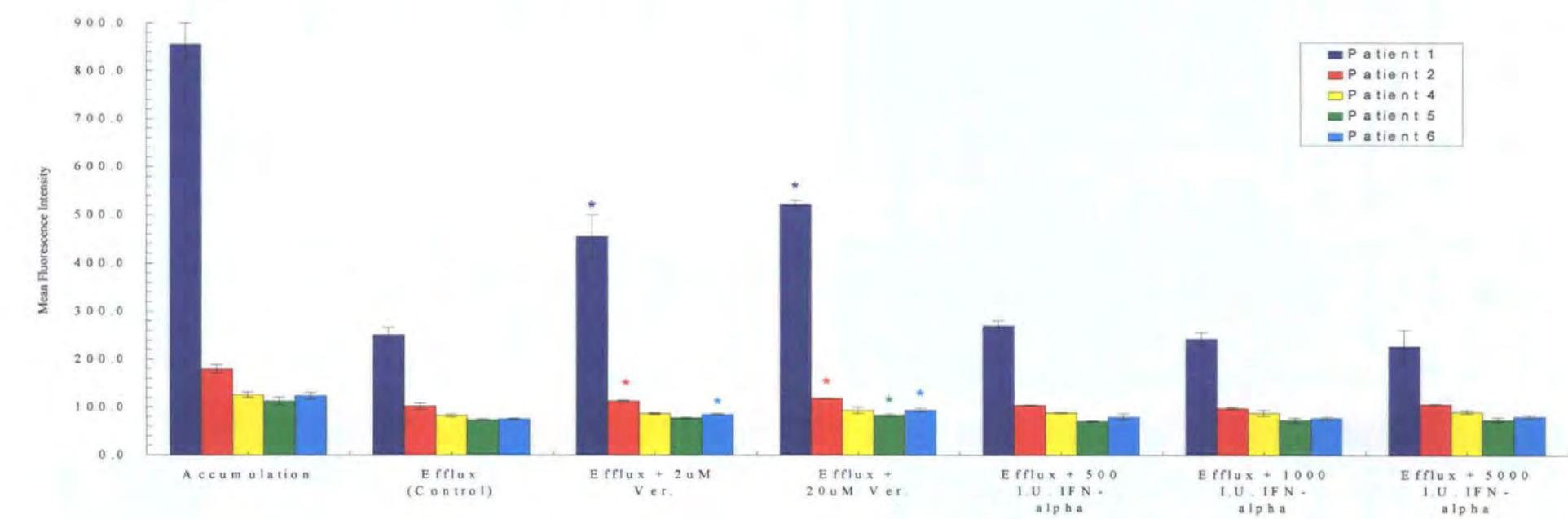
Fig. 4.18 *: statistically significant difference compared to efflux control. (P < 0.01 or 0.05 using ANOVA followed by Dunnett's post-hoc analysis).

4.5.2.2.2.2   Single agent treated patients

Five patients were treated for B-CLL with either chlorambucil or fludarabine. Typical results from a treated patient are presented in Fig 4.19 (for other patient data, see Appendix J), and a summary of data from treated patients presented in Fig 4.20.

Fig. 4.19 Daunorubicin accumulation, efflux, and P-gp modulation in patient # 8

[A]

Legend:
- Control - unstained cells - 2.9 mf
- Daunorubicin load/accumulation - 199.9 mf
- Daunorubicin efflux - 72.3 mf
- Efflux + 2uM verapamil - 93.5 mf
- Efflux + 20uM verapamil - 94.9 mf

Events / Daunorubicin Fluorescence Intensity

[B]

Legend:
- Control - unstained cells - 2.9 mf
- Daunorubicin load/accumulation - 199.9 mf
- Daunorubicin efflux - 72.3 mf
- Efflux + 500 I.U. IFN-alpha - 80.6 mf
- Efflux + 1000 I.U. IFN-alpha - 83.6 mf
- Efflux + 5000 I.U. IFN-alpha - 82.0 mf

Events / Daunorubicin Fluorescence Intensity

Fig. 4.19 Patient B-lymphocytes were simultaneously exposed to [A] 2µg/ml daunorubicin +/- verapamil (2µM & 20µM) and [B] 2µg/ml daunorubicin +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2µg/ml daunorubicin in the absence of modulators. Significant modulation was achieved with 2µM & 20µM verapamil ($P < 0.01$ using ANOVA followed by Dunnett's post-hoc analysis). as well as all concentrations of IFN-α (500I.U./ml, $P < 0.05$; 1000I.U./ml & 5000I.U./ml, $p < 0.01$).

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Fig. 4.20 Summary of daunorubicin accumulation, efflux, and P-gp modulation in treated patients
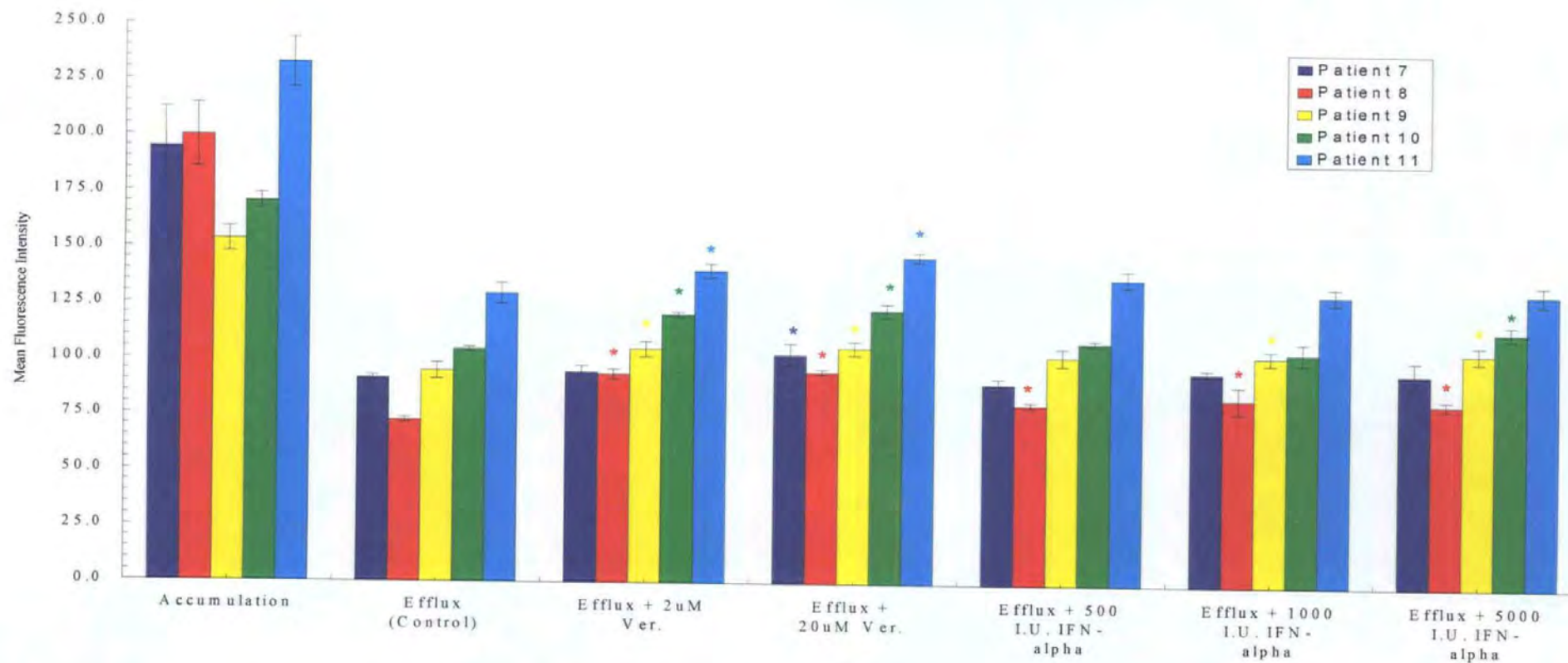
Fig. 4.20 *: statistically significant difference compared to efflux control. (P < 0.01 or 0.05 using ANOVA followed by Dunnett's post-hoc analysis).

4.6   DISCUSSION

In 1991, Scala *et al.* indicated that IFN-α could have a potential role in the modulation of P-gp-associated MDR, avoiding the high concentrations traditionally associated with classical P-gp modulators such as verapamil. For this reason, the objective of this study was primarily to evaluate the potential of IFN-α as a P-gp modulator in B-CLL including analysis of P-gp functionality in an attempt to clarify P-gp presence on isolated CLL B-lymphocytes.

Initial studies on the toxicity of IFN-α to the doxorubicin-sensitive and doxorubicin-resistant cell lines (MESSA and MESSA/Dx-5, respectively) demonstrated that 500 I.U./ml IFN-α had the least toxic effect on cell survival and also demonstrated that the MESSA/Dx-5 cells were more susceptible overall to the effects of IFN-α (Fig. 4.2, p205). This increased IFN-α toxicity in the resistant cell line was unexpected but may be explained by a recent publication demonstrating that IFN-α treatment abrogated cell proliferation in a cell line expressing high levels of P-gp (KTCTL-26) whereas proliferation of a cell line expressing low levels of P-gp (KTCTL-2) was only partially inhibited (Frank and Pomer, 1999). These findings suggest that IFN-α-induced antiproliferative activity requires P-gp-mediated transport. The findings also suggest that susceptibility to the antiproliferative effects of IFN-α may correlate with P-gp expression, therefore, the increased IFN-α toxicity observed for the MESSA/Dx-5 cell line could be due to P-gp overexpression.

This phenomenon was not thought to pose a problem with further analysis of P-gp function and its modulation using IFN-α, as at 500 I.U./ml IFN-α toxicity was similar in both cell

lines. It is interesting, however, that Scala *et al.* (1991) reported that both the doxorubicin-sensitive and doxorubicin-resistant cells used in their laboratory (LoVo & LoVo/Dx, respectively) had a similar sensitivity to IFN-α in general, over a concentration range of 50 I.U./ml to 10 000 I.U./ml. The fact that the resistant cell line used by Scala *et al.* (LoVo/Dx) showed little difference in IFN-α sensitivity compared to the sensitive cell line (LoVo) suggests differences in the function of P-gp protein expressed in MESSA/Dx-5 cells compared to LoVo/Dx, although this would need further investigation.

MTT analysis of P-gp modulation was performed by comparing the effect of verapamil (2μM and 20μM) with a non-toxic concentration of IFN-α (500 I.U./ml), and showed that verapamil increased the sensitivity of resistant cells to daunorubicin toxicity, while IFN-α had no effect (Fig. 4.5, p212). Flow cytometric analysis showed this increase in sensitivity to be due to an increase in drug accumulation, *i.e.* an alteration in P-gp functionality, while no such drug accumulation was detected using IFN-α (Figs. 4.10, p220 & 4.11, p221). The *in vitro* results suggested an inability of IFN-α, at 500 I.U./ml, to modulate P-gp function. Therefore, in these cell lines, IFN-α did not give the same promising results described by Scala *et al.*(1991).However, not only were different cell types used in this investigation but, due to this fact, different cell culture media was also used. A delay in cell culture medium change has been reported to alter the function of P-gp (Hegewisch-Becker *et al.* 1996), therefore, changes in culture media could potentially interfere with any functional alteration exerted by IFN-α and lead to conflicting conclusions.

As the mechanism by which IFN-α exerted the modulatory effects reported by Scala *et al.* (1991) is yet unknown and there were differences in experimental conditions which may have influenced the effect of IFN-α on P-gp function, investigation into the effects of

verapamil and IFN-α on patient B-lymphocytes was continued. It was possible that an IFN-α concentration of 500 I.U./ml did not modulate P-gp function in the cell lines MESSA & MESSA/Dx-5, but was effective at modulating P-gp expressed by patients' B-lymphocytes. Modulation with higher concentrations of IFN-α was also investigated after it had been suggested that patients could tolerate higher concentrations of IFN-α *in vivo* than originally thought (personal communication from Dr. A Prentice, 2000), thus leading to a greater potential for modulation.

Eleven patients were analysed fully for P-gp functionality. These patients were analysed for rhodamine 123 and daunorubicin efflux where efflux was defined as a loss in cell fluorescence that could be reversed by verapamil, either 2μM or 20μM.

Out of the eleven patients studied, ten demonstrated a loss in rhodamine fluorescence intensity that could be significantly altered by 2μM or 20μM verapamil (Figs. 4.13 and 4.15). Considering the affinity of verapamil for P-gp and also the trend of verapamil treatment returning the fluorescence intensity towards the levels seen before initial drug efflux (Figs. 4.12 & 4.14), *i.e.* fluorescence intensities associated with drug accumulation, the results strongly suggested that rhodamine was being actively expelled by the action of P-gp and that this action was being modulated by the addition of verapamil. This implied that, in those patients with detectable P-gp levels (Table 4.6, p240), the protein was also functional.

However, 4 out of the 10 patients demonstrating rhodamine efflux had been classed as negative for P-gp by flow cytometry analysis (Table 4.6, below). It is possible that another efflux mechanism was responsible for the decrease in rhodamine levels in these patients. However, the practice of denoting thresholds to differentiate between negative and positive

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in experimental samples (Beck *et al.* 1996) is arbitrary and may incorrectly suggest the absence of an important cellular component. For example, the four patients (patient nos 1, 2, 4 & 10) who were reported as negative for the presence of P-gp all had values just below the 10% cut-off (range 7 to 9%) but nevertheless effluxed rhodamine (Table 4.6and Figs. 4.12 & 4.17). This indicated the need for a different definition of P-gp expression such that P-gp presence could be described in grades rather than in absolutes. Instead of yes or no definitions for P-gp expression, in these patients it would be more accurate to describe P-gp expression as low or high while still retaining the 10% cut-off point for these descriptions. With this new definition 10 patients analysed by rhodamine efflux would be classed as expressing functional P-gp (6 high expressers and 4 low expressers) with only one patient out of the eleven not expressing functional P-gp.

Patient #	P-gp expression	rhodamine efflux	Modulation. of rhodamine efflux					dauno. efflux	Modulation of daunorubicin efflux				
			Modn. - ver.		Modn. - IFN-α				Modn. - ver.		Modn. - IFN-α		
			2 µM	20 µM	500 I.U	1000 I.U	5000 I.U		2 µM	20 µM	500 I.U	1000 I.U	5000 LU
1	no (8.3%)	yes	yes	yes	no	no	no	yes	yes	yes	no	no	no
2	no (7.3%)	yes	yes	yes	no	no	no	yes	yes	yes	no	no	no
3	yes (44.9%)	yes	yes	yes	yes	no	no	no data	no data	no data	no data	no data	no data
4	no (9.4%)	yes	yes	yes	yes	no	no	no	no	no	no	no	no
5	yes (28.6%)	yes	yes	yes	no	no	no	yes	no	yes	no	no	no
6	yes (29.1%)	yes	yes	yes	no	no	no	yes	yes	yes	no	no	no
7	yes (37.5%)	yes	yes	yes	no	no	no	yes	no	yes	no	no	no
8	yes (57.0%)	yes	yes	yes	no	no	no	yes	yes	yes	yes	yes	yes
9	yes (13.4%)	no	no	no	no	yes	yes	yes	yes	yes	no	yes	yes
10	no (7.2%)	yes	yes	yes	no	no	no	yes	yes	yes	no	no	yes
11	yes (20.5%)	yes	yes	yes	no	no	no	yes	yes	yes	no	no	no
12	no (8.9%)	yes	yes	yes	no	no data	no data	no data	no data	no data	no data	no data	no data

Table 4.6 P-gp expression, function and modulation in patient group Patient Number: 1-6 untreated, 7-11 previously treated, 12 treated with combination therapy.

Patients 1-11 were fully analysed with rhodamine efflux and verapamil modulation, while patient 12 was only partially analysed.

In the same patient sample, only 10 out the eleven patients were able to be analysed by daunorubicin efflux. Nine patients demonstrated a loss in daunorubicin fluorescence intensity that could be significantly altered by 2μM or 20μM verapamil (Figs. 4.18 and 4.20) again suggesting that, in these patients, daunorubicin was being actively expelled by the action of P-gp. As anthracycline drugs are known P-gp substrates, these results not only indicated the presence of functional P-gp but also suggested a potential drug resistance problem for treatment regimens incorporating P-gp substrates such as CHOP.

In light of this it would have been advantageous to analyse patients on a combination regimen to compare with the above patient sample. This type of therapy could potentially increase the possibility of P-gp-associated resistance, not only by inducing P-gp expression but also by promoting a selective clonal expansion of these P-gp expressing cells.

Isolated B-lymphocytes from one such patient (patient # 12) were able to be examined although full analysis was not possible. Consequently only rhodamine efflux and modulation were examined using 2μM and 20μM verapamil as well as 500 I.U./ml IFN-α (Fig. 4.16 & Table 4.6). Cells from this patient were negative for P-gp when analysed by flow cytometry but expressed MDR1 mRNA when analysed by RT-PCR. When analysed functionally, these cells exhibited efflux of rhodamine 123 and on analysis with verapamil, similar results to the other patients' samples were obtained, *i.e.* verapamil was effective at modulating this efflux. In a similar fashion to the other P-gp negative patients, expression was just below the 10% threshold (8.9%) but as rhodamine efflux was detected from these cells this patient was also classed as a low functional expresser for P-gp.

As the efflux pattern did not differ from the other patients expelling rhodamine 123 and as P-gp expression was low, treatment with CHOP, in this case, did not seem to support the

theory that anthracycline-containing regimens could alter P-gp expression or affect clonal expansion and thus potentially increase the chances of drug resistance. However, this analysis was performed on only one patient and to reach any conclusion about the relationship between anthracycline treatment and the progression of drug resistance, a much larger analysis would need to be performed (Ferry, 1998).

On the other hand, even if treatment with an anthracycline did not result in increased chances of resistance, the above results demonstrate that low and high expression of P-gp can occur in B-CLL patients and that, in cases of both high and low expression, rhodamine 123 as well as daunorubicin efflux can be detected. Therefore, in the event of treatment with regimens incorporating a P-gp substrate, use of a P-gp modulator exhibiting low toxicity could be beneficial.

In view of this, analysis of rhodamine 123 and daunorubicin efflux in the 11 patient sample indicated that, in the presence of differing concentrations of IFN-α, effective P-gp modulation occurred in some cases.

Rhodamine efflux in cells of 2 out of 10 patients was reversed by 500 I.U./ml of IFN-α and in the case of daunorubicin efflux, cells in 1 out of 9 nine patients responded to 500 I.U/ml of IFN-α. If including higher concentrations of IFN-α, these statistics become 3 out of 10 for rhodamine modulation and 3 out of 9 for daunorubicin. Although it is possible that higher concentrations of IFN-α were needed in these cases, it is likely that the higher concentrations of IFN-α had an effect due to a toxicity exerted on the cells rather than any direct effect on P-gp function *e.g.* for patient 9, incubation with 1000 I.U/ml and 5000 I.U./ml of IFN-α returned the decreased fluorescence intensity for rhodamine back towards the values for rhodamine accumulation (Fig. 6.8 [Appendix J], p286) even in the absence

of verapamil modulation. It seems unlikely that a P-gp-specific modulator such as verapamil would have had no effect at all on P-gp function but that a high concentration of IFN-α would exert a specific effect on this membrane pump. In addition, if it were only a matter of modulator concentration then reversal of P-gp function should have been observed for each patient at the higher concentrations of IFN-α. Similarly, for the patient treated with CHOP no modulation of rhodamine flux was detected with 500 I.U./ml IFN-α (Fig. 4.16), however it may be that higher concentrations were needed.

