INDUCTION OF DRUG RESISTANCE AND DIFFERENTIATION IN HUMAN LEUKAEMIA CELL LINES

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ABSTRACT

The ability of low, clinically relevant levels of the chemotherapeutic drugs epirubicin and vinblastine to induce drug resistance was examined in the K562. U937, KG-1a and HEL human leukaemia cell lines. Treatment with epirubicin and vinblastine induced the MDR phenotype and P-glycoprotein expression in K562 and U937 cells. However this treatment had no effect on drug resistance in the P-glycoprotein expressing KG-1a and HEL cells. In the U937 cells, drug resistant cells were not only MDR but were also resistant to other drugs including cisplatinum and chlorambucil which are not normally associated with MDR. The drug resistant U937 sublines were also sensitised to doxorubicin, cisplatinum and chlorambucil by buthionine sulphoximine (BSO), suggesting that glutathione-related mechanisms also contributed to resistance in these sublines. The U937 sublines also had an increased DNA content and an increased ability to recover from DNA damage, as determined by cell cycle analysis, indicating that the broad cross-resistance exhibited by these cells was due to the co-existence of multiple resistance mechanisms. Drug treatment induced changes in expression of differentiation associated antigens in all four cell lines.

Treatment with inducers of differentiation (TPA, sodium butyrate, granulocyte-macrophage colony-stimulating factor; GM-CSF). Treatment of K562 and K562/E15B cells with TPA induced megakaryocytic differentiation, with increases in drug resistance, and increased P-glycoprotein expression in the K562/E15B subline. TPA induced monocytic differentiation in the U937 cells but not the U937/E15 subline, with increased P-glycoprotein expression and function in the U937/E15 cells but not the U937 cells. Staurosporine, an inhibitor of PKC, inhibited differentiation in these cell lines, but did not inhibit increases in P-glycoprotein expression, suggesting drug resistance was not mediated by PKC.

Sodium butyrate induced erythroid differentiation, and increased P-glycoprotein expression in the K562/E15B cells. However at a higher concentration (15 mM) this was not accompanied by increased drug resistance. Granulocyte monocyte colony stimulating factor (GM-CSF) did not induce differentiation in the K562 cells or K562/E15B subline, although the K562/E15B cells became more drug resistant after treatment with GM-CSF. Treatment with GM-CSF induced differentiation in the U937/E15 subline but did not change drug resistance in either the U937 cells or the U937/E15 subline.

Therefore the P-glycoprotein expressing K562/E15B and U937/E15 sublines were more responsive to inducers of differentiation than the parental cell lines. Induction of differentiation therefore induced increases in P-glycoprotein expression and drug resistance, suggesting that expression of P-glycoprotein or a multidrug resistance phenotype was associated with differentiation.

Abbreviations

Abbreviation	Full Name
AML	acute myeloid leukaemia
ALL	acute lymphocytic leukaemia
APAAP	alkaline phosphatase anti-alkaline phosphatase
BCIP	5-bromo,4-chloro,3-indolyphosphate
BSO	buthionine sulphoximine
CML	chronic myeloid leukaemia
CLL	chronic lymphocytic leukaemia
COL	colchicine
DMSO	dimethylsulfoxide
DNR	daunorubicin
DOX	doxorubicin
DTT	dithiothreitol
EDTA	ethylene diamine triacetic acid
EPR	epirubicin
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSH	glutathione (reduced)
GST	glutathione-S-Transferase
h	hour
IDA	idarubicin
MDR	multidrug resistance
min	minutes
MTT	3-4,5-dimethylthiazol-2,5 diphenyl tetrazolium bromide
NBT	nitro blue-tetrazolium
PAGE	polyacrylamide gel electrophoresis
PI	propidium iodide
PBS	phosphate buffered saline
PKC	protein kinase C
Rh123	rhodamine 123
SDS	sodium dodecyl sulphate
STP	staurosporine
TBS	tris buffered saline
TEMED	tetramethylethylenediamine
topo II	topoisomerase II

TPA	12-O-tetradecanoylphorbol-13-acetate
VCR	vincristine
VER	verapamil
VLB	vinblastine
VP-16	etoposide

PUBLICATIONS

1. Denese C. Marks, Larissa Belov, Mary W. Davey, Ross A. Davey and Antony D. Kidman. (1992) The MTT cell viability assay for cytotoxicity testing in multidrug resistant human leukemic cells. *Leukemia Research*, **16**;1165-1173.

2. Denese C. Marks, Mary W. Davey, Ross A. Davey and Antony D. Kidman. (1993) Differentiation and multidrug resistance in response to drug treatment in the K562 human leukaemia cell line. *British Journal of Haematology*, **84**;.83-89.

ABSTRACTS AND PRESENTATIONS

1. Poster presentation entitled "The establishment of a colorimetric (MTT) assay for cytotoxicity testing" at the Australian Society for Biochemistry and Molecular Biology. Sydney, Australia. September, 1990.

2. Poster presentation entitled "Development and characterisation of drug resistant K562 cell lines" at the American Association for Cancer Research Special Conference: Membrane transport in multidrug resistance, development and disease. Banff, Alberta, Canada. March, 1991.

3. Poster presentation entitled "Is P-glycoprotien clinically relevant" at the Australian Society for Medical Research. Canberra, Australia. December, 1991.

4. Poster presentation entitled "Induction of haematopoietic differentiation and multidrug resistance in human leukaemic cell lines"at the Australian Society for Medical Research conference. Brisbane, Australia. December, 1992.

5. Poster presentation entitled "Induction of haematopoietic differentiation and multidrug resistance in human leukaemic cell lines" at the General Motors Cancer Research Foundation International Symposium "Resistance against anticancer drugs: Molecular mechanisms and clinical opportunities". Toronto, Canada. May, 1993.

6. Oral presentation entitled "Induction of megakaryocytic differentiation and drug resistance in human leukaemic K562 cell lines" at the Royal North Shore Hospital/University of Technology, Sydney Annual Scientific Meeting. Sydney, Australia. November, 1993.

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RESULTS AND DISCUSSION

INTRODUCTION

Many cancers do not respond to chemotherapy, and other cancers which initially respond later become resistant to chemotherapeutic drugs. *In vitro* cell culture has allowed an understanding of the molecular and biochemical basis of this drug resistance. Such studies have identified several mechanisms by which malignant cells aquire drug resistance. The most well characterised form of drug resistance is multidrug resistance (MDR). The characteristics of MDR are cross-resistance to a wide variety of natural product drugs, decreased drug accumulation, expression of a membrane glycoprotein called P-glycoprotein, and reversal of resistance by agents such as verapamil. Other mechanisms of drug resistance include a variety of biochemical mechanisms, such as increased drug detoxification, altered intracellular drug distribution so that drug does not interact with its target, increased concentration of target site and increased repair of damaged target.

1 Characteristics of P-glycoprotein and Multidrug Resistance

There are many membrane transporters which can be grouped into families, in which members have a common evolutionary origin and are related to each other by DNA sequence and molecular mechanisms. One such family is the ATP-binding cassette (ABC) superfamily. The characteristic feature of this superfamily is the highly conserved ATP-binding cassette (Higgins, 1992). ABC transporters are found in both eukaryotes and prokaryotes, and typically utilise energy generated from ATP hydrolysis to pump substrate across the membrane against a concentration gradient. Some members of the ABC superfamily include haemolysin B (*HlyB*) in *Eschericia coli* (Femlee *et al*, 1985), the pheromone transporter *STE6* in the yeast species *Saccharomyces cerevisiae* (McGrath and Varshavsky, 1989), the MHC class II peptide (Trowsdale *et al*, 1990), the cystic fibrosis transmembrane conductance regulator (CFTR; Riordan *et al*, 1989), the recently characterised multidrug resistance-associated protein (MRP; Cole *et al*, 1992) and P-glycoprotein (reviewed by Gottesman and Pastan, 1993).