The variability of IFN-α in the patient analysis together with the total absence of modulation in the cell line analyses not only conflict with the results published by Scala *et al.* (1991) but also vary from the published IFN-α enhancement of MRK16 (MAb against P-gp) circumvention of MDR (Fogler *et al.* 1995). However, in 1993 Kang & Perry reported no change in Chinese hamster ovary cell doxorubicin retention by IFN-α and it has been reported that, rather than having a toxic effect on B-lymphocytes, IFN-α can up-regulate bcl-2 expression and protects B-CLL cells from cell death (apoptosis) *in vitro* and *in vivo* (Jewell *et al.* 1994; Panayiotidis *et al.* 1994).

Although the results presented in this chapter regarding the modulatory actions of IFN-α seem to show that IFN-α is not a very good reversing agent, this discussion demonstrates the complexity surrounding the many reported actions of IFN-α and, in light of such conflicting information and the small sample size of this investigation, the potential of IFN-α as a P-gp modulator still cannot be dismissed.

Quesada *et al.* (1996) offer an explanation for the differing results regarding the ability of IFN-α to modulate P-gp function. Resistant cells, identified by exposure to increasing

concentrations of a single anticancer drug, may not only acquire cross-resistance but may also undergo a phenomenon termed 'collateral sensitivity' to other drugs including some chemosensitising agents such as verapamil. With this phenomenon there may be no correlation between the cytotoxic effects of chemosensitising agents on MDR cells and their ability to potentiate the accumulation of anticancer agents. P-gp has been suggested to be the target molecule in collateral sensitivity to verapamil and in that case compounds such as verapamil are toxic to the MDR cells at concentrations much lower than those toxic to the parental cells. Therefore, anticancer drugs exhibiting increased toxicity in a cytotoxicity assay might not be doing so due to an inhibition of P-gp. Instead increased toxicity would be due to a selective toxicity of the chemosensitising compound to the resistant cells.

Quesada *et al* (1996) also showed that the LoVo/Dx cells used by Scala *et al.*(1991) demonstrated a sensitivity to verapamil suggesting the collateral sensitivity phenomenon. Such an effect was not observed in LoVo parental cells. Therefore the positive cytotoxicity result obtained by Scala *et al* (1991) for IFN-α may not have been due to P-gp modulation but could have been due to the phenomenon of collateral sensitivity. This explanation would therefore support the data in Fig. 4.5 (p212), showing that IFN-α does not have a modulatory action on P-gp in comparison to a modulator such as verapamil. This theory is unlikely however, as Scala *et al.* (1991) had also demonstrated that the IFN-α effects which they observed were coupled to an increased accumulation of doxorubicin, negating the possibility of collateral sensitivity being responsible for the IFN-α effects in this cell line and supporting the theory of P-gp modulation.

Another explanation for the interlaboratory variation is the ability of P-gp to be phosphorylated. The 170 kDa P-gp has been reported to be phosphorylated in its basal state

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by protein kinase C (PKC) and it has been suggested that this phosphorylation of P-gp might be essential for drug transport (Germann, 1996a). Similarly, Sato *et al.* (1990) and Chambers *et al.* (1990 & 1992), theorised that phosphorylation of P-gp could activate it as inhibition of PKC increased drug accumulation and decreased drug resistance. Studies using vincristine resistant HL 60 cells demonstrated that another membrane associated protein kinase (PK1), which also phosphorylates P-gp on its serine and threonine residues, may regulate levels of multidrug resistance (Chambers *et al.* 1990; Staats *et al.* 1990). In contrast, two different groups showed that a mutation of the major phosphorylation sites within P-gp did not affect its transportation function (Germann *et al.* 1996b; Goodfellow *et al.* 1996). It has also been demonstrated that staurosporine and H7, which are inhibitors of protein kinase C and cAMP dependent protein kinase activity, do not affect overall P-gp phosphorylation (Harris, 1992). In addition, verapamil treatment which inhibits P-gp function and increases drug accumulation in MDR cells, results in P-gp hyperphosphorylation (Hamada, 1987).

This paradox suggests a complex role for phosphorylation in relation to P-gp function. Assuming, however, that phosphorylation of P-gp activates the protein and therefore allows it to act as an efflux pump, anything altering this phosphorylation should inhibit P-gp function. IFN-α has been reported to activate protein kinases *e.g.* tyrosine kinases (Uddin *et al.* 1998; Yan *et al.* 1998). Protein kinases are responsible for the phosphorylation of many cell proteins and are involved in complex signal transduction pathways. It is possible therefore that IFN-α P-gp modulation may vary depending on the levels & types of protein kinases within different cell lines as well as the signal transduction mechanisms in which these kinases are involved.

Another explanation involves the drug stability data presented in Chapter 2 (p72) where the different physical processes identified in the *in vitro* experimental system could have interfered with IFN-α P-gp modulation. Scala *et al.* (1991) suggested that IFN-α affected drug accumulation by altering P-gp function rather than expression. Modulation involving direct IFN-α interaction with the P-gp molecule could be possible and, in view of the stability studies that identified the degradation of daunorubicin (and possibly rhodamine 123), lack of modulatory action could be explained by the presence of degradation products. For example, it is known that verapamil modulates P-gp function by competing with substrates for the P-gp binding site (Doppenschmitt *et al.* 1999; Neuhoff *et al.* 2000). IFN-α may have a binding site different to that of verapamil but similar or identical to that of degradation products from daunorubicin and perhaps rhodamine. It is possible that daunorubicin degradation products establish higher binding affinities to IFN-α binding sites than for verapamil binding sites, meaning that degradative products could be competitively inhibiting the IFN-α from binding to P-gp, thereby preventing modulation of P-gp function. The likelihood of this is remote however as IFN-α acts via specific receptors (section 1.4.2.1, p39) and the binding affinity of IFN-α for its own receptors would be expected to be higher than for P-gp.

4.7   CONCLUSION

The functionality investigations presented in this chapter suggest that an efflux mechanism resembling P-gp in activity and modulation is expressed in CLL B-lymphocytes to varying degrees. Furthermore, this study also demonstrates that, depending on the techniques used and the analytical thresholds applied, the occurrence of P-gp-associated MDR in B-CLL could be misrepresented.

In addition, the variability of IFN-α modulation demonstrated in these patients suggests that IFN-α would not be a suitable candidate for clinical use as a drug-resistance reversing agent. However, the complexity surrounding the actions of IFN-α as well as the conflicting inter-laboratory *in vitro* results suggest that the ability of this cytokine to modulate P-gp function cannot be dismissed until further investigations have been performed with a larger sample group. In addition, a study examining the toxicity of IFN-α to isolated B-lymphocytes would be needed to determine the usefulness of increasing IFN-α concentrations in an attempt to achieve P-gp modulation.

5　　OVERALL DISCUSSION, CONCLUSION AND FUTURE WORK

5.1   DISCUSSION

The discovery that MDR1 and P-gp were responsible for clinical resistance to several classes of chemotherapeutic drugs led to a major effort to find agents able to inhibit P-gp-mediated MDR. In clinical trials most of these modulators have produced major side-effects such as cardiotoxicity (verapamil) or immunosuppression (cyclosporin A), which limit their clinical use at the concentrations needed to achieve inhibition of MDR (Eytan and Kuchel, 1999; Mechetner, 1992; Tan *et al.* 2000). Therefore, the finding by Scala *et al.* (1991) that IFN-α could modulate P-gp function at a concentration tolerated *in vivo* together with several conflicting reports regarding the level of expression of the MDR1 gene & P-gp protein in B-CLL (Arai *et al.* 1997; Ribrag *et al.* 1996; Sparrow *et al.* 1993; Webb *et al.* 1998), prompted this investigation into both P-gp associated anthracycline resistance in B-CLL patients and the ability of IFN-α to modulate P-gp function.

Initially, the stability of the anthracycline daunorubicin was examined, and it was found that there was decreased drug concentration at $37^{\circ}C$ over time (a 49.8% reduction over 2h) due to both adsorption and degradation (section 2.6, p 117). This novel finding not only suggested that drug availability *in vitro* could be overestimated, but also illustrated that degradation products could exist in an analytical culture system, potentially interfering with functionality experiments during analysis of cell efflux mechanisms (section 4.6, p 236). These two findings may have implications for the accuracy and validity of previous studies on drug sensitivity *in vitro*.

Following the drug stability analysis, flow cytometric analysis of the patient population's cells showed a low frequency of P-gp expression (section 3.5, p171) when compared to

expression of MDR1 mRNA by RT-PCR. Many studies have investigated P-gp expression in B-CLL using different techniques (Arai *et al.* 1997; Bosanquet *et al.* 1996; Maynadie *et al.* 1997; Michieli *et al.* 1991; Ribrag *et al.* 1996; Sparrow *et al.* 1993; Webb *et al.* 1998) but have reported conflicting findings as to the presence and relevance of P-gp in B-CLL. The somewhat conflicting results obtained using flow cytometry and RT-PCR were therefore not uncommon and corroborate the findings of Beck *et al.* (1996) who warned of the need to combine functional analysis with P-gp expression analysis when investigating the issue of P-gp-associated MDR.

This situation led to further investigation of P-gp functionality. Analysis of both rhodamine 123 and daunorubicin efflux was continued in a sample of 11 patients (either untreated or treated with a non-P-gp-transportable single agent), and an analysis of rhodamine efflux was performed in one patient treated with an anthracycline containing regimen (CHOP). The B-lymphocytes from the majority of patients sampled demonstrated a loss in rhodamine 123 (11/12 patients) and daunorubicin (9/10 patients) fluorescence intensity that could be significantly altered by exposing the cells to 2μM or 20μM verapamil (Figs. 4.13, 4.15, 4.16, 4.18 and 4.20). This strongly suggested that, in these patients, both rhodamine and daunorubicin were being actively expelled by the action of P-gp and that this action was being modulated by the addition of verapamil. Consequently, this implied that, in addition to being expressed (presence of mRNA and membrane protein) in these B-lymphocytes, the protein was also functional even at low levels of expression (section 4.6, p236)

Furthermore, analysis of B-cells from the patient treated with an anthracycline-containing regimen (CHOP) showed a similar rhodamine efflux pattern to that observed in the other 10 patient samples. The B-cells expelled rhodamine 123 and this expulsion was modulated

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by verapamil even though P-gp was not as strongly expressed as in some of the untreated patients or others on single agent therapy.

As already mentioned, P-gp expression, whether analysed by detection of protein or MDR1 mRNA, has been investigated by many groups but the results have been conflicting. While some have reported a possible relationship between increased P-gp expression levels, drug resistance and treatment response (Svoboda-Beusan *et al.* 2000; Webb *et al.* 1998) others have reported no change in P-gp expression levels and no clinical relevance to B-CLL (Ribrag *et al.* 1996) or have described P-gp levels of expression which do not correlate with Rai stage of disease, with *ex vivo* drug sensitivity, or with patient survival (Bosanquet *et al.* 1996).

However, many of these reports do not include results of study of the functionality of the protein and this alone may explain the findings in the literature. While the levels of P-gp expression may be an important factor in drug resistance, protein expression may not necessarily correlate with function. For this reason the results for P-gp expression and function from this study were compared to determine the relationship between P-gp expression and rhodamine or daunorubicin efflux. (Figs. 5.1 & 5.2).

A linear regression was performed in each case and a trend was observed for both rhodamine efflux (Fig. 5.1) and daunorubicin efflux (Fig. 5.2), both increasing with P-gp expression. As the percentage of P-gp expression increased so did the percentage of drug efflux. This was more obvious for daunorubicin efflux, although one atypical patient's analysis showed a far greater efflux compared to protein expression (Fig. 5.2).

Fig. 5.1 Relationship between %P-gp expression and %rhodamine efflux in patient B-lymphocytes.
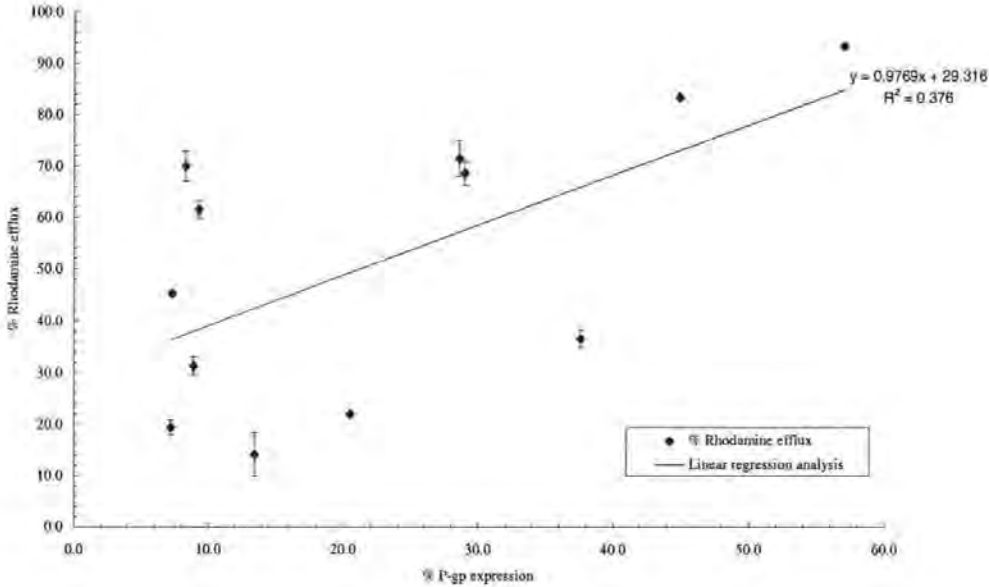
Fig. 5.2 Relationship between %P-gp expression and %daunorubicin efflux in patient B-lymphocytes.
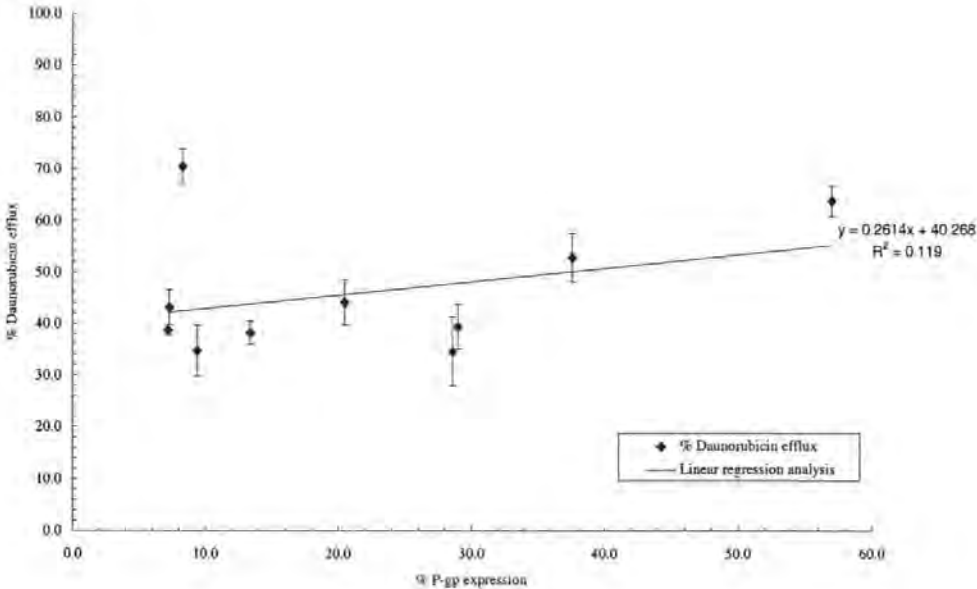
This observed relationship between increasing P-gp expression and increasing drug efflux suggests that P-gp expression in B-CLL could be a factor in treatment failure when using P-gp transportable drugs. Interestingly in this case, P-gp expression and therefore drug efflux did not seem to correlate with either age, sex or stage of disease (Appendix K, Table 5.1 & 5.2), suggesting that these factors did not influence P-gp expression nor were they paramount to the efficacy of the pump activity. Of course a larger sample population would have to be obtained to permit a full evaluation of the influence of patient age, patient sex and stage of disease on P-gp expression and activity.

It has been shown recently that P-gp overexpression can be down-regulated in some B-CLL patients by P-gp-independent drugs (Svoboda-Beusan *et al.* 2000). However, even when P-gp expression remains unchanged in other patients given P-gp independent drugs, successful outcome of therapy still correlates with P-gp expression (Svoboda-Beusan *et al.* 2000). This correlation, plus the finding that both the patient treated with CHOP and the other patients expelling rhodamine 123 had similar efflux, even with low P-gp expression, does not support the recent suggestion that P-gp effects drug resistance only in patients treated with P-gp transportable drugs (Webb *et al.* 1998). Neither does the result from the CHOP treated patient support the theory that anthracycline-containing regimens could alter P-gp expression or affect clonal expansion and thus potentially increase the chances of drug resistance. However, this analysis was performed on only one patient and to reach any conclusion about the relationship between anthracycline treatment and the progression of drug resistance, a much larger analysis is needed (Ferry, 1998).