1.1 Putative Structure of P-glycoprotein

Human P-glycoprotein is a 170-180kD membrane protein expressed in both drug resistant cells and intrinsically in some normal tissues. The cDNA structure of *mdr*1, the gene which encodes P-glycoprotein, indicates an internal duplication in the polypeptide of approximately 500 amino acids, with 2 nucleotide binding domains (Gros *et al*, 1986). There is strong homology between *mdr*1 and *HlyB*, a component of the *E.coli* haemolysin transport system, suggesting the basic structural element used by energy dependent bacterial membrane transport proteins is conserved in the *mdr* gene (Gerlach *et al*, 1986; Gros *et al*, 1986). The two transmembrane domains were originally thought to have arisen from a gene duplication event (Chen *et al*, 1986). However, more recently it

has been proposed that the primordial proteins corresponding to the two halves formed independently by fusion of genes coding for the nucleotide binding domains with genes for different transmembrane proteins, which may or may not have been closely related. The fusion of these two independently evolved genes resulted in the formation of the P-glycoprotein gene. The high degree of homology and the positions of the introns indicate a common origin for the nucleotide-binding domain (Chen *et al*, 1990).

Sequence data and hydropathy profiles predict that P-glycoprotein, like most ABC-transporters, consists of six membrane spanning α-helical segments per domain. P-glycoprotein has a large cytoplasmic domain with a highly conserved primary sequence while the membrane-bound domains are not conserved. The hydrophobic amino acids allow interdigitation of the protein within the cell membrane and hydrophilic amino acids allow contact with both the intracellular and extracellular environments (Yoshimura *et al*, 1989). The C-terminal hydrophilic regions in each half of the molecule contain the consensus sequence for two folds forming the nucleotide-binding sites, which are intracellular (Zhang and Ling, 1991) and are also found in other nucleotide-binding proteins.

Recent data conflicts with the general model of six membrane-spanning α-helices. Instead the sequences believed to be between transmembrane domains 8 and 9, and between transmembrane domains 9 and 10 are now thought to be orientated outside the membrane (Zhang and Ling, 1991). It is difficult to explain this as the hydrophobic segment of the predicted transmembrane domain 8 would be extracellular, and the question arises as to how stable this segment would be in a hydrophilic extracellular environment. However this model is attractive as the external hydrophobic portions could serve as sites where hydrophobic drugs could interact and bind to P-glycoprotein.

Cross-linking studies have shown that P-glycoprotein can exist as a 340kD homodimer as well as a 180kD monomer. [^{3}H]-azidopine photolabeling of the dimer could be inhibited by vincristine, doxorubicin and verapamil, suggesting the dimeric form is also functional (Naito and Tsuruo, 1992). Mapping of the epitopes to which the monoclonal antibody MRK16 binds suggests that P-glycoprotein may arrange in the membrane as a dimer (Georges *et al*, 1993).

1.2 Detection of P-glycoprotein

Several antibodies have been raised against different epitopes of the P-glycoprotein molecule, including C219 and C494 (Kartner *et al*, 1985), MRK16 and MRK17 (Hamada and Tsuruo, 1986), JSB-1 (Scheper *et al*, 1988), and UIC-2 (Mechetner and Roninson, 1992). C219 and MRK16 are most commonly used. C219 reacts with a highly conserved amino acid sequence which forms an internal epitope on P-glycoprotein (Georges *et al*, 1990), and is useful for Western blotting and immunocytochemistry.

MRK16 reacts with a discontinuous external epitope composed of at least the first and fourth extracellular loops of P-glycoprotein (Georges *et al*, 1993) and is useful for flow cytometry.

Problems have been encountered using MRK16 to detect P-glycoprotein in leukaemia cells. Cumber *et al* (1990) found that while 53% of samples from chronic lymphocytic leukaemia (CLL) patients had increased *mdr*1 mRNA expression, only 12% of samples from CLL were positive when MRK16 was used to detect P-glycoprotein. When sialic acid residues were removed by neuraminidase the proportion of P-glycoprotein positive cells increased to 52%. P-glycoprotein is glycosylated on the first external loop (Zhang and Ling, 1991), and part of the epitope with which MRK16 reacts includes this loop, so it is possible that post-translational modifications could affect reactivity of an antibody such as MRK16 with P-glycoprotein. Norris *et al* (1989) found that Western blot analysis with C219, but not flow-cytometric analysis with MRK16, detected P-glycoprotein in methotrexate-resistant cells.