The above results demonstrate that varying degrees of expression of P-gp can occur in B-CLL patients and that, in cases of both high and low expression, rhodamine 123 as well as daunorubicin efflux can be detected. Therefore, in the event of treatment with regimens

Patient #	Stage of disease	Sex of patient	Age of patient (years)	P-gp expression	rhodamine efflux	Modulation of rhodamine efflux (% of total calculated efflux)				
						Modn. - ver.		Modn. - IFN-α		
						2 µM	20 µM	500 I.U	1000 I.U	5000 I.U
1	II (B)	F	58	no (8.3%)	yes (70.0%)	yes (61.0%)	yes (67.6%)	no (3.2%)	no (0.0%)	no (0.0%)
2	0 (A)	F	76	no (7.3%)	yes (45.3%)	yes (81.6%)	yes (94.1%)	no (0.0%)	no (0.0%)	no (0.0%)
3	no data	M	no data	yes (44.9%)	yes (83.5%)	yes (53.3%)	yes (64.1%)	yes (5.0%)	no (2.3%)	no (1.9%)
4	0 (A)	M	47	no (9.4%)	yes (61.5%)	yes (67.1%)	yes (78.5%)	yes (7.2%)	no (4.3%)	no (0.5%)
5	0 (A)	M	72	yes (28.6%)	yes (71.6%)	yes (95.7%)	yes (100.0%)	no (0.0%)	no (0.0%)	no (0.5%)
6	0 (A)	F	54	yes (29.1%)	yes (68.7%)	yes (100.0%)	yes (100.0%)	no (3.0%)	no (0.0%)	no (2.2%)
7	II (A)	M	86	yes (37.5%)	yes (36.6%)	yes (19.8%)	yes (22.1%)	no (2.1%)	no (5.4%)	no (3.2%)
8	0 (A)	M	66	yes (57.0%)	yes (93.5%)	yes (48.1%)	yes (68.7%)	no (1.3%)	no (0.0%)	no (0.0%)
9	0 (A)	M	66	yes (13.4%)	no (14.1%)	no (64.3%)	no (35.1%)	no (0.0%)	yes (72.6%)	yes (95.7%)
10	III (C)	M	59	no (7.2%)	yes (19.4%)	yes (65.7%)	yes (87.9%)	no (5.2%)	no (0.0%)	no (0.0%)
11	IV (C)	M	72	yes (20.5%)	yes (22.0%)	yes (18.5%)	yes (23.0%)	no (0.0%)	no (0.0%)	no (0.0%)
12	II (B)	M	52	no (8.9%)	yes (31.3%)	yes (79.9%)	yes (64.2%)	no (0.0%)	no data	no data

Table 5.1 Clinical data, P-gp expression, function and modulation in patient group using rhodamine efflux Patient Number: 1-6 untreated, 7-11 previously treated, 12 treated with combination therapy

Patient #	Stage of disease	Sex of patient	Age of patient (years)	P-gp expression	Dauno efflux	Modulation. of daunorubicin efflux (% of total calculated efflux)				
						Modn. - ver.		Modn. - IFN-α		
						2 μM	20 μM	500 I.U	1000 I.U	5000 I.U
1	II (B)	F	58	no (8.3%)	yes (70.5%)	yes (16.6%)	yes (27.1%)	no (4.5%)	no (2.5%)	no (4.9%)
2	0 (A)	F	76	no (7.3%)	yes (43.1%)	yes (13.6%)	yes (21.4%)	no (2.6%)	no (0.0%)	no (5.8%)
3	no data	M	no data	yes (44.9%)	no data	no data	no data	no data	no data	no data
4	0 (A)	M	47	no (9.4%)	no (34.6%)	no (8.6%)	no (23.9%)	no (13.4%)	no (14.5%)	no (19.0%)
5	0 (A)	M	72	yes (28.6%)	yes (39.1%)	no (20.0%)	yes (33.0%)	no (0.0%)	no (0.0%)	no (0.0%)
6	0 (A)	F	54	yes (29.1%)	yes (39.4%)	yes (19.8%)	yes (40.2%)	no (2.7%)	no (5.1%)	no (4.6%)
7	II (A)	M	86	yes (37.5%)	yes (52.8%)	no (3.2%)	yes (11.1%)	no (0.0%)	no (4.5%)	no (4.9%)
8	0 (A)	M	66	yes (57.0%)	yes (63.7%)	yes (16.9%)	yes (17.8%)	yes (6.5%)	yes (8.8%)	yes (7.6%)
9	0 (A)	M	66	yes (13.4%)	yes (38.2%)	yes (17.0%)	yes (18.6%)	no (1.5%)	yes (13.8%)	yes (17.6%)
10	III (C)	M	59	no (7.2%)	yes (38.7%)	yes (23.9%)	yes (27.5%)	no (6.3%)	no (0.5%)	yes (16.1%)
11	IV (C)	M	72	yes (20.5%)	yes (44.1%)	yes (10.1%)	yes (16.6%)	no (2.6%)	no (0.4%)	no (1.7%)
12	II (B)	M	52	no (8.9%)	no data	no data	no data	no data	no data	no data

Table 5.2 Clinical data, P-gp expression, function and modulation in patient group using daunorubicin efflux Patient Number: 1-6 untreated, 7-11 previously treated, 12 treated with combination therapy

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incorporating a P-gp substrate, use of a P-gp modulator exhibiting low toxicity could be beneficial.

Analysis of rhodamine 123 and daunorubicin efflux in the 11 patient sample demonstrated that effective P-gp modulation occurred in nearly all cases with verapamil and also in a few cases in the presence of differing concentrations of IFN-α (section 4.6). However, in the presence of verapamil and IFN-α, an alteration in the efflux pattern of rhodamine and daunorubicin (depicted by the graphical representations of the flow analysis) in some patients was not visibly apparent even though the statistics showed otherwise. For the rhodamine efflux profiles where the modulation with verapamil was unquestionable in most cases, in patients 7, 10 and 11 the changes in fluorescence intensity were not easy to visualise from Figs. 6.6, 6.9 & 4.14, respectively. This was also apparent when examining the profiles for rhodamine in the presence and absence of interferon at different concentrations. Similarly, from the graphical profiles, it was difficult to see how some of the changes in fluorescence intensity for daunorubicin efflux and modulation could be found to be statistically significant.

For this reason, the raw data for each patient was analysed twice with two different statistical packages (PRISM and SPSS). The statistics were found to be the same in each case, but this was not surprising considering that most statistical packages use the same algorithms for their analyses. As the calculated statistical significances between the efflux and modulation values (Figs. 4.12, 4.14, 4.16, 4.17, 4.19 & Appendix J) were indisputable, this statistical analysis denotes a significant difference in values and upon further evaluation it was theorised that this graphical presentation of the flow analysis may not have been the best way to present the drug flux profiles for each patient. Using the available flow cytometry software, the x-axis fluorescent values of these graphs were

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plotted on a logarithmic scale while the statistical analysis correctly took the actual triplicate values of each analysis and proceeded with a linear scale analysis. Of course, when analysing similar values on a linear scale, small numerical differences will be more apparent than if attempting to depict these same values visually on a logarithmic scale. Additionally, the role of a statistical analysis is to help find differences in situations where a large biological difference may not be immediately apparent and which may therefore be difficult to discern.

Paradoxically, the question of whether the statistically significant numerical differences reflect a clinical significance remains unanswered. It would be encouraging to witness a relationship between degree of modulation versus percent efflux and percent P-gp expression and somehow relate that to disease progression and prognosis. However, a high percent rhodamine modulation with verapamil was found in patients with low P-gp expression and high rhodamine efflux (Patient #1, Table 5.1, Fig. 6.1) as well as patients with very little visible modulation in the graphical representations (Patient #10, Table 5.1, Fig. 6.9). Modulation with IFN-α occurred to a lesser degree and the degree of modulation was also less. This was also true of the efflux and modulation of daunorubicin (Table. 5.2), suggesting that the ability to modulate the function of P-gp is not a uniform inter-patient phenomenon but rather a very distinct action varying from patient to patient and probably depending on a number of inter-related factors.

Taking into consideration this critical evaluation, it would be interesting to have been able to proceed with a follow-up investigation to determine if those patients not exhibiting an immediately obvious efflux and modulatory pattern, developed a different profile over time.

In the absence of this opportunity, the analytical correlation between the two statistical packages was viewed as satisfactory evidence to argue that, in addition to demonstrating that P-gp is present in B-CLL patient B-lymphocytes and that the protein can be functional, (posing a potential drug resistance problem for treatment regimens incorporating anthracycline P-gp substrates such as doxorubicin or idarubicin (Gahn *et al.* 2000)), P-gp function was also subject to modulation to varying degrees in the presence of verapamil and IFN-α. Even so, compared to verapamil, the overall inability of 500I.U./mL IFN-α to modulate drug efflux in the cell line, MESSA/Dx-5, coupled with the sparse patient response even at higher concentrations, suggests that IFN-α is unsuitable for use as a P-gp regulatory agent.

There are many possible explanations for the inactivity of 500I.U./ml IFN-α in modulating P-gp function in the resistant cell line, MESSA/Dx-5 and the majority of patient B-lymphocytes. One explanation not presented in chapter 5 concerns the stability of IFN-α. Although the stability of daunorubicin under cell culture conditions was investigated (section 2.5.2, p 106), the stability of IFN-α was not. Even though IFN-α exerts its effects via binding to its receptor, Scala *et al.* (1991) hypothesised that IFN-α may exert a modulatory action by binding directly to P-gp. However, if IFN-α is not stable in the required culture conditions, changes in its concentration and structure could affect the ability of IFN-α to bind to P-gp directly, therefore preventing a potential modulatory action. Scala *et al.* (1991) used different cell culture medium for the cell lines, LoVo & LoVo(Dx), and it may be that the RPMI medium used in this investigation affected the stability of IFN-α leading to the variation in the results regarding P-gp modulation.

The limited IFN-α-mediated modulation observed in this study may not be directly related to problems with IFN-α binding, stability or mechanism of action, but may instead result from the many actions of P-gp. P-gp can act as a chloride ion channel regulator (Vanoye *et al.* 1997) as well as an efflux pump (Arai *et al.* 1997; Dalton *et al.* 1997; Webb *et al.* 1998) and has recently been reported to have a role in regulating cell death (Johnstone *et al.* 1999). If the P-gp protein is capable of manipulating other cellular mechanisms, such as cell death, it could also be interfering with the potential antiproliferative effects of IFN-α. The issue of IFN-α modulating P-gp function becomes even more complex with the data from Frank and Pomer (1999) showing an increased antiproliferative effect for IFN-α on a cell line expressing P-gp at high levels compared to a cell line expressing low levels. In the absence of a chemotherapeutic agent this suggests that the antiproliferative effects of IFN-α may correlate with P-gp expression and that P-gp facilitates the transmembrane transport of IFN-α. Although the interferons are known to interact with membrane receptors (Jonasch and Haluska, 2001; Mandelli *et al.* 1994), Drach *et al.* (1996) concluded that P-gp also participates in the transport of cytokines (IL-2, IL-4 and IFN-gamma) in normal peripheral T lymphocytes.

Thus the issue of IFN-α modulation of P-gp-associated MDR in B-CLL is complex and requires further definition. The possibility of P-gp modulation by cytokines is being pursued, and in 1998 Tambur *et al.* demonstrated that the expression and function of P-gp activity on NK cells (leukaemic and normal) could be reversed with IL-4.

However, based on the efficacy of P-gp being dependent on its pumping capacity, affinity toward the particular drug, the rate of transmembrane diffusion of the drug, the affinity of the pharmacological target in the cell for the drug, and the affinity of intracellular trapping sites for the drug, the ideal modulator should exhibit all of the following properties: high

affinity of the active site(s) of P-gp; a fast rate of transmembrane exchange; low toxicity and side effects; low affinity of cellular components, other than P-gp (Eytan and Kuchel, 1999). It is not known whether IFN-α fulfils these criteria.

Given its wide distribution, modulation of P-gp has not been without problems. Modulation of P-gp in normal tissues can affect the pharmacokinetics and, thus, the toxicity of associated chemotherapeutic agents (Covelli 1999; Silverman, 1999; Tan *et al*, 2000) and most agents have produced severe toxic effects at doses required to block P-gp function. The relationships between the structure and MDR reversal effects of potential modulators are under investigation. A variety of studies have been performed on the identification of structure-activity relationships (SARs) and quantitative SARs (QSARs) of different MDR reversing agents concluding that the careful selection of relevant structural and biological data processed with appropriate QSAR and 3D-QSAR methods is a promising approach to structure-activity studies of MDR reversers (Wiese and Pajeva, 2001). Unfortunately, even with these efforts, a possible drawback of finding a specific P-gp inhibitor, in terms of circumventing MDR in patients with B-CLL and malignancy in general, is that it may not necessarily be an efficient inhibitor of alternative MDR mechanisms that may be present such as MRP, LRP, MDR3, or as yet unidentified mechanisms (Tan *et al*, 2000).

Alternative approaches to combat MDR are therefore necessary and have included the design and synthesis of new non-cross-resistant drugs with physicochemical properties that favour the uptake of such drugs by resistant cells. Garnier-Suillerot *et al.,* (2001) have discussed the improvement of drug cytotoxicity in resistant cells by increasing drug lipophilicity thereby avoiding the P-gp resistance pump. Consequently, they have referred to the increased efficacy of idarubicin against MDR tumour cell lines *in vitro* compared to

daunorubicin and doxorubicin. Gahn *et al.* (2000), published the results of a multicenter phase II study of oral idarubicin in treated and untreated patients with B-CLL. While they demonstrated that as a single agent idarubicin was of limited effectiveness in B-CLL, they acknowledged the need for further studies to assess different doses and schedules of oral idarubicin and to test it in combination with other therapeutic agents. Newer lipophilic anthracyclines are also being investigated such as annamycin which has an even greater lipophilicity than idarubicin which, although more effective than daunorubicin and doxorubicin, may still be affected by the presence of efflux pumps such as P-gp (Ross *et al.* 1995). Studies are also being undertaken to analyse the effectiveness of MDR1-targeted antisense oligonucleotides in the treatment of B-CLL (Dassow *et al*, 2000).

Alternatively, the particular and consistent dysregulation of apoptosis in B-CLL makes the cell death pathway an attractive target for treatment. There is even greater dysregulation of apoptosis in advanced and chemotherapy-resistant disease (Bentley and Pepper, 2000) perhaps contributing to the reason for pleiotropic multidrug resistance in this disease. Advances in the understanding of the complexities of the apoptotic pathway have lead to therapeutic opportunities for the modulation of this pathway as a target for specific treatment. Investigation of this has included the targeting of cell surface receptors (Kato *et al.* 1998), Bcl-2 family members (Hirsch *et al.* 1998; Pepper *et al.* 1999a & b) and caspases (Chandra *et al.* 1998; Masdehors *et al.* 1999).

These novel treatment strategies are encouraging. However, the mechanism of MDR resistance in B-CLL is still far from clear. Other efflux proteins have been identified in B-CLL patients. Multidrug resistance protein (MRP) has been reported to be highly expressed in B-CLL (Burger *et al.* 1994). The expression of different MDR genes encoding separate P-gp isoforms may also be a mechanism for generating diversity of response, this concept

being emphasised by the detection of MDR3 as well as MDR1 in B-CLL samples (Arai *et al.* 1997). Analysis of MDR3 expression in B-cell malignancies has revealed that the expression is associated with the maturation stage of the neoplasm (Herweijner *et al.* 1990). Previous investigations have reported that MDR1-associated P-gp, MDR2/3 gene product and MRP are all able to be expressed in malignancies. However, while transfection of MDR2/3 did not confer resistance, the transfection of the other two genes did (Marie, 1995). Even so, high levels of MDR3 expression have been found in CLL (Herweijner *et al.* 1990) and, despite Sonneveld *et al.* (1992) finding that these high levels were not responsible for drug resistance, Arai *et al.* (1997) saw increased accumulation of rho123 in P-gp -ve, MDR3 +ve cells exposed to the modulator, cyclosporin A, demonstrating an efflux function for the MDR3 product. Although the role of MDR3 in B-CLL remains undefined, the ability of the MDR3 product to act as an efflux mechanism means that it too may be involved in malignant B-cells' drug resistance and its presence may need to be investigated simultaneously with MDR1 mRNA, P-gp and MRP. This is especially important when evaluating the potential of novel P-gp modulators. Clearly, modulation of the MDR1 P-gp would be erroneously reported if the investigation had unintentionally used a model primarily expressing the MDR3 gene product. MAbs to P-gp have been found to bind to the MDR3 gene product in a non-specific manner (section 3.1.1.3.1, p133) and the MDR3 gene product may also respond to the same array of modulators as the MDR1 P-gp.

Even with intense investigation into the expression and function of an ever increasing number of efflux proteins, MDR in B-CLL remains a complex matter and it is unlikely that efflux mechanisms alone will account for all of the cellular resistance mechanisms involved. This investigation has not touched on the other potential mechanisms mentioned in section 1.5 (p 43) such as increased GST levels, but it is probable that part of the drug resistance in B-CLL is associated with other MDR mechanisms such as increased GST

levels and topo II mutations (section 1.5, p 43). Additionally, it is likely that there are many obstacles to apoptosis intrinsic to the B-CLL cell, many of which will not be influenced by modulation of MDR (Haraguchi *et al.* 2000; Tamm *et al.* 2000; Zhang *et al.* 2000).

Identification and quantification of the relative contributions of each mechanism causing resistance at any one moment is essential in research in the field of MDR. However, other challenges within this area also include the use of modulators where low potency and non-specificity result in a dose-limiting toxicity. In addition to the fact that P-gp modulators can alter anticancer drug pharmacokinetics, P-gp is expressed in many normal tissues, including parts of the liver, kidneys, intestines, and at the blood-brain barrier (Fromm, 2000). As a result, drugs that affect the activity of P-gp can increase the toxicity of anticancer drugs to normal tissues, so that the dosage of anticancer drugs must be reduced when the two are given in combination. In addition to these challenges, there is the possibility that a modulating agent can suppress one or more mechanisms of drug resistance while enhancing others (Dalton *et al.* 1997).

Current strategies in overcoming the problem of MDR and associated modulator toxicity encompass the design of better targeted, less toxic agents such as PSC833 which is active in MDR1 expressing cell lines *in vitro* and is in clinical trials (Beketic-Oreskovic *et al.* 1995). Gene therapy is another potential avenue of investigation by activation of the MDR1 gene in blood and bone marrow stem cells with a view to protecting them selectively from cytotoxic drugs so that these cells are able to withstand high-dose chemotherapy. The use of anticancer drugs such as the newer anthracyclines that are not as susceptible to the actions of P-gp (idarubicin) or that bypass the P-gp resistance mechanism altogether (annamycin) is another possibility (Consoli *et al.* 1996).

Although many alternatives are being studied, treatment of patients exhibiting P-gp-associated MDR would still benefit from a modulator devoid of the *in vivo* toxicity associated with inhibiting agents. Although the results of this study are not favourable towards the use of IFN-α as a P-gp modulator, the uncertainty about the mechanisms of IFN-α action means that it would be presumptuous to conclude that IFN-α is not a suitable P-gp modulator in B-CLL. However, it does seem likely that elevated concentrations of IFN-α may be required in some cases whereby dose-related toxicity could be a limiting factor in its clinical use as a P-gp modulator.

5.2   CONCLUSION

Daunorubicin is not a stable anthracycline in the cell culture conditions used for P-gp analysis, thus leading to a decreased drug availability in the *in vitro* experimental system. This drug loss is due to both adsorption and degradation resulting in the presence of degradation products in the analytical culture system which could potentially interfere with HPLC analysis and flow cytometry analysis of P-gp functionality. Thus, the stability of components in a culture system, *e.g.* drug and protein modulator, is an important factor and should be taken into consideration.

The results presented by this investigation help resolve the issue of P-gp expression in B-CLL where protein expression is demonstrated in patients to varying degrees. In addition, the active expulsion of P-gp substrates such as rhodamine 123 and daunorubicin from patient B-lymphocytes suggests a role for the expressed P-gp in the efflux of these drugs and anthracyclines in general. Further analysis of P-gp expression and function suggests the presence of a P-gp-associated efflux mechanism in B-CLL which may be only partially

regulated by 500I.U./ml IFN-α, questioning the initial idea that this cytokine may be a potential candidate for use as a P-gp modulator in the clinic.

This study also proposes that this form of MDR is easy to misrepresent depending on which analytical techniques are employed during investigation. Consequently, this investigation shows that P-gp is present and functional in B-CLL but, due to variations in analytical techniques, could have been so far underestimated. This possibility, coupled to the varying interlaboratory results, reinforces the need to integrate a variety of analytical methods to gain a more accurate understanding of P-gp-associated anthracycline resistance in B-CLL and other tumours. Analyses should include measurement of MDR1 mRNA, of P-gp protein (monoclonal antibody detection and protein isolation) and of P-gp functionality. The combined use of such would allow the confirmation of at least one resistance mechanism at work in B-CLL.

5.3   FUTURE WORK

If deemed clinically appropriate, determining why IFN-α did not modulate P-gp function to a greater degree in the *in vitro* B-lymphocyte culture could include investigations into the stability of this cytokine which should be undertaken before continuing with a more extensive patient analysis of P-gp function and modulation using IFN-α. Analysis could look at IFN-α stability in various culture media and at different temperatures. However, investigations on a hybrid interferon-alpha (BDBB) have reported that pH plays a key role in its degradation, therefore, studies on the stability of IFN-α in RPMI at physiological pH would be beneficial for continued study into P-gp modulation in B-CLL. Possible analytical techniques include HPLC and SDS-PAGE (Allen *et al.* 1999)

In addition, the fact that more ATP-binding cassette proteins are becoming associated with MDR in B-CLL (Arai *et al.* 1997; Webb *et al.* 1998) necessitates continued evaluation of resistance-inducing efflux mechanisms in this disease. Future investigations should include a wider spectrum of analysis including the detection of proteins such as MRP and the MDR3-associated gene product. For ease and speed of analysis, flow cytometry is still a practical technique although not without its problems, *e.g.* low sensitivity depending on choice of monoclonal antibody. However, the conflicting reports regarding P-gp expression in B-CLL, demonstrate the importance of isolating and purifying the protein. Consequently it is promising that there are recent reports of such isolation and purification of P-gp proteins (Figler *et al.*, 2000, Julien *et al.*, 2000, Lerner-Marmarosh *et al.*, 1999) and other proteins such as MRP (Mao *et al.*, 1999) from cell membranes. In addition there are reports regarding the use of reconstituted P-gp in proteoliposomes to further investigate its functional properties with a view to gaining a better understanding of function and modulation (Lu *et al.* 2001).

Although only speculative in nature, the theory that daunorubicin degradation and metabolic products could have impeded a potential IFN-α modulatory effect is an interesting explanation for the disappointing IFN-α results on P-gp modulation (section 4.6, p236) and one that may result in discovery of more clinically useful data if further investigated. Analysis of protein–protein interactions between P-gp and IFN-α could potentially resolve the question of whether IFN-α can exert a direct modulatory effect on P-gp.

In the longer term, identification, isolation and analysis of structure of P-gp isotypes may increase the understanding of the resistance mechanisms at work in B-CLL. Similarly, further study of P-gp isoforms may lead to the identification of a disease-specific P-gp,

continued analysis of which could result in the synthesis of specific modulators with minimal toxicity *in vivo*.

6   APPENDIX

6.1   APPENDIX A

Sample calculation of final stock daunorubicin concentration:

Weight of dry, silanised, glass volumetric flask + lid   =   30g

Weight of dry, silanised, glass volumetric flask + lid + daunorubicin powder =   30.010g

Weight of daunorubicin powder transferred = $30.010 - 30g = 0.010g$

Final volume in flask =   25ml

Concentration of daunorubicin   =   0.010g/25ml

                                    =   0.0004g/ml

                                    =   0.4mg/ml

                                    =   400µg/ml

6.2   APPENDIX B

Stability indication procedure:

1]   Take six, silanised, 10ml volumetric flasks and label (a)-(f).

2]   Add 1ml of 10μg/ml daunorubicin stock solution to each.

3]   Add the following to each:

      flask (a)   1ml distilled water

      flask (b)   1ml distilled water

      flask (c)   1ml RPMI

      flask (d)   N/1 HCl (1ml)

      flask (e)   N/1 NaOH (1ml)

      flask (f)   20vol H_2O_2 (1ml)

4]   Immerse flasks (b)-(f) in a boiling water bath for 20min and maintain flask (a) at fridge temperature as a control.

5]   Cool the flasks and then neutralise (d) & (e) with 1ml of NaOH or HCl respectively.

6]   Adjust all flasks to volume with 18MΩ distilled water and assay (HPLC).

6.3   APPENDIX C

Positive CD19 dynabead/DETACHaBEAD cell isolation:

Dynabead washing

All Dynabeads should be washed before use. A general washing procedure is as follows;

1] Resuspend the Dynabeads M-450 in the vial by gentle vortexing and/or shaking.

2] Transfer the required amount of Dynabeads M-450 to a washing tube.

3] Place the washing tube on a Dynal Magnetic Particle Concentrator for two minutes, and pipette off the fluid.

4] Remove the tube from the Dynal MPC, and resuspend in an excess volume of washing buffer (PBS pH 7.2).

5] Repeat step 3 and resuspend the washed Dynabeads M-450 in washing buffer.

Dynabead separation technique

Cool buffers and suspensions and keep at $4^{o}C$ at all times to prevent non-specific phagocytosis of Dynabeads M-450

1] Prewash a calculated number of Dynabeads M-450 CD19 according to above procedure and cool whole blood sample to $2\text{-}4^{o}C$.

2] Add Dynabeads to the cell suspension containing target cells (e.g. whole blood).

3] Incubate at $4^{o}C$ with slow tilting and rotation for 30min for whole blood samples.

4] Isolate the rosetted B cells by placing the tube in a Dynal MPC for 2min.

5] Pour or pipette the supernatant from the tube while the rosetted cells are attached to the wall of the test tube by the Dynal MPC.

6] Remove the test tube from the dynal MPC and resuspend the rosetted cells in a washing buffer. Avoid resuspension using vigorous pipetting or vortexing as the exerted shear forces may cause damage to the cells. Repeat steps 4-6 five times, and then resuspend the cells in the desired volume of an appropriate buffer or cell-culture media.

Detachment protocol using DETACHaBEAD

1] Perform cell isolation with dynabeads CD19 as recommended above.

2] Resuspend the rosetted cells in 300μl of cell culture medium.

3] Add five units (50μl) DETACHaBEAD to the rosetted cells

4] Incubate for 45-60min at ambient temperature on an apparatus that provides both gentle tilting and rotation

5] Remove the released beads by placing the test tube in the Dynal MPC for 2min.

6] Pipette the cell suspension from the test tube while the beads are attached to the wall of the tube by the Dynal MPC.

7] To obtain the residual cells, wash the detached beads 2-3 times in cell culture medium, and collect the supernatant.

8] Wash the detached cells thoroughly by resuspending the cells and centrifugation for 10min at 800 x gav, 2-3 times, to remove DETACHaBEAD from the solution.

9] Resuspend the cells in the desired volume of an appropriate buffer.

The cells can now be used for several research applications.

6.4   APPENDIX D

Isolation of PBMCs:

1] Extract patient blood into Sodium Heparin tubes at room temperature.

2] Mix blood by inverting (to prevent clotting) and transfer to universal flask.

3] Dilute blood with equal volume of RPMI - 10:10ml = 20ml.

4] Add 10ml of density gradient medium (lymphoprep) to each of two universal flasks.

5] Layer equal volume (10ml) of diluted blood onto lymphoprep.

6] Centrifuge for 20mins at 400g (2000rpm).

7] Harvest interface PBMC layer into universal flask.

8] Dilute with RPMI to 20ml and mix well.

9] Centrifuge 10mins at 240g (1200rpm).

10] Discard supernatant and resuspend pellet in 5ml of RPMI.

11] Wash x 2 in RPMI (5 min at 240g) and resuspend in 5-10ml RPMI culture medium.

12] Count cells in Haemocytometer.

6.5   APPENDIX E

Trizol RNA extraction:

1] Remove the aliquot of mononuclear cells to be analysed from the liquid nitrogen store DIRECTLY to ICE.

2] Add 1ml of TRIZOL to the cell pellet as soon as possible while the pellet is still frozen.

3] Pipette to allow full cell lysis.

4] Transfer the cell/reagent mix to a fresh 1.5ml tube and incubate at room temperature for 5 min.

5] Add 200µl chloroform and shake vigorously for 15s. Do not vortex. Incubate at room temperature for a further 3min.

6] Centrifuge at approx. 10,800g at 4°C for 15min.

7] Transfer the aqueous (top) phase to a fresh 1.5ml tube, 100µl at a time avoiding the interface layer.

8] Add 500µl isopropanol to the RNA solution.

9] Invert the tube several times to mix and incubate at room temperature for 10min to precipitate the RNA.

10] Centrifuge at 10,800g at 4°C for 10min. CAREFULLY pour off the supernatant.

11] Wash the RNA pellet by adding 1ml 70% ethanol and centrifuging at 7,400g at 4°C for 5min.

12] Carefully pour off the supernatant. Add a further 1ml 100% ethanol to the RNA pellet and centrifuge at 7,400g at 4°C for 5min.

13] Pour off the supernatant. Cover the open tube with a piece of parafilm and pierce the film 2 to 3 times with a needle.

14] Dry the pellet of RNA under vacuum. DO NOT OVERDRY THE RNA PELLET.

15] Resuspend the RNA pellet in RNase-free H_2O and store at -20°C for later analysis.

6.6   APPENDIX F

'Access' RT-PCR Kit Protocol

1] Following the table below, prepare the reaction mix tubes by combining the volumes of nuclease-free water, AMV/Tfl 5X reaction buffer, dNTP Mix and 25mM MgSO$_4$ in thin-walled 0.5ml reaction tubes on ice.

Components	Vol. in reaction mix (µl)	Vol. template and primers (µl)
Nuclease-free water	26.4	
AMV/Tfl 5X reaction buffer	10	
dNTP mix	1	
Downstream primer		3.3 (50pmol)
Upstream primer		3.3 (50pmol)
25mM MgSO$_4$	2	
AMV reverse transcriptase	1	
Tfl DNA polymerase	1	
RNA sample or control		2

2] Make corresponding individual tubes of RNA sample/primers for denaturing as shown in table above.

3] Denature RNA sample/primer tubes at 94°C for 2 min.

4] During denaturing, fetch enzymes on ice.

5] Put enzymes in reaction mix and keep on ice.

6] Remove denatured tubes to ice and spin briefly before adding contents to corresponding reaction mix tubes.

7] Run following RT-PCR profile -

1 cycle	48°C for 45min	reverse transcription
1 cycle	94°C for 2min	AMV RT inactivation and RNA/cDNA/primer denaturation
40 cycle	94°C for 30sec	denaturation
	60°C for 1min	annealing
	68°C for 2min	extension
1 cycle	4°C	soak

8] Run samples on 2% agarose gel

6.7 APPENDIX G

Restriction Endonuclease Mae I:

1] Turn on water bath to 45°C and allow to heat.

2] Briefly centrifuge the samples to collect any condensation.

3] Aliquot 5μl of each sample into 0.5ml microfuge tubes and label.

4] Add 5μl of enzyme reaction buffer to each tube.

3] Remove the enzyme form the freezer DIRECTLY ONTO ICE.

4] Add 1 unit of enzyme (0.5μl of a 2unit/μl stock) to each sample, using a fresh pipette tip for each one. Keep on ice until all samples are ready for incubation.

5] Briefly centrifuge to mix and place in a float in the water bath for 2h.

6] When digested, briefly centrifuge the samples to bring down any condensation and load 10μl of each (plus 1μl Ficoll DNA loading buffer) onto a 10% polyacrylamide gel and run at 20mA for approximately 30min then check the position of the bromophenol blue dye front. Keep checking until the gel has run far enough.

7] At the end of the run stain with ethidium bromide for 5min and visualise with a 3 UV™ Transilluminator UVP U.V. box.

Blast searches

6.8.1   PGP +VE CELL LINE CONTROL, MESSA/DX-5, SENSE SEQUENCE

BLASTN 2.2.1 |Apr-13-2001|

RID: 1012185135-76-27242

Query= MDR1S
         (120 letters)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS,
GSS, or phase 0, 1 or 2 HTGS sequences)
         1,079,142 sequences; 4,647,298,756 total letters

Alignments

1] Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 1
         (ABCB1), mRNA
         Length = 4549

Score = 210 bits (105), Expect = 1e-52
Identities = 117/121 (96%), Gaps = 1/121 (0%)
Strand = Plus / Plus

```
Query: 1     tgttgtctggaca-gcayygaaagataagaaagaactagaaggttctgcgaagatcgcta
59
             |||||||||||||| |||   ||||||||||||||||||||||||||| ||||||||||||
Sbjct: 2960  tgttgtctggacaagcactgaaagataagaaagaactagaaggttctgggaagatcgcta
3019


Query: 60    ctgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
119
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3020  ctgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
3079


Query: 120   a 120
             |
Sbjct: 3080  a 3080
```

2] Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 1
(ABCB1), mRNA
Length = 4643

Score = 202 bits (101), Expect = 4e-50
Identities = 116/121 (95%), Gaps = 1/121 (0%)
Strand = Plus / Plus

Query: 1   tgttgtctggaca-gcayygaaagataagaaagaactagaaggttctgcgaagatcgcta
59
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Sbjct: 3054   tgttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgcta
3113

Query: 60   ctgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
119
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3114   ctgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
3173

Query: 120   a 120
              |
Sbjct: 3174   a 3174

3] Homo sapiens P-glycoprotein (PGY1) mRNA, complete cds
Length = 4646

Score = 202 bits (101), Expect = 4e-50
Identities = 116/121 (95%), Gaps = 1/121 (0%)
Strand = Plus / Plus

Query: 1   tgttgtctggaca-gcayygaaagataagaaagaactagaaggttctgcgaagatcgcta
59
              ||||||||||||| ||| ||||||||||||||||||||||||| ||| |||||||||||
Sbjct: 3057   tgttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgcta
3116

Query: 60   ctgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
119
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3117   ctgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
3176

Query: 120   a 120
              |
Sbjct: 3177   a 3177

278

6.8.2   PATIENT 3 SENSE SEQUENCE

BLASTN 2.2.1 |Apr-13-2001|

RID: 1012185587-3428-18719

Query= Patient 3
          (119 letters)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS,
GSS, or phase 0, 1 or 2 HTGS sequences)
          1,079,142 sequences; 4,647,298,756 total letters

Alignments

1] Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 1
          (ABCB1), mRNA
          Length = 4643

Score = 236 bits (119), Expect = 3e-60
Identities = 119/119 (100%)
Strand = Plus / Plus

Query: 1   gttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgctac
60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3055 gttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgctac
3114

Query: 61   tgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
119
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3115 tgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
3173

2] Homo sapiens P-glycoprotein (PGY1) mRNA, complete cds
          Length = 4646

Score = 236 bits (119), Expect = 3e-60
Identities = 119/119 (100%)
Strand = Plus / Plus

Query: 1   gttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgctac
60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3058 gttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgctac
3117

Query: 61   tgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
119
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3118 tgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
3176

279

3] Homo sapiens P-glycoprotein (mdr1) mRNA, complete cds
                Length = 4192

Score = 236 bits (119), Expect = 3e-60
Identities = 119/119 (100%)
Strand = Plus / Plus

Query: 1   gttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgctac
60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2750 gttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgctac
2809

Query: 61   tgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
119
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Sbjct: 2810 tgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
2868

6.8.3 PATIENT 4 SENSE SEQUENCE

BLASTN 2.2.1 [Apr-13-2001]

RID: 1012185815-5202-10736

Query= Patient 4
          (117 letters)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS,
GSS, or phase 0, 1 or 2 HTGS sequences)
          1,079,142 sequences; 4,647,298,756 total letters

Alignments

1] Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 1
          (ABCB1), mRNA
          Length = 4643

Score = 218 bits (110), Expect = 7e-55
Identities = 117/118 (99%), Gaps = 1/118 (0%)
Strand = Plus / Plus

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Query: 1     ttgtctggaca-gcactgaaagataagaaagaactagaaggtgctgggaagatcgctact
59
             |||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3056  ttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgctact
3115


Query: 60    gaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
117
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3116  gaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
3173
```

2] Homo sapiens P-glycoprotein (PGY1) mRNA, complete cds
          Length = 4646

Score = 218 bits (110), Expect = 7e-55
Identities = 117/118 (99%), Gaps = 1/118 (0%)
Strand = Plus / Plus

```
Query: 1     ttgtctggaca-gcactgaaagataagaaagaactagaaggtgctgggaagatcgctact
59
             |||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3059  ttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgctact
3118


Query: 60    gaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
117
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3119  gaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
3176
```

3] Homo sapiens P-glycoprotein (mdr1) mRNA, complete cds
          Length = 4192

Score = 218 bits (110), Expect = 7e-55
Identities = 117/118 (99%), Gaps = 1/118 (0%)
Strand = Plus / Plus

Query: 1   ttgtctggaca-gcactgaaagataagaaagaactagaaggtgctgggaagatcgctact
59
             |||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2751 ttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgctact
2810

Query: 60   gaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
117
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2811 gaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttgaac
2868

6.8.4 PATIENT 8 SENSE SEQUENCE

BLASTN 2.2.1 [Apr-13-2001]

RID: 1012186004-6832-1536

Query= Patient 8
          (122 letters)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS,
GSS, or phase 0, 1 or 2 HTGS sequences)
          1,079,142 sequences; 4,647,298,756 total letters

<div align="center">

Alignments

</div>

1] Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 1
          (ABCB1), mRNA
          Length = 4643

Score = 242 bits (122), Expect = 5e-62
Identities = 122/122 (100%)
Strand = Plus / Plus


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Query: 1     aatgttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgc
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             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3052  aatgttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgc
3111


Query: 61    tactgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttga
120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3112  tactgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttga
3171


Query: 121   ac 122
             ||
Sbjct: 3172  ac 3173
```

2] Homo sapiens P-glycoprotein (PGY1) mRNA, complete cds
            Length = 4646

Score = 242 bits (122), Expect = 5e-62
Identities = 122/122 (100%)
Strand = Plus / Plus

Query: 1   aatgttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgc
60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3055 aatgttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgc
3114

Query: 61   tactgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttga
120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3115 tactgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttga
3174

Query: 121   ac 122
             ||
Sbjct: 3175 ac 3176

3] Homo sapiens P-glycoprotein (mdr1) mRNA, complete cds
            Length = 4192

Score = 242 bits (122), Expect = 5e-62
Identities = 122/122 (100%)
Strand = Plus / Plus

Query: 1   aatgttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgc
60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2747 aatgttgtctggacaagcactgaaagataagaaagaactagaaggtgctgggaagatcgc
2806

Query: 61   tactgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttga
120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2807 tactgaagcaatagaaaacttccgaaccgttgtttctttgactcaggagcagaagtttga
2866

Query: 121   ac 122
             ||
Sbjct: 2867 ac 2868

MTT Assay

1] Setup cells in 96-well plates and treat as required.

2] Set up blank wells that has medium alone, without cells.

3] After required period of growth and exposure to experimental agent, wash wells 2 x with fresh medium and add 1/10 vol. of MTT reagent (5mg/ml).

4] Incubate at $37^{o}C$ for 3-4h. Keep incubation time the same for cell lines that are going to be compared against each other.

5] If working with adherent cells, remove medium completely and add 0.2ml of solubilisation solution, *i.e.* acidic isopropanol (0.04-0.1N HCl in absolute isopropanol). Pipette up and down thoroughly to dissolve crystals. If working with suspension cells the dye is added directly and dissolution is accomplished by trituration.

6] Measure OD of converted dye in cell-containing wells at 570nm v the blank wells without cells, *i.e.* subtract the blank from all experimental values.

7] Calculate % survival fraction as shown below.

$$\% \text{ Survival fraction} = \frac{\text{OD reading for experimental sample}}{\text{OD reading for control}} \times \frac{100}{1}$$

For example,

$$\frac{\text{OD reading for cells} + 500 \text{ I.U./ml IFN-}\alpha}{\text{OD reading for cells without IFN-}\alpha} \times \frac{100}{1}$$

6.10   APPENDIX J

6.10.1 PATIENTS' RHODAMINE EFFLUX & MODULATION PROFILES

6.10.1.1 Untreated patients

See Fig. 6.1 onwards.

Fig. 6.1 Rhodamine accumulation, efflux, and P-gp modulation in patient # 1

Fig. 6.1 Patient B-lymphocytes were simultaneously exposed to [A] 2μM rhodamine 123 +/- verapamil (2μM & 20μM) and [B] 2μM rhodamine 123 +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μM rhodamine123 in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil ($P < 0.01$ using ANOVA followed by Dunnett's post-hoc analysis), while IFN-α had no significant effect ($P > 0.05$).