Rh123 accumulation has recently been adopted as an assay of MDR in human tumour samples, and is useful as the ability of verapamil to increase Rh123 accumulation in cells can be used as an assay of P-glycoprotein function. Rh123 is accumulated by sensitive cells but not drug resistant cells (Bucana *et al*, 1990). Rh123 has been shown to be more versatile and sensitive than fluorescent drugs such as daunorubicin (Ludescher *et al*, 1991; Ludescher *et al*, 1992). A study of acute myeloid leukaemia (AML) cells, three *mdr*1 positive patients had reduced Rh123 accumulation, whereas only one case was detected by MRK16 (Ludescher *et al*, 1992). Therefore it appears that methods such as Rh123 are ideal for detecting MDR, as it is sensitive and able to assess P-glycoprotein function. Further, as pointed out by Baccarani *et al* (1993) comparison between functional assays and expression levels are important, especially when dealing with cells expressing low levels of P-glycoprotein.

There are also several methods by which P-glycoprotein can be detected. Expression of *mdr*1 mRNA by Northern blotting or expression of P-glycoprotein itself by Western blotting are two commonly used methods which utilise bulk tissue or cell preparation, and give an overall indication of expression. However, these methods do not give an indication of P-glycoprotein expression in individual cells, and are not useful for populations where not all of the cells may be expressing P-glycoprotein. This is particularly important in malignant tissue samples, where the background stroma and cells may have intrinsic P-glycoprotein expression, and would contaminate the tumour sample, giving a false positive. Immunocytochemistry and flow cytometry are therefore more useful methods in such circumstances.

1.3 The *mdr* genes

There are several different mammalian genes which encode P-glycoprotein. The genes are divided into 2 classes, according to their ability to mediate drug resistance. Class I genes include human *mdr*1, which is closely related to two mouse genes *mdr*1a , also known as *mdr*3, and *mdr*1b also known as *mdr*1. Class I genes are involved in drug resistance. Class II genes include human *mdr*3 and mouse *mdr*2, and are generally not responsible for drug resistance. *mdr*1 has been shown to encode P-glycoprotein in humans (Roninson *et al*, 1986; Ueda *et al*, 1986; Chin *et al*, 1989). The function of the *mdr*3 gene is not as well understood.

1.3.1 Regulation of the *mdr1* gene

In order to fully understand MDR it is important to understand the regulation of P-glycoprotein expression. The *mdr*1 gene promoter region is located in the region 19 to 83 bases upstream from the translation initiation codon (Raymond and Gros, 1990). A second promoter has also been isolated from drug-selected cells which is not active in normal human tissues or other cell lines (Ueda *et al*, 1987). Studies using deletion constructs have identified sequences necessary for transcription of *mdr*1 *in vivo* (Madden *et al*, 1993). A 10-base pair sequence in the 5' flanking region of *mdr*1 has been identified, with 100% homology to MHC class II gene promoters, to which nucleoproteins can bind, and is essential to expression of the *mdr*1 (Goldsmith *et al*, 1993). The human *mdr*1 gene promoter also contains an AP-1 binding site, which encodes a protein kinase C (PKC) consensus phosphorylation site (Hsu *et al*, 1990). This site may be important in the regulation of the *mdr*1 gene.

The human *mdr*1 gene promoter has been shown to be a target for the cHa-Ras-1 oncogene and mutant p53 tumour suppressor gene product, which may explain the regulation of *mdr*1 expression during tumour progression (Chin *et al*, 1992). An understanding of the way in which these promoters are activated will no doubt provide information regarding P-glycoprotein expression and its control.

*mdr*1 expression is also regulated by differentiating agents (Mickley *et al*, 1989), heat shock and heavy metals (Chin *et al*, 1990a), serum starvation (Tanimura *et al*, 1992), components of the extracellular matrix (Schuetz and Schuetz, 1993), as well as cytotoxic drugs (Chin *et al*, 1990b), all of which suggest that the induction of P-glycoprotein expression may be a stress-induced response.

1.3.2 The *mdr*3 gene

The *mdr*3 gene shows 85% homology with *mdr*1, and is co-expressed with *mdr*1 in liver, kidney, adrenal and spleen. However, Buschman *et al* (1992) reported that expression of *mdr*3 is restricted to the bile canalicular membrane. This evidence suggests a physiological role for the *mdr*3 gene product in transport of toxins or peptides.

*mdr*3 was not at first thought to confer drug resistance, and selection of *mdr*3-transfected cells with MDR drugs did not yield drug resistant clones. Functional expression of *mdr*3 has more recently been detected in B-cell leukaemias and chronic myeloid leukaemia (CML) using an *mdr*3 gene fragment as a probe, in which *mdr*3-positive cells showed reduced accumulation of daunorubicin which was modulated by verapamil and cyclosporin A (Herwejer *et al*, 1990). This suggests that *mdr*3 may also play a role in resistance to chemotherpaeutic drugs encountered in patients.

1.3.3 Functional Studies Using *mdr*1 and *mdr*3 Mutants

Studies comparing *mdr*3 with *mdr*1 help to clarify which domains on P-glycoprotein are important for drug binding and transport. Analysis of chimeric mouse *mdr*1b/*mdr*2 proteins, which should correspond to human *mdr*1/*mdr*3 chimeras, have shown that insertion of the *mdr*2 ATP-binding sites into *mdr*1 gene results in a protein which retains its transport function (Buschman *et al*, 1991). Substitution of amino acids 165, 166, 168 and 169, located in the first transmembrane loop of P-glycoprotein, from *mdr*3 into *mdr*1, results in a construct which does not confer drug resistance, indicating the first transmembrane loop is important in P-glycoprotein function (Currier, 1992). Replacement of a serine with phenylalanine in the putative transmembrane 11 domain of *mdr*1 produced a mutant protein with loss of resistance to doxorubicin, vinblastine and colchicine. When phenylalanine was replaced serine in the *mdr*3 gene, the cells became more resistant to actinomycin-D, colchicine, vinblastine and doxorubicin (Gros *et al*, 1991). This suggests that the predicted transmembrane 11 domain of P-glycoprotein is also important in substrate recognition and transport. Together, these studies suggest that several different regions of P-glycoprotein are involved in its ability to mediate drug resistance.

1.4 Post-translational Modifications of P-glycoprotein

1.4.1 Glycosylation of P-glycoprotein

P-glycoprotein is post-translationally modified by glycosylation and phosphorylation. The N-terminal half of P-glycoprotein contains 4 glycosylation sites on the first extracellular loop of the protein, at Asn residues -73, -91, -96 and -103. Another large glycosylated loop also links transmembrane domains 8 and 9, and is glycosylated at Asn-807. Amino acid sequence studies shows that this site is conserved in all P-glycoprotein

isoforms of human, mouse and Chinese hamster, suggesting it may be of functional importance (Zhang and Ling, 1991). Deletion of glycosylation sites on P-glycoprotein does not effect drug resistance *per se*, but decreases the ability to generate drug resistant clones, suggesting N-glycosylation is important in the proper routing or stability of P-glycoprotein in the membrane (Schinkel *et al*, 1993).