Fig. 6.2 Rhodamine accumulation, efflux, and P-gp modulation in patient # 2

Fig. 6.2 Patient B-lymphocytes were simultaneously exposed to [A] 2μM rhodamine123 +/- verapamil (2μM & 20μM) and [B] 2μM rhodamine123 +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μM rhodamine123 in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil (P < 0.01 using ANOVA followed by Dunnett's post-hoc analysis), while IFN-α had no significant effect (P > 0.05).

Fig. 6.3 Rhodamine accumulation, efflux, and P-gp modulation in patient # 3

Fig. 6.3 Patient B-lymphocytes were simultaneously exposed to [A] 2µM rhodamine 123 +/- verapamil (2µM & 20µM) and [B] 2µM rhodamine 123 +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2µM rhodamine123 in the absence of modulators. Significant modulation was achieved with 2µM & 20µM verapamil as well as 500I.U/ml IFN-α (P < 0.01 using ANOVA followed by Dunnett's post-hoc analysis). 1000I.U/ml & 5000I.U/ml IFN-α had no significant effect (P > 0.05).

Fig. 6.4 Rhodamine accumulation, efflux, and P-gp modulation in patient # 5
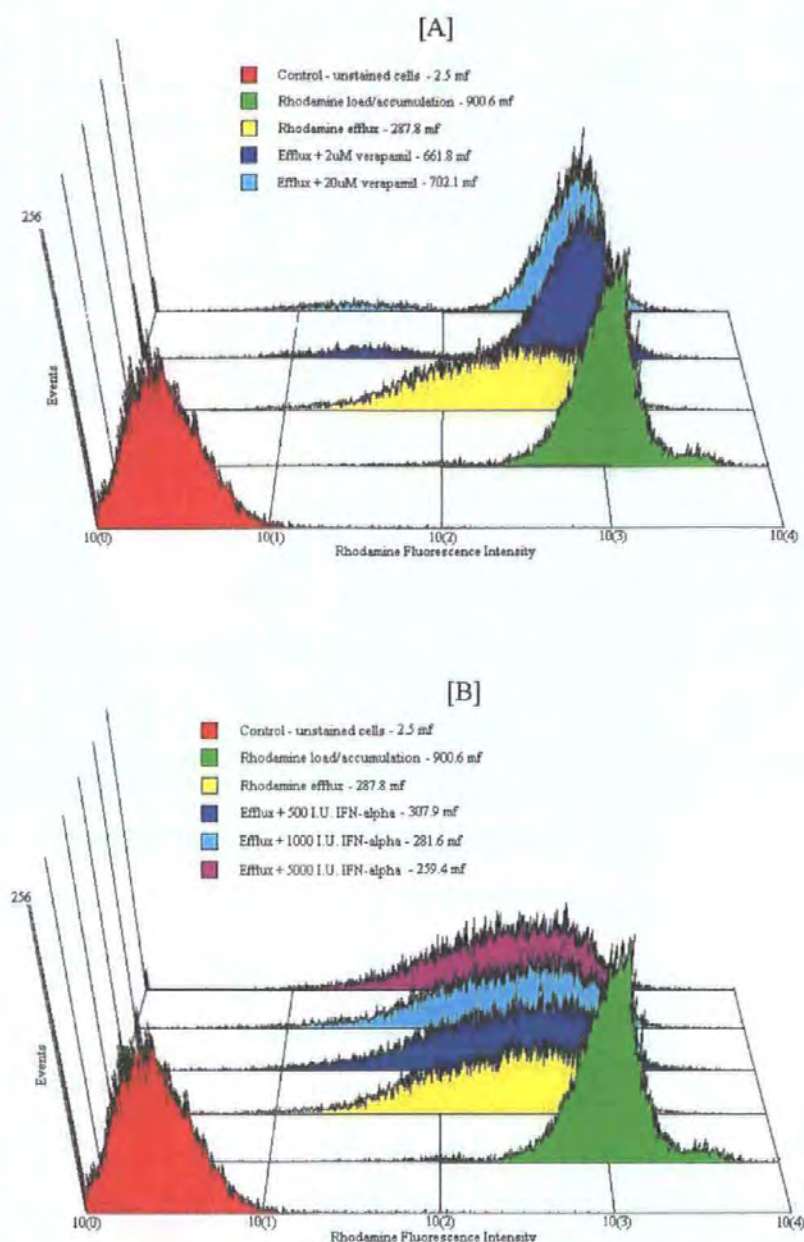

Fig. 6.4 Patient B-lymphocytes were simultaneously exposed to [A] 2μM rhodamine 123 +/- verapamil (2μM & 20μM) and [B] 2μM rhodamine 123 +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μM rhodamine123 in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil ($P < 0.01$ using ANOVA followed by Dunnett's post-hoc analysis). IFN-α had no significant effect ($P > 0.05$).

Fig. 6.5 Rhodamine accumulation, efflux, and P-gp modulation in patient # 6
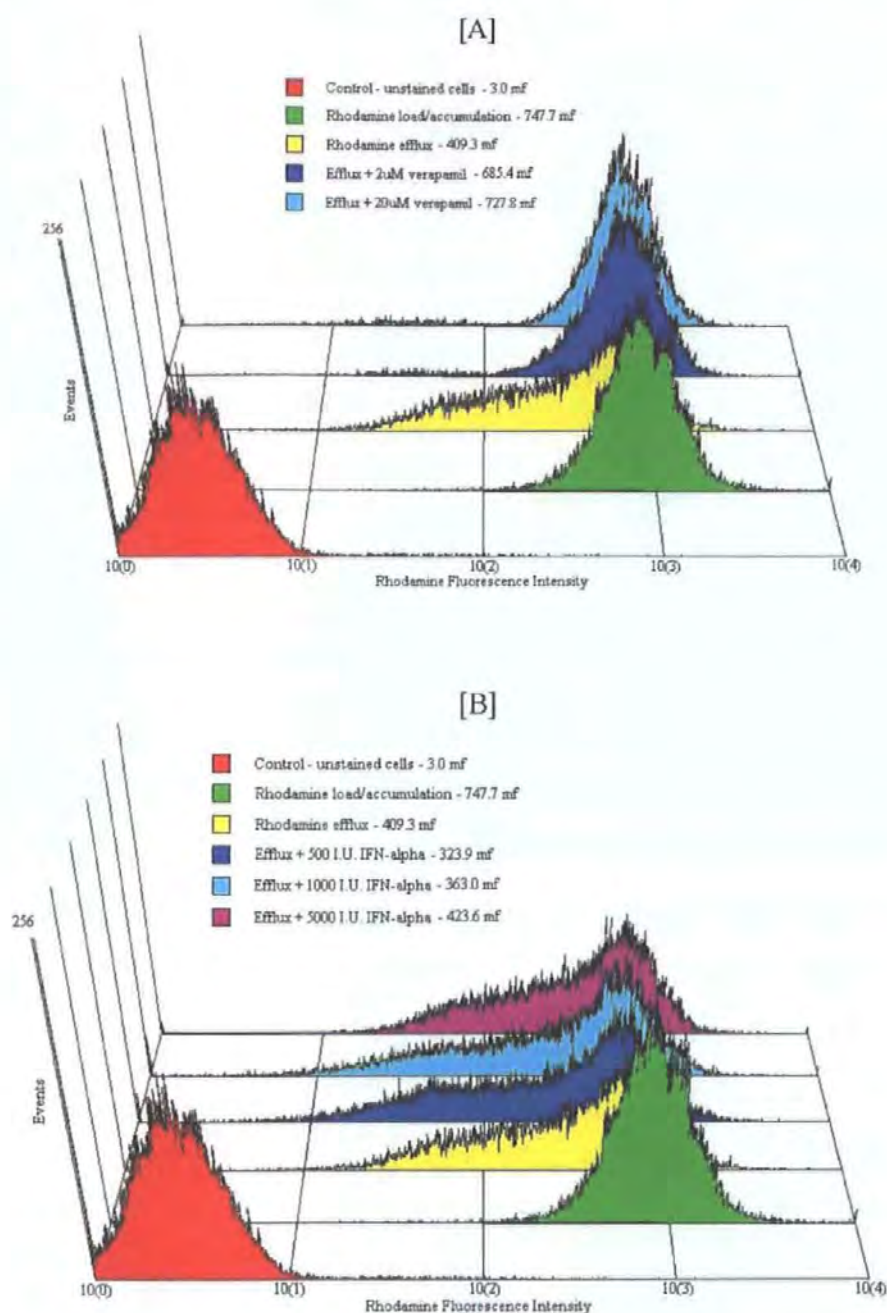

Fig. 6.5 Patient B-lymphocytes were simultaneously exposed to [A] 2μM rhodamine 123 +/- verapamil (2μM & 20μM) and [B] 2μM rhodamine 123 +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μM rhodamine123 in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil ($P < 0.01$ using ANOVA followed by Dunnett's post-hoc analysis). IFN-α had no significant effect ($P > 0.05$).

6.10.1.2 Single agent treated patients

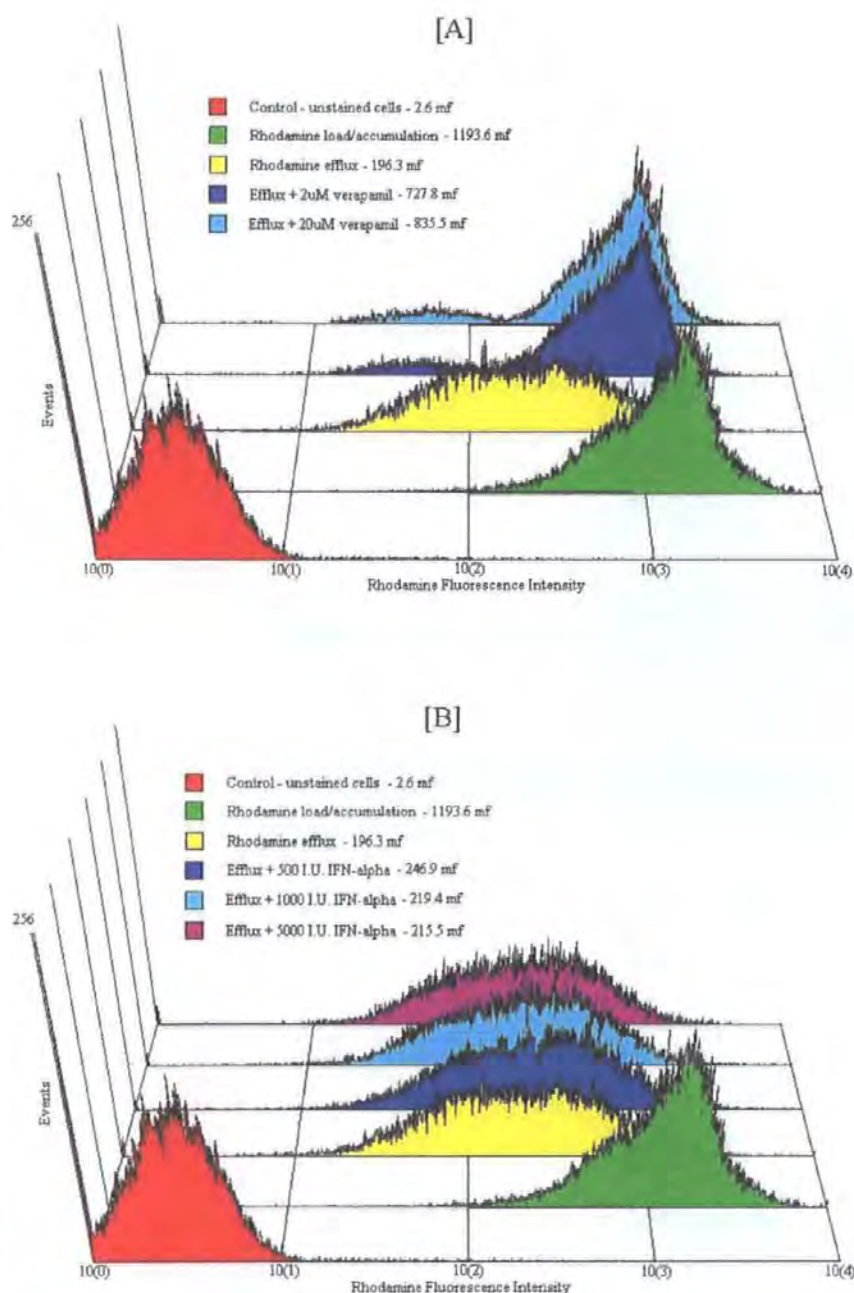## Fig. 6.6 Rhodamine accumulation, efflux, and P-gp modulation in patient # 7

Fig. 6.6 Patient B-lymphocytes were simultaneously exposed to [A] 2μM rhodamine 123 +/- verapamil (2μM & 20μM) and [B] 2μM rhodamine 123 +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μM rhodamine123 in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil (P < 0.01 using ANOVA followed by Dunnett's post-hoc analysis). IFN-α had no significant effect (P > 0.05).

Fig. 6.7 Rhodamine accumulation, efflux, and P-gp modulation in patient # 8

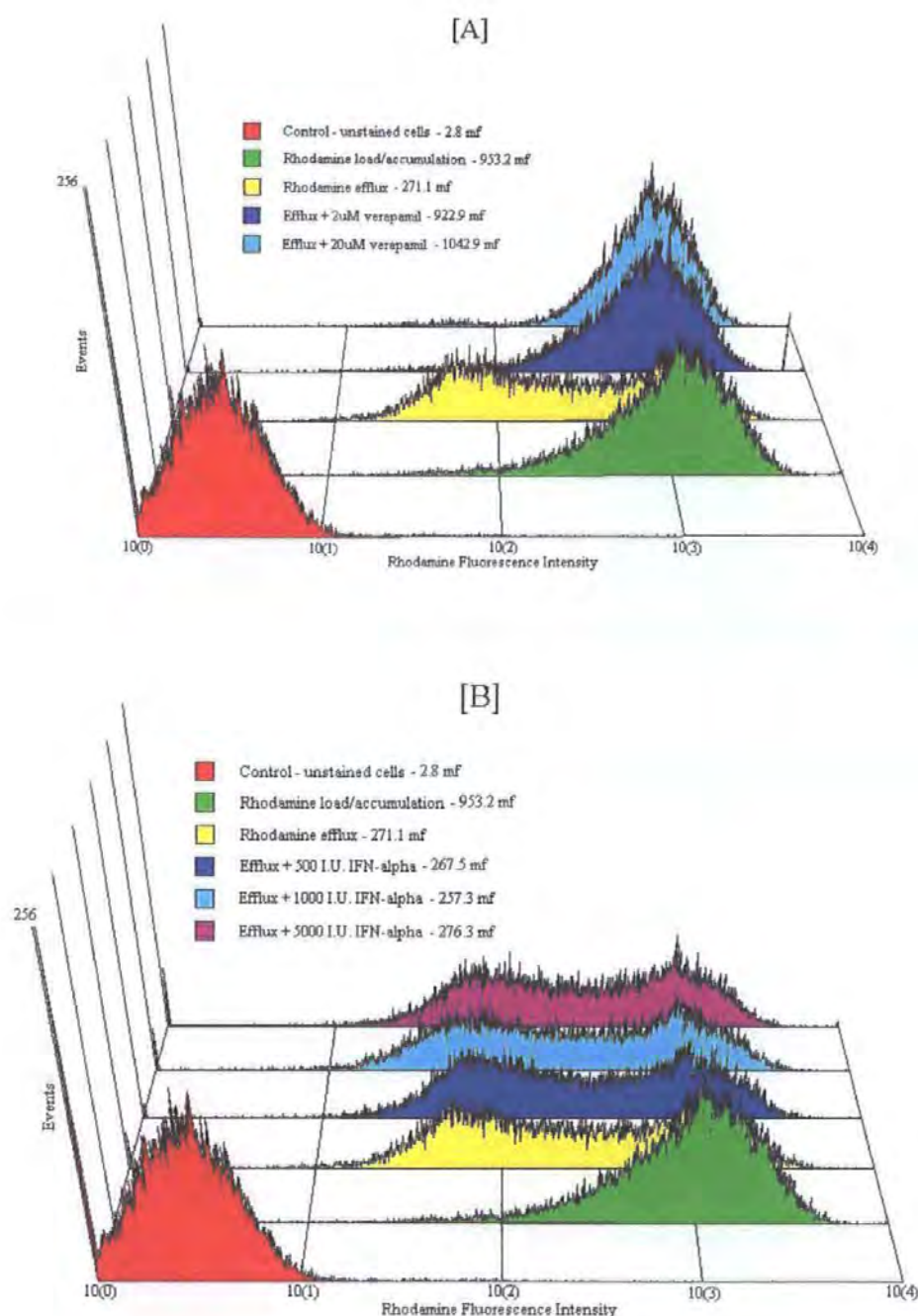Fig. 6.7 Patient B-lymphocytes were simultaneously exposed to [A] 2μM rhodamine 123 +/- verapamil (2μM & 20μM) and [B] 2μM rhodamine 123 +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μM rhodamine123 in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil (P < 0.01 using ANOVA followed by Dunnett's post-hoc analysis). IFN-α had no significant effect (P > 0.05).

Fig. 6.8 Rhodamine accumulation, efflux, and P-gp modulation in patient # 9

[A]

[B]
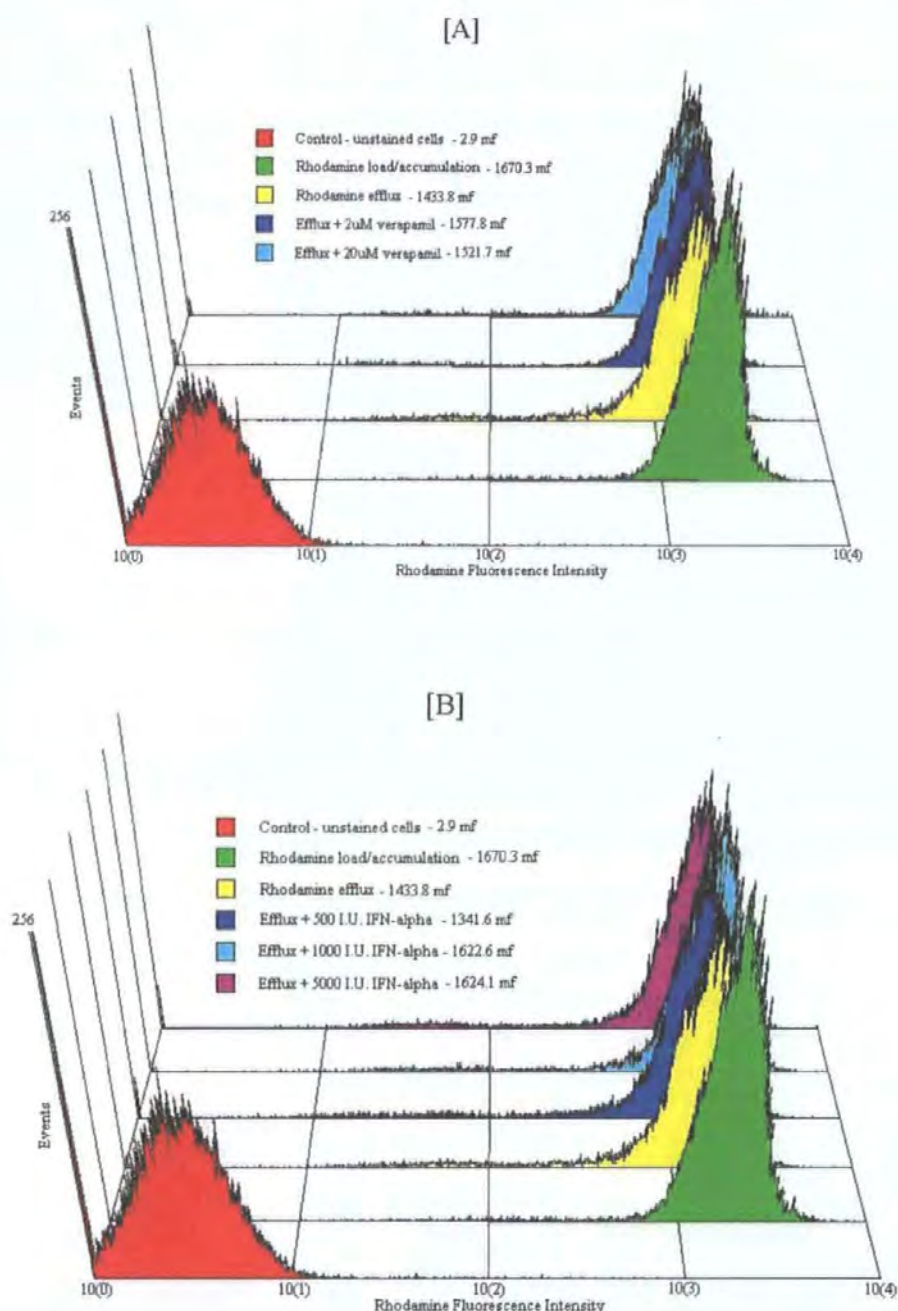
<u>Fig. 6.8</u> Patient B-lymphocytes were simultaneously exposed to [A] 2μM rhodamine 123 +/- verapamil (2μM & 20μM) and [B] 2μM rhodamine 123 +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μM rhodamine123 in the absence of modulators. Significant modulation was achieved with 1000I.U./ml & 5000I.U./ml IFN-α ($P < 0.05$ using ANOVA followed by Dunnett's post-hoc analysis). 2μM & 20μM verapamil as well as 500I.U./ml IFN-α had no significant effect ($P > 0.05$).