1.4.2 Phosphorylation of P-glycoprotein: The Role of Protein Kinase C

P-glycoprotein is subject to cycles of phosphorylation and dephosphorylation mediated by protein kinase C (PKC), and this is thought to play an important role in the mechanism of action of P-glycoprotein. PKC inhibitors such as staurosporine inhibit phosphorylation of P-glycoprotein, but do not alter dephosphorylation (Ma *et al*, 1991). Sequence analysis has identified specific serine residues (661, 671 and one or more of 667, 675 and 683) on P-glycoprotein which are phosphorylated by PKC. These sites are clustered in the linker region between the two homologous domains of P-glycoprotein which is analogous to the regulatory ("R") domain of the cystic fibrosis transmembrane conductance regulator. The regulatory domain on the cystic fibrosis protein is a target for phosphorylation by PKC and regulates chloride channel activity. This analogy suggests that phosphorylation also regulates P-glycoprotein function (Chambers *et al*, 1993).

Increased phosphorylation of P-glycoprotein correlates with increased drug transport and drug resistance (Chambers *et al*, 1990). Transfection studies show that the PKCα isoform is responsible for changes in drug resistance while the PKCγ isoform is not likely to be involved (Ahmad *et al*, 1992).

1.5 P-glycoprotein Function

1.5.2 ATPase Activity of P-glycoprotein

P-glycoprotein has ATPase activity, which is thought to be used to actively efflux drugs (Hamada and Tsuruo, 1988a). The ATPase activity of partially purified P-glycoprotein can be stimulated by the drugs vinblastine, doxorubicin, daunorubicin, actinomycin D and colchicine, as well as the reversal agent verapamil (Ambudkar *et al*, 1992). Expression of the human *mdr*1 gene in insect cells results in a vanadate-sensitive membrane ATPase, which is detectable by C219, and which can be stimulated by drugs such as vinblastine and verapamil. These experiments provide direct evidence that P-glycoprotein has drug-stimulated, magnesium-dependent ATPase activity (Sarkadi e*t al*, 1992). Interestingly, P-glycoprotein in drug resistant cell lines has also been reported to act as a channel for ATP release from the cell (Abraham *et al*, 1993).

1.5.1 Drug Binding Sites

Photoaffinity labeled chemotherapeutic drugs and their analogues have been used in order to gain insight into the drug binding and transport domains on P-glycoprotein. Experiments using the photoreactive vinblastine analogue N-(p-azido-[3-^{125}]-N'-(b-aminoethyl)vindesine (vindesine) show that it binds to drug resistant cells but not drug sensitive cells (Cornwell *et al*, 1986). The photoactive dihydropyridine calcium channel blocker azidopine also binds to P-glycoprotein (Safa *et al*, 1987). Vinblastine non-competitively inhibits azidopine binding to P-glycoprotein, indicating that azidopine binds at a different site to vinblastine (Tamai and Safa, 1991). Another photoactive compound [^{125}I]iodoaryl azidoprazosin, which also has an arylazido group and is structurally related to azidopine, binds to transmembrane domains 6 and 12 of P-glycoprotein, adjacent to the ATP-binding site, with another minor photolabeling domain between transmembrane domains 4 and 6 (Greenberger, 1993). This data indicates that a portion of this drug binding domain is near the ATP-binding site, as well as a region in each cassette of P-glycoprotein.

Iodomycin, a photoaffinity analogue of daunorubicin also binds to a 150-170kD protein in drug resistant cells, and its binding is inhibited by vinblastine, verapamil, daunorubicin and colchicine in a dose-dependent manner (Busche *et al*, 1989; Friche *et al*, 1993). The most lipophilic anthracyclines such as aclacinomycin A produced the most iodomycin inhibition. Furthermore, the detergents Cremophor EL and Tween 80 inhibited iodomycin labeling, suggesting that disruption of the lipid bilayer as well as drug binding inhibits iodomycin photolabeling of P-glycoprotein (Friche *et al*, 1993)

It is yet to be determined as to whether there are several drug binding sites on P-glycoprotein or whether there is a single multidrug-binding site.

1.6 Reversal of MDR

Another characteristic of MDR is the ability of resistant cells to be sensitised to chemotherapeutic drugs by a variety of agents. Drugs which reverse multidrug resistance are referred to as chemosensitisers. Verapamil, a calcium channel antagonist, trifluoperazine a calmodulin inhibitor, and cyclosporin A, an immunosuppressive drug are three reversal agents which have been trialed clinically. Generally, the most effective modulators are hydrophobic molecules with planar aromatic rings and a tertiary basic nitrogen atom allowing them to interact with the P-glycoprotein molecule (Rothenberg and Ling, 1989).