Fig. 6.9 Rhodamine accumulation, efflux, and P-gp modulation in patient # 10
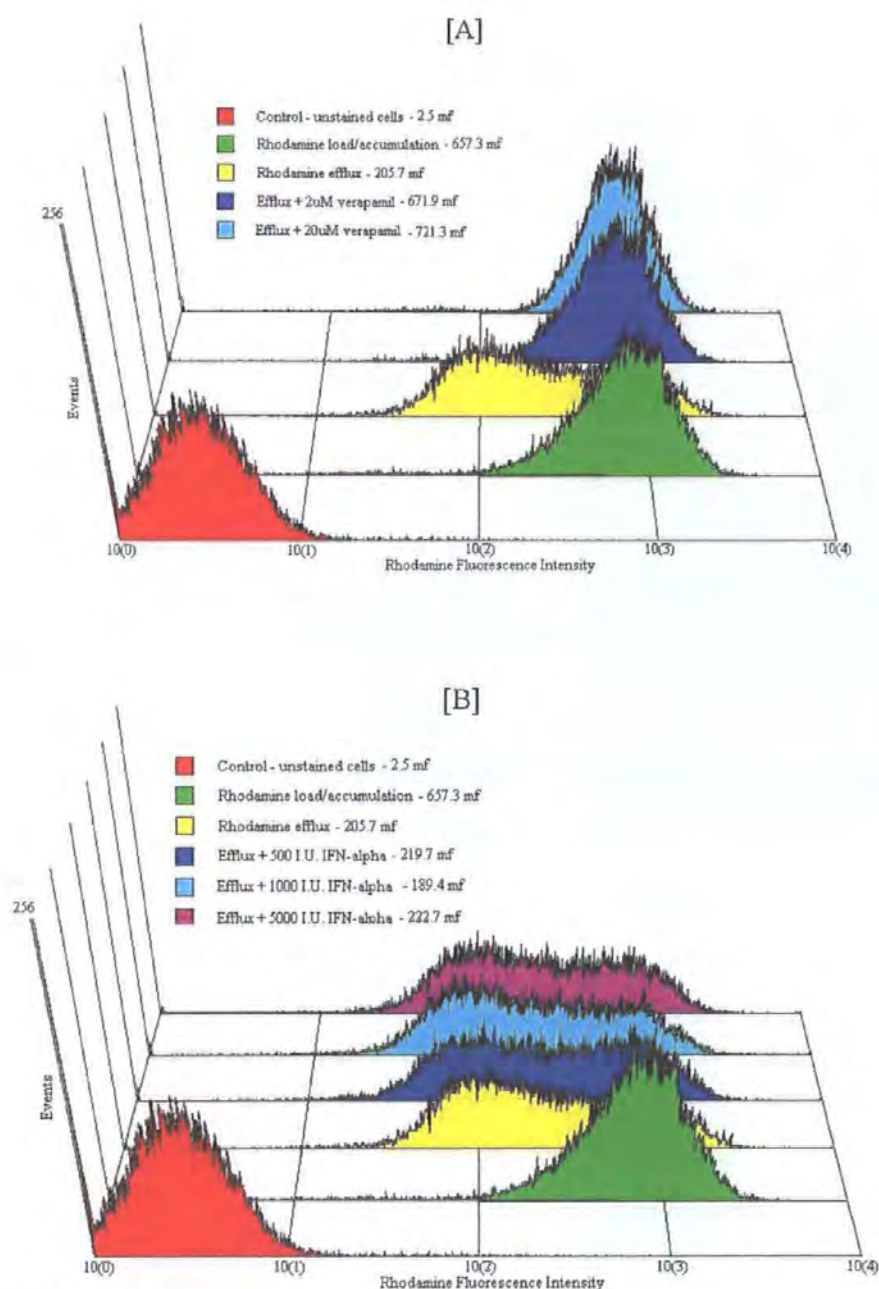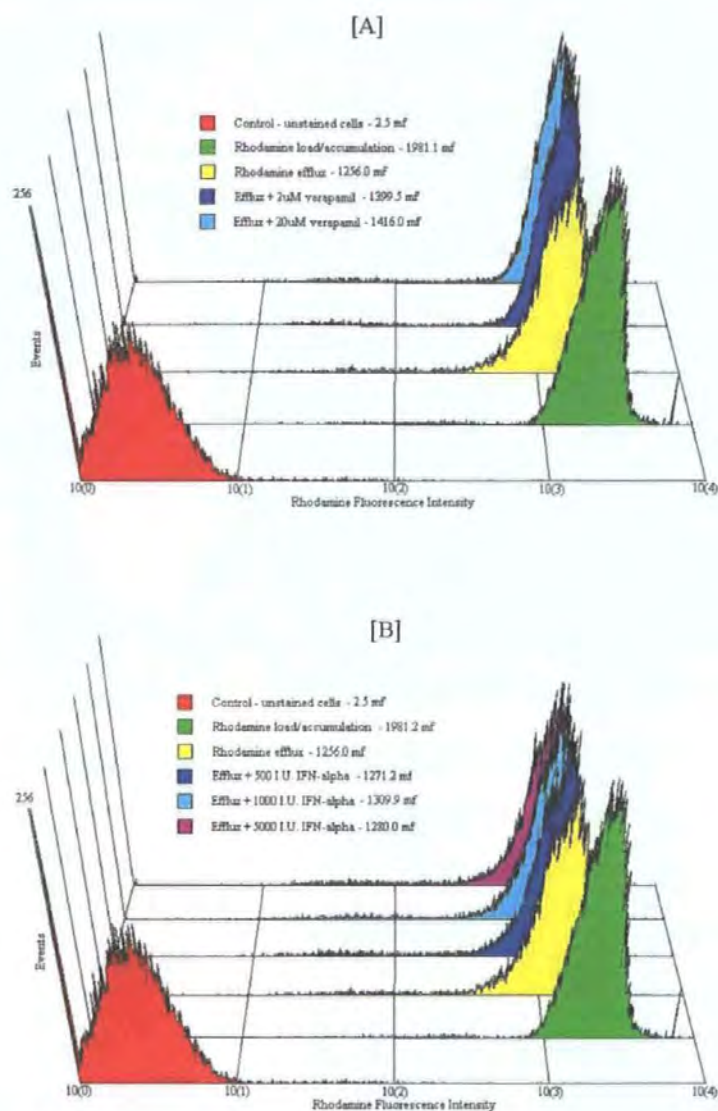
[A]

[B]

Fig. 6.9 Patient B-lymphocytes were simultaneously exposed to [A] 2μM rhodamine 123 +/- verapamil (2μM & 20μM) and [B] 2μM rhodamine 123 +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μM rhodamine123 in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil (P < 0.01 using ANOVA followed by Dunnett's post-hoc analysis). IFN-α had no significant effect (P > 0.05).

6.10.2.1 Untreated patients

Fig. 6.10 Daunorubicin accumulation, efflux, and P-gp modulation in patient # 1

Fig. 6.10 Patient B-lymphocytes were simultaneously exposed to [A] 2µg/ml daunorubicin +/- verapamil (2µM & 20µM) and [B] 2µg/ml daunorubicin +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2µg/ml daunorubicin in the absence of modulators. Significant modulation was achieved with 2µM & 20µM verapamil (P < 0.01 using ANOVA followed by Dunnett's post-hoc analysis). IFN-α had no significant effect (P > 0.05).

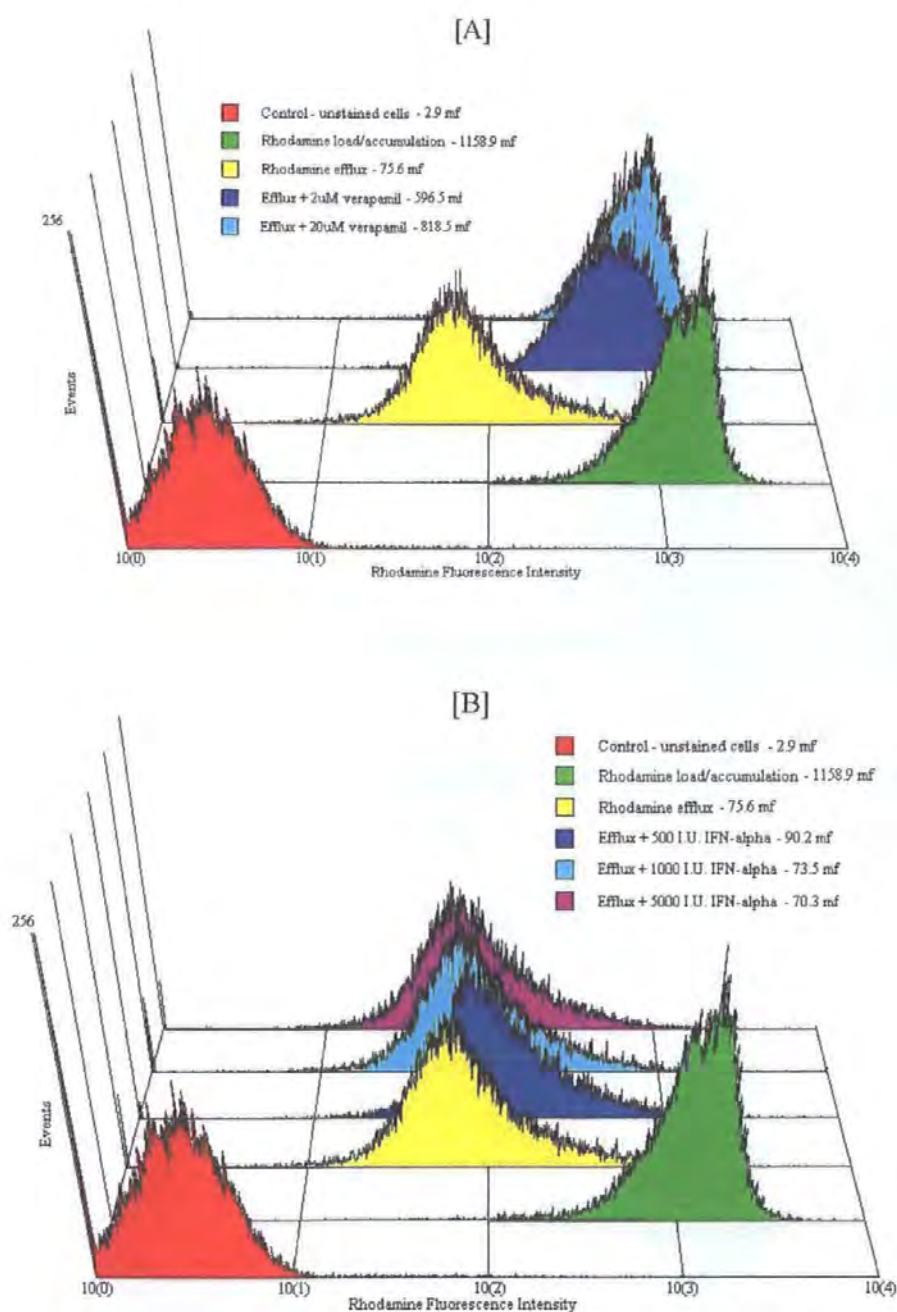# Fig. 6.11 Daunorubicin accumulation, efflux, and P-gp modulation in patient # 4

<u>Fig. 6.11</u> Patient B-lymphocytes were simultaneously exposed to [A] 2μg/ml daunorubicin +/- verapamil (2μM & 20μM) and [B] 2μg/ml daunorubicin +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μg/ml daunorubicin in the absence of modulators. Significant modulation was not achieved with either verapamil or IFN-α (P > 0.05 using ANOVA)

Fig. 6.12 Daunorubicin accumulation, efflux, and P-gp modulation in patient # 5

Fig. 6.12 Patient B-lymphocytes were simultaneously exposed to [A] 2μg/ml daunorubicin +/- verapamil (2μM & 20μM) and [B] 2μg/ml daunorubicin +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μg/ml daunorubicin in the absence of modulators. Significant modulation was achieved with 20μM verapamil ($P < 0.05$ using ANOVA followed by Dunnett's post-hoc analysis). 2μM verapamil and IFN-α had no significant effect ($P > 0.05$).

Fig. 6.13 Daunorubicin accumulation, efflux, and P-gp modulation in patient # 6

[A]

- Control - unstained cells - 2.8 mf
- Daunorubicin load/accumulation - 123.7 mf
- Daunorubicin efflux - 74.7 mf
- Efflux + 2uM verapamil - 84.4 mf
- Efflux + 20uM verapamil - 93.8 mf

[B]

- Control - unstained cells - 2.8 mf
- Daunorubicin load/accumulation - 123.7 mf
- Daunorubicin efflux - 74.7 mf
- Efflux + 500 I.U. IFN-alpha - 79.4 mf
- Efflux + 1000 I.U. IFN-alpha - 76.9 mf
- Efflux + 5000 I.U. IFN-alpha - 80.0 mf

Fig. 6.13 Patient B-lymphocytes were simultaneously exposed to [A] 2μg/ml daunorubicin +/- verapamil (2μM & 20μM) and [B] 2μg/ml daunorubicin +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μg/ml daunorubicin in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil (P < 0.05 and 0.01, respectively, using ANOVA followed by Dunnett's post-hoc analysis). IFN-α had no significant effect (P > 0.05).

Fig. 6.14 Daunorubicin accumulation, efflux, and P-gp modulation in patient # 7
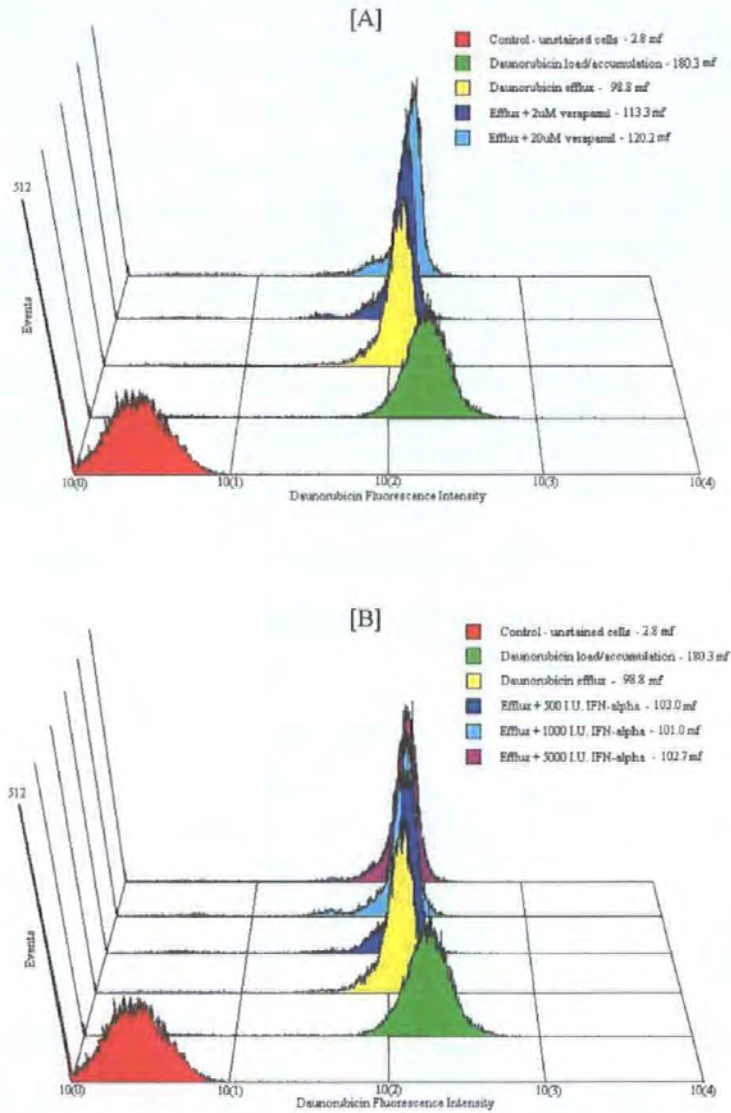

Fig. 6.14 Patient B-lymphocytes were simultaneously exposed to [A] 2μg/ml daunorubicin +/- verapamil (2μM & 20μM) and [B] 2μg/ml daunorubicin +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μg/ml daunorubicin in the absence of modulators. Significant modulation was achieved with 20μM verapamil (P < 0.01 using ANOVA followed by Dunnett's post-hoc analysis). 2μM verapamil and IFN-α had no significant effect (P > 0.05).

Fig. 6.15 Daunorubicin accumulation, efflux, and P-gp modulation in patient # 9
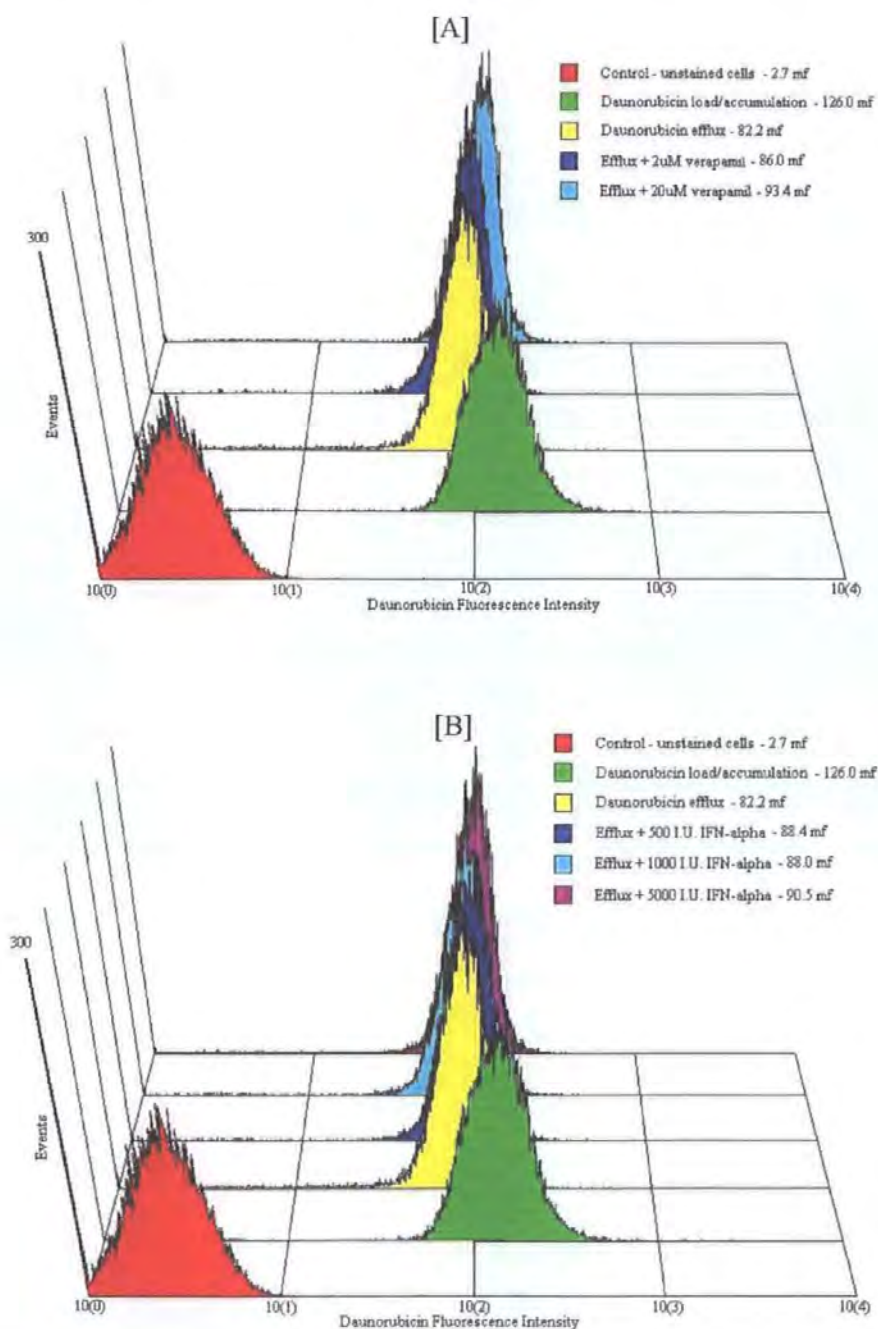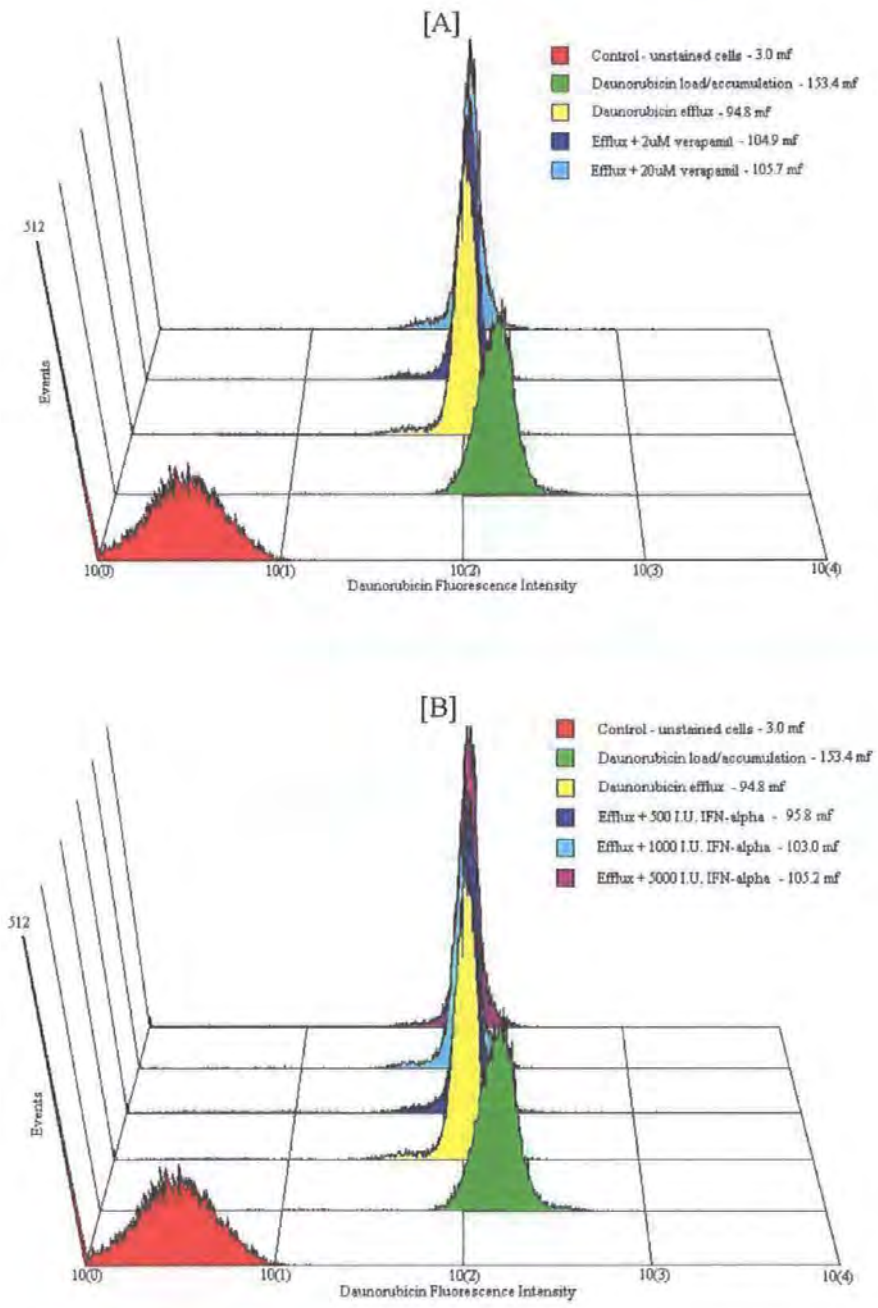

Fig. 6.15 Patient B-lymphocytes were simultaneously exposed to [A] 2μg/ml daunorubicin +/- verapamil (2μM & 20μM) and [B] 2μg/ml daunorubicin +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μg/ml daunorubicin in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil (P < 0.05 & 0.01, respectively, using ANOVA followed by Dunnett's post-hoc analysis). as well as 1000I.U/ml & 5000I.U./ml of IFN-α (P < 0.05). 500I.U./ml IFN-α had no significant effect (p > 0.05).

Fig. 6.16 Daunorubicin accumulation, efflux, and P-gp modulation in patient # 10
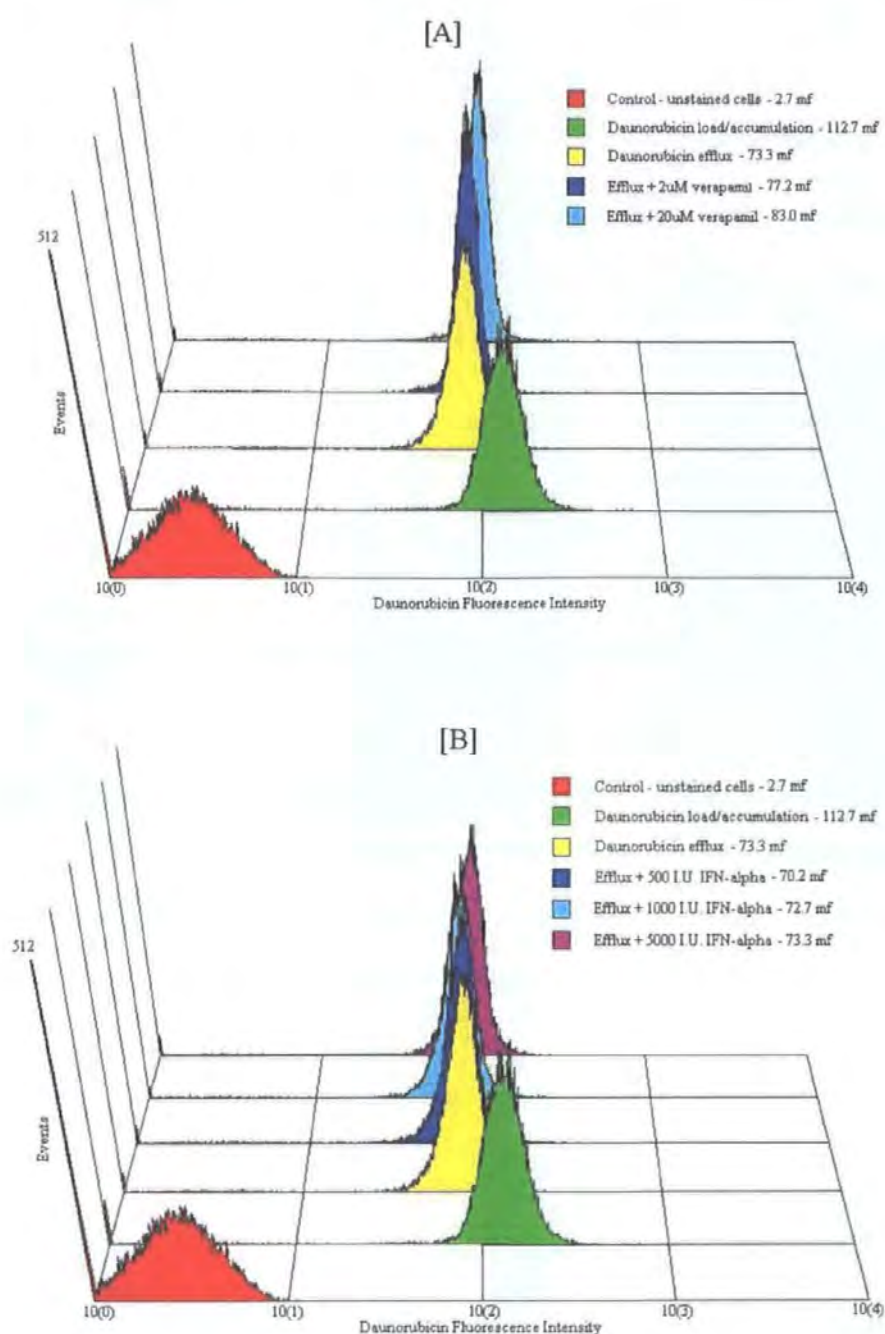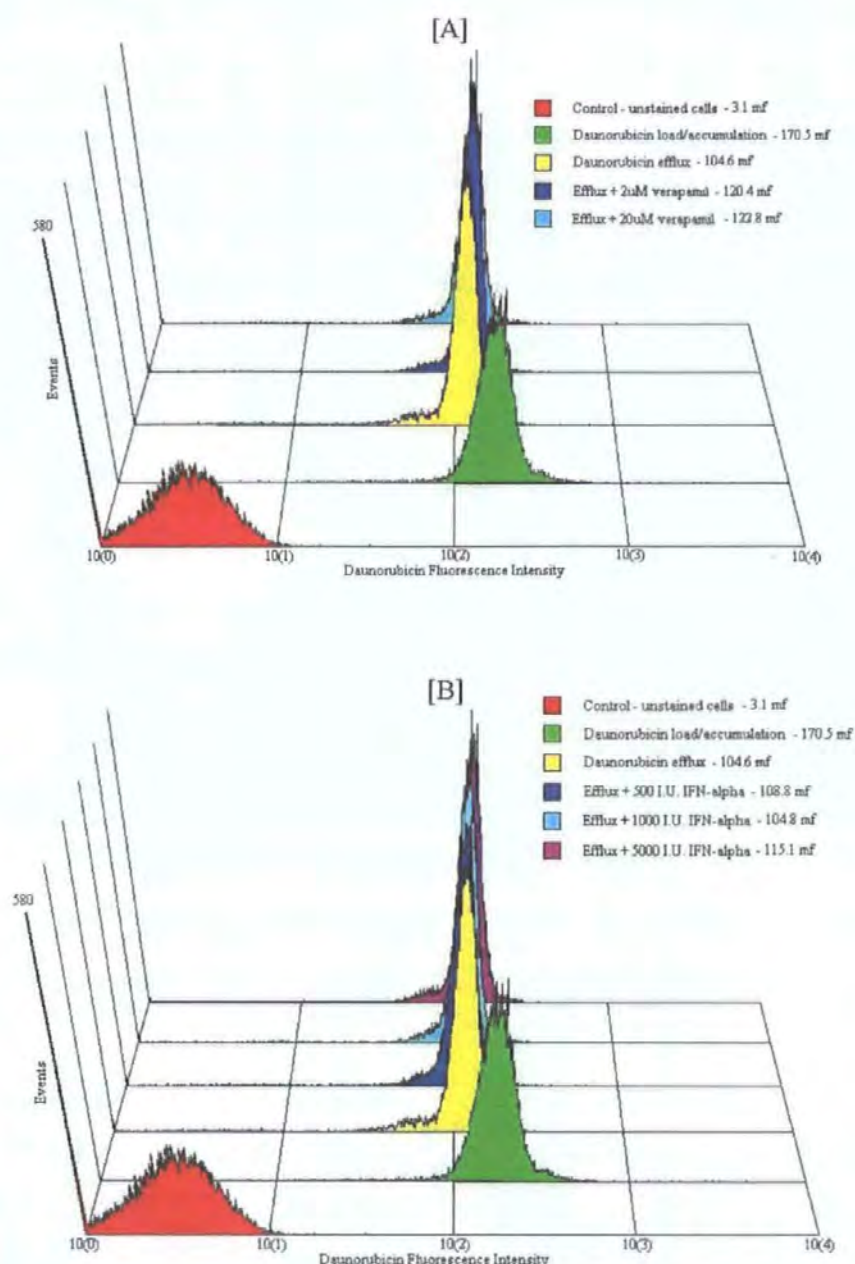

Fig. 6.16 Patient B-lymphocytes were simultaneously exposed to [A] 2μg/ml daunorubicin +/- verapamil (2μM & 20μM) and [B] 2μg/ml daunorubicin +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μg/ml daunorubicin in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil ($P < 0.01$ using ANOVA followed by Dunnett's post-hoc analysis). as well as 5000I.U./ml of IFN-α ($P < 0.01$). 500I.U./ml & 1000I.U/ml IFN-α had no significant effect ($p > 0.05$).

Fig. 6.17 Daunorubicin accumulation, efflux, and P-gp modulation in patient # 11

[A]

Control - unstained cells - 3.0 mf
Daunorubicin load/accumulation - 232.6 mf
Daunorubicin efflux - 129.7 mf
Efflux + 2uM verapamil - 140.3 mf
Efflux + 20uM verapamil - 146.8 mf

[B]

Control - unstained cells - 3.0 mf
Daunorubicin load/accumulation - 232.6 mf
Daunorubicin efflux - 129.7 mf
Efflux + 500 I.U. IFN-alpha - 137.6 mf
Efflux + 1000 I.U. IFN-alpha - 130.8 mf
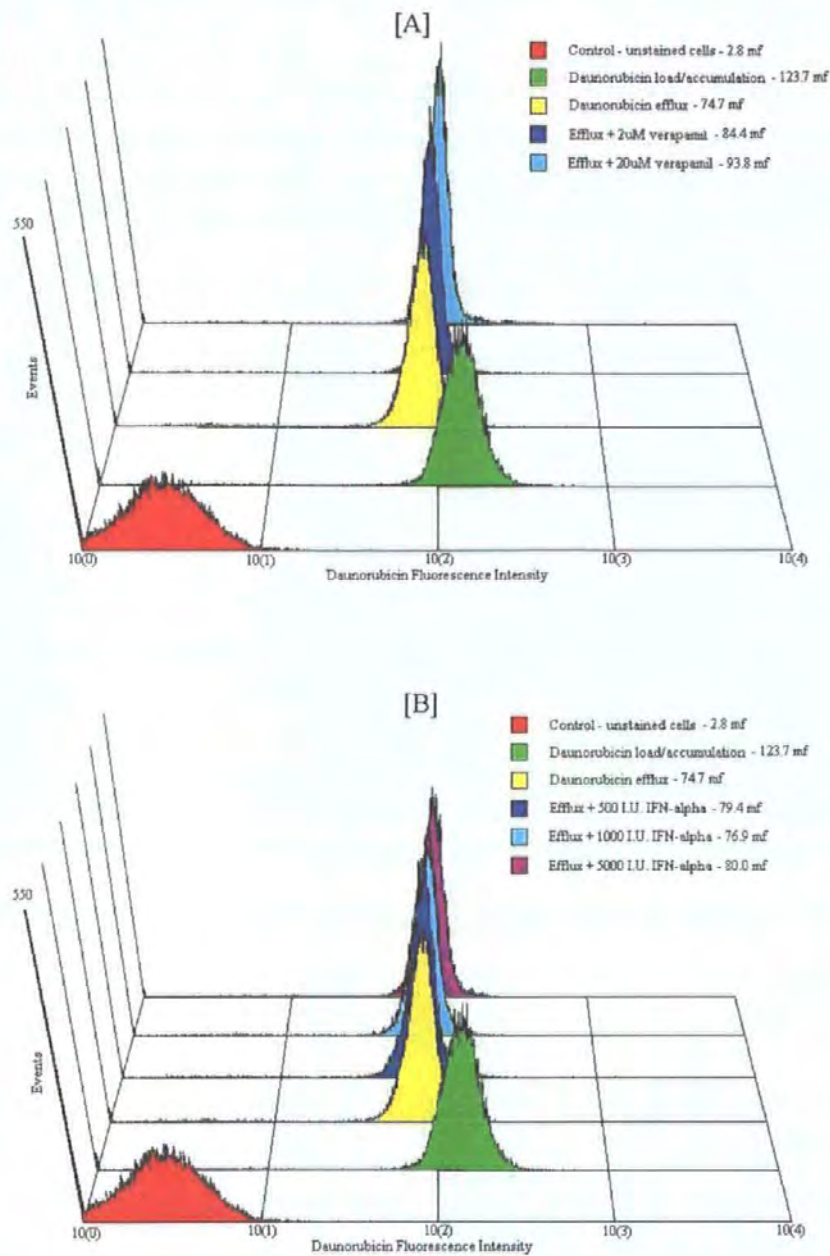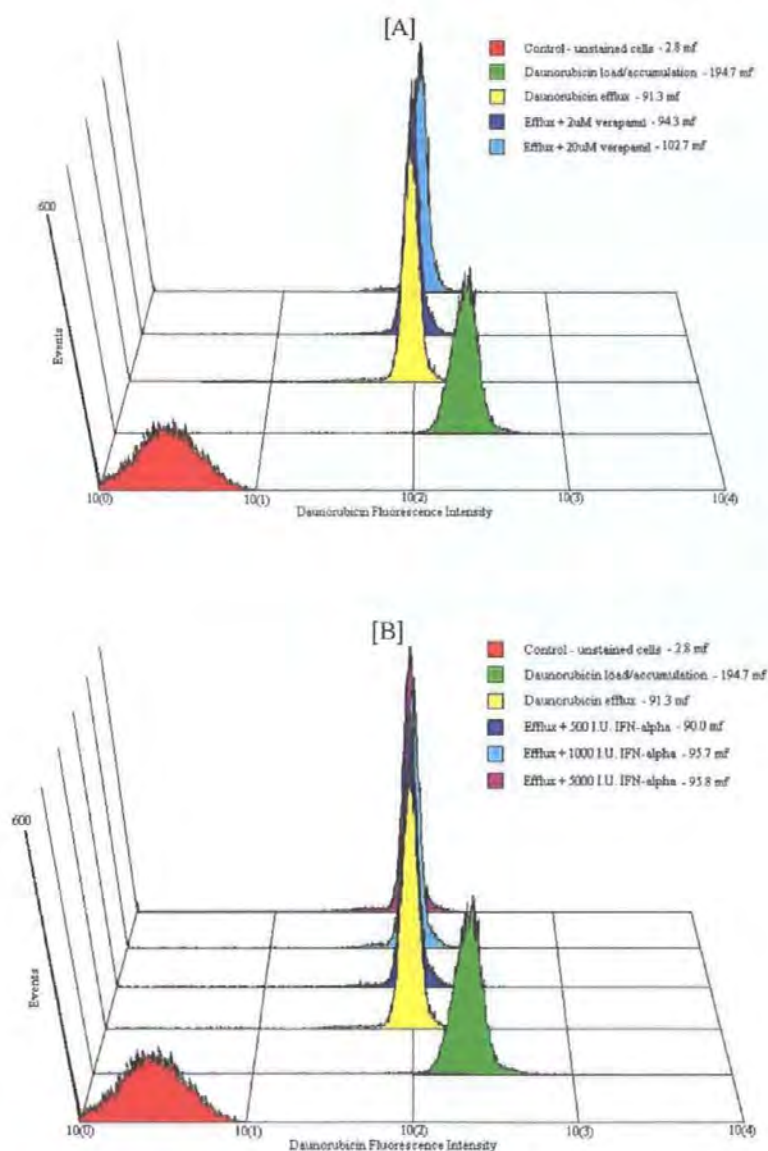Efflux + 5000 I.U. IFN-alpha - 131.9 mf
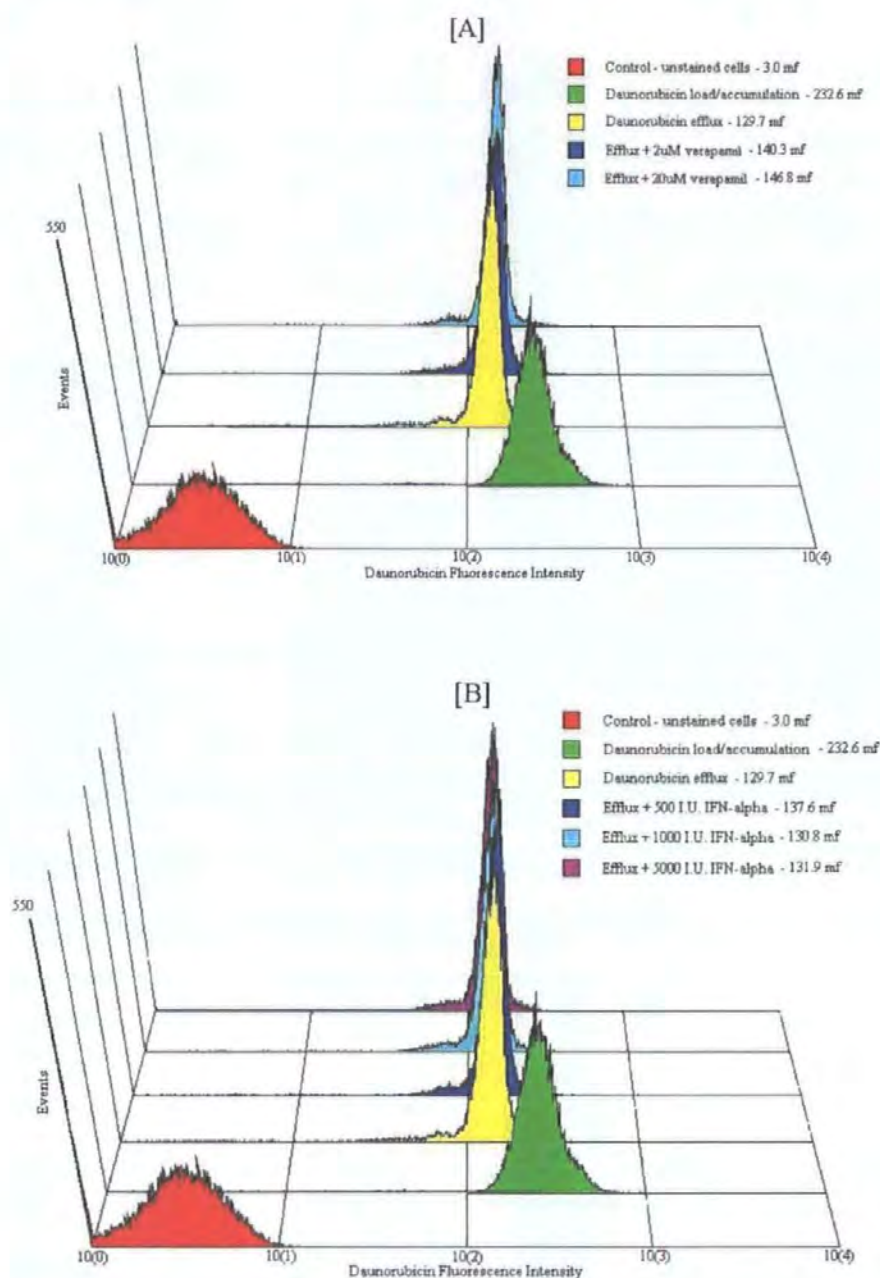
Fig. 6.17 Patient B-lymphocytes were simultaneously exposed to [A] 2μg/ml daunorubicin +/- verapamil (2μM & 20μM) and [B] 2μg/ml daunorubicin +/- IFN-α (500I.U./ml, 1000I.U./ml and 5000I.U./ml) then left to incubate at 37°C for 90min (efflux). Intracellular fluorescence intensities were compared to those of B-lymphocytes having effluxed 2μg/ml daunorubicin in the absence of modulators. Significant modulation was achieved with 2μM & 20μM verapamil ($P < 0.05$ & 0.01, respectively, using ANOVA followed by Dunnett's post-hoc analysis). IFN-α had no significant effect ($P < 0.01$).

Fig. 6.18 Relationship between %P-gp expression and patient age and sex
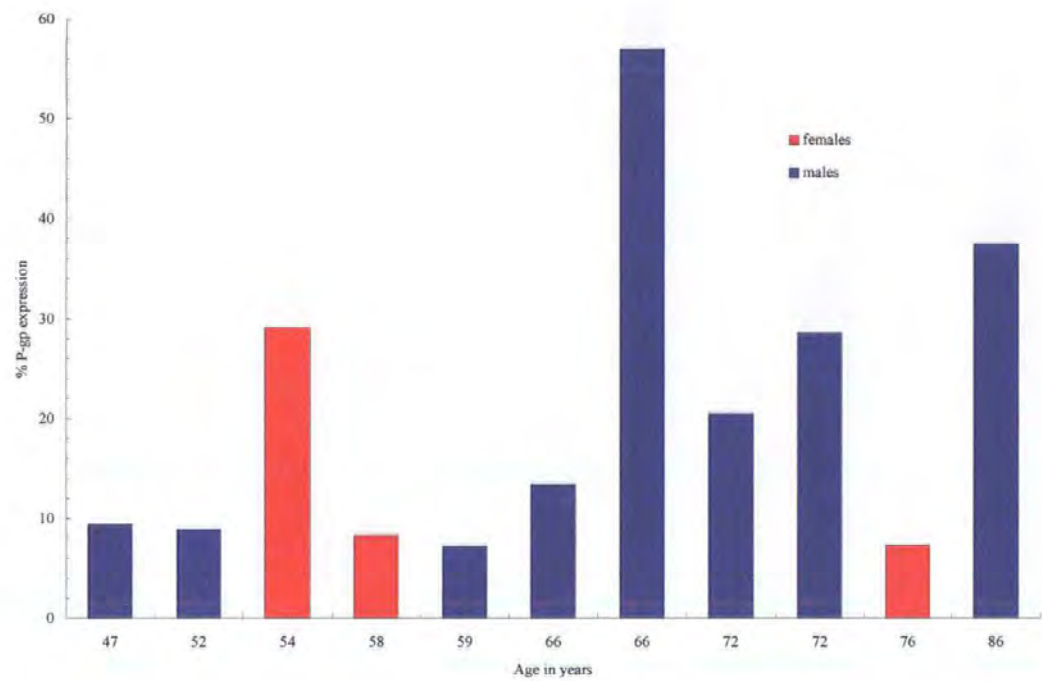
Fig. 6.19 Relationship between %P-gp expression and stage of disease
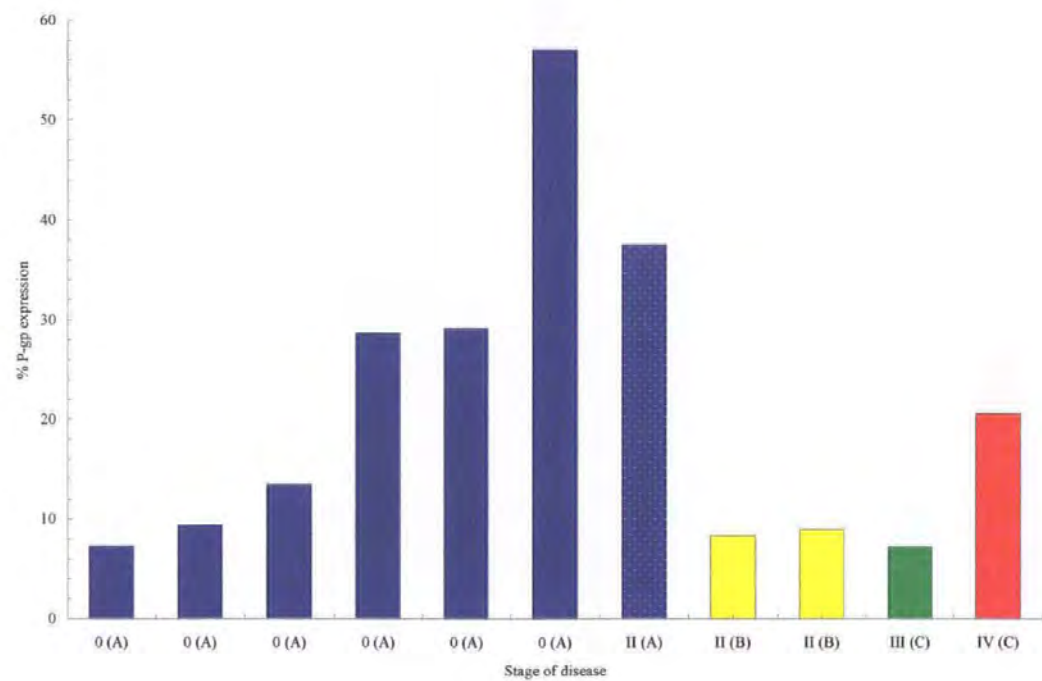
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8. PUBLICATIONS

Abstract

Loss of Daunorubicin from *in vitro* systems used in drug resistance studies

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Introduction and Objective: Daunorubicin has been studied extensively in research into anthracycline-related multidrug resistance (MDR). There are no published reports, however, on the drug's concentration after *in vitro* alterations in physico-chemical variables which may affect the stability of daunorubicin. This study reports the effect of time, temperature, culture media, and pH on daunorubicin concentration in an *in vitro* system initially devoid of cellular material.

Method: Aqueous solutions of daunorubicin (100ng/ml) were incubated in cell culture plates at 4°C, 25°C & 37°C, sampled at various time intervals over 24h and subjected to HPLC assay. Subsequently, 100ng/ml solutions of daunorubicin in various culture media (RPMI, DMEM and PBS) and H_2O (control) were incubated in culture plates at 37°C and sampled for assay after 0.5h. Solutions of daunorubicin in RPMI (100ng/ml) were then buffered to pH values of 6, 7, and 7.5 and incubated in culture plates at 37°C for 0.5h prior to assay using a validated LC method. Parallel solvent elution experiments were performed to quantify drug adsorbed to the culture plates.

Results: Over a period of 2h daunorubicin loss was 23%, 35% and 70% when incubated at 37°C, 4°C and 25°C, respectively. The drug loss after incubation at 37°C was less than expected because of a favourable pH change to more acidic conditions due to the 5% CO_2 environment in the incubator. Drug loss between the different diluent types was 17.5%, 38.7%, 43.5% and 50.5% for H_2O, PBS, RPMI and DMEM, respectively. In PBS, RPMI and DMEM there was an instantaneous drug loss with further loss occurring upon incubation. Loss during incubation occurred by both degradation and adsorption to the culture plate. Greatest stability was observed at a pH of 6 where drug loss was solely due to adsorption (38.5%), whereas at pH 7 and 7.5 there was significant loss due to degradation (82.6% and 93.4% total loss, respectively).

Conclusion: Under typical *in vitro* experimental conditions significant loss of daunorubicin occurred over short (0.5h) incubation periods. Drug loss was attributable to simultaneous adsorption and pH-dependant degradation processes. These phenomena could exert a profound effect on *in vitro* MDR studies and must be considered in experimental design. Techniques to avoid drug loss prior to cellular assimilation could be employed although this approach may also influence the MDR process.

Abstract

IMPLICATIONS OF ANTHRACYCLINE STABILITY IN *IN VITRO* SYSTEMS WHEN INVESTIGATING DRUG RESISTANCE. V. Munoz*, M. Priston*, A. Prentice*, G. Sewell*. (Intr. by J. A. Copplestone) *Departments of Pharmacy and Haematology, Plymouth Postgraduate Medical School , University of Plymouth; Derriford Hospital, Plymouth; Department of Pharmacy and Pharmacology, University of Bath.*

Research into the multidrug resistance (MDR) associated with P-glycoprotein (P-gp), has involved the use of many agents including Daunorubicin (DNR). There are no published reports, however, on the drug's concentration after *in vitro* alterations in physico-chemico variables which may affect the stability of DNR. This study reports the effect of time, temperature, culture media, pH and protein binding on DNR concentration in an *in vitro* system devoid of cellular material. It also highlights the effects of DNR cytotoxicity, as well as one of its derivatives, Daunorubicinol (DOL), on a sensitive and MDR1 expressing cell line. All analysis was performed, using a validated Liquid Chromatography method, on protein free DNR solutions (100ng/ml) or deproteinated DNR filtrates (1μg/ml). Over a period of 2h DNR loss (in RPMI) was 23%, 35% and 70% when incubated at 37°C, 4°C and 25°C respectively. The drug loss after incubation at 37°C was less than expected because of a favourable pH change to more acidic conditions due to the 5% CO_2 environment in the incubator. The increased loss at 4°C and 25°C was attributed to an unfavourable pH change. Drug loss between the different diluent types was 17.5%, 38.7%, 43.5% and 50.5% after 0.5h incubation for H_2O, PBS, RPMI and DMEM, respectively. In PBS, RPMI and DMEM there was an instantaneous drug loss with further loss occurring upon incubation. Loss during incubation occurred by both degradation and adsorption to the culture plate. Greatest stability was observed at a pH of 6 where drug loss was solely due to adsorption (38.5%), whereas at pH 7 and 7.5 there was significant loss due to degradation (82.6% and 93.4% total loss, respectively). Protein binding after incubating a 1μg/ml DNR solution at 37°C in RPMI, resulted in a final 16.9% loss. Investigations into the cytotoxic effects of DNR and DOL show that both agents are toxic to cells. Although DNR is more potent than DOL, they both exhibit the same trend, showing a greater cell kill on the sensitive cells v the resistant cells. Under typical *in vitro* experimental conditions significant loss of DNR occurred over short (0.5h) incubation periods where drug loss was attributable to simultaneous adsorption and pH-dependent degradative processes. DNR is known to be a substrate for P-gp however little work has been done to identify the interactions of its degradation and metabolic products with P-gp. In B-cell Chronic Lymphocytic Leukaemia, we have found P-gp expression to be minimal, but for those haematological malignancies in which P-gp is a major contributor to MDR, the instability of the parent anthracycline and the presence of the resulting degradation and metabolic products could exert a profound effect on *in vitro* MDR/P-gp studies and must be considered in experimental design.

Drug Resistance Studies - Implications of Daunorubicin Stability in *in vitro* Systems.

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Extensive research into anthracycline-related multidrug resistance (MDR) has involved the use of many agents including daunorubicin (DNR). There are no published reports, however, on the drug's concentration after *in vitro* alterations in physico-chemical variables which may affect the stability of DNR. Similarly, current reports on multidrug resistance comment solely on the cytotoxic effects of the parent anthracycline, neglecting the potential interference from degradation products. This study reports the effect of time, temperature, culture media, pH and protein binding on DNR concentration in an *in vitro* system devoid of cellular material. It also highlights the effects of DNR cytotoxicity, as well as one of its derivatives, on a sensitive and MDR1 expressing cell line.

All analysis was performed, using a validated LC method, on protein free DNR solutions (100ng/ml) or deproteinated DNR filtrates (1μg/ml).

Over a period of 2h DNR loss (in RPMI) was 23%, 35% and 70% when incubated at 37°C, 4°C and 25°C, respectively. The drug loss after incubation at 37°C was less than expected because of a favourable pH change to more acidic conditions due to the 5% CO_2 environment in the incubator. The increased loss at 4°C and 25°C was attributed to an unfavourable pH change. Drug loss between the different diluent types was 17.5%, 38.7%, 43.5% and 50.5% after 0.5h incubation for H_2O, PBS, RPMI and DMEM, respectively. In PBS, RPMI and DMEM there was an instantaneous drug loss with further loss occurring upon incubation. Loss during incubation occurred by both degradation and adsorption to the culture

plate. Greatest stability was observed at a pH of 6 where drug loss was solely due to adsorption (38.5%), whereas at pH 7 and 7.5 there was significant loss due to degradation (82.6% and 93.4% total loss, respectively). Protein binding after incubating a 1μg/ml DNR solution at 37°C in RPMI, resulted in a final 16.9% loss - see Fig.1.
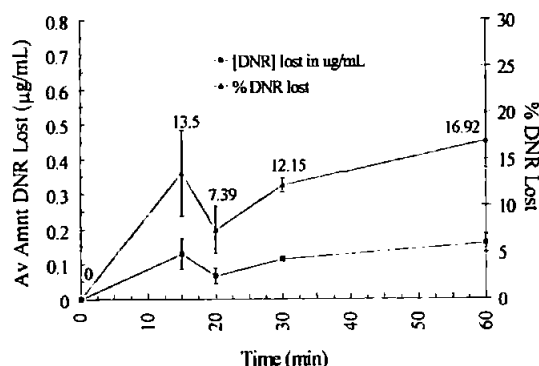

Fig.1 Protein Binding for 1μg/mL Daunorubicin at 37°C N=3

The investigations into the cytotoxic effects of DNR and its derivative are currently ongoing.

Under typical *in vitro* experimental conditions significant loss of DNR occurred over short (0.5h) incubation periods. Drug loss was attributable to simultaneous adsorption and pH-dependant degradation processes. This loss, including the presence of degradation products, could exert a profound effect on *in vitro* MDR studies and must be considered in experimental design.