1.6.1 Verapamil

Verapamil is the drug most widely used to investigate the reversal of multidrug resistance, and it has several modes of action. Verapamil interacts directly with P-glycoprotein, as shown by binding studies using photoreactive verapamil analogues (Yusa and Tsuruo, 1989; Qian and Beck, 1990). Verapamil has been shown to be most

effective in the reversal of resistance to *Vinca* alkaloids (Beck *et al*, 1986a), whereas drugs such as colchicine, doxorubicin and tenoposide do not compete as avidly with verapamil for binding to P-glycoprotein, as they have a lower affinity than *Vinca* alkaloids for the verapamil binding site (Qian and Beck, 1990). Verapamil itself has also been shown to induce P-glycoprotein expression *in vitro* (Herzog *et al*, 1993).

Verapamil also competes with photoreactive drug analogues for binding to P-glycoprotein (Akiyama *et al*, 1988; Busche *et al*, 1989). This suggests that verapamil reverses MDR through inhibiting drug binding to P-glycoprotein, hence allowing the drug to accumulate within the cell. Verapamil is also thought to modulate drug resistance through increasing the phosphorylation of P-glycoprotein, via the enzyme protein kinase C (Hamada *et al*, 1987). Increased phosphorylation is thought to increase the activity of P-glycoprotein (Chambers *et al*, 1993).

Verapamil has been found to change the distribution of anthracyclines in hydrophilic/hydrophobic compartments in a cell-free system, moving anthracyclines from the aqueous to the hydrophobic phase (Hindenburg *et al*, 1987). Verapamil also alters the intracellular drug distribution of anthracyclines in resistant cells, returning it to that of the sensitive phenotype (Schuurhuis *et al*, 1989). Therefore verapamil appears not only to bind to P-glycoprotein but also alters membrane lipophilicity.

1.6.2 Other Agents

Trifluoperazine, an inhibitor of calmodulin-dependent enzymes, has been shown to reverse drug resistance *in vitro* through its ability to alter intracellular drug compartmentalisation (Ganapathi *et al*, 1986; Ganapathi *et al*, 1988). Cyclosporin A, an immunosuppressive agent, also modifies drug resistance, probably through altering the biophysical state of the plasma membrane, allowing drug to enter more readily (Twentyman *et al*, 1987). Cyclosporin A also directly interacts with P-glycoprotein, inhibiting [^{3}H]-vinblastine photoaffinity labeling (Tamai and Safa, 1990). Cyclosporin A interacts with the same binding site on P-glycoprotein as verapamil and the *Vinca* alkaloids, and is transported by P-glycoprotein (Saeki *et al*, 1993).

1.6.3 Clinical Trials with Modulators of MDR

Verapamil has been used in clinical trials with combination chemotherapy to overcome drug resistance in patients with B-cell neoplasms (Durie and Dalton, 1988; Dalton *et al*, 1989), with a clinical response in three of eight myeloma and non-Hodgkin's lymphoma patients previously refractory to treatment with doxorubicin and vincristine (Dalton *et al*, 1989). However, some P-glycoprotein positive patients did not respond to verapamil in combination with standard chemotherapy (Dalton *et al*, 1989). Verapamil has also been used in conjunction with VP-16 and vinblastine in paediatric tumours, with partial

responses (Cairo *et al*, 1989). However, side effects include cardiac arrhythmia, congestive heart failure and hypotension due to the high concentrations needed to achieve a useful plasma level. Verapamil has also been used to modulate MDR in ovarian cancer patients, but with little success due to side effects (Ozols *et al*, 1987). However, verapamil has also been shown to chemosensitise transgenic mice which express the human *mdr*1 gene in bone marrow cells, without increasing the toxic side effects of the drugs (Mickish *et al*, 1991).

Trifluoperazine has been used in clinical trials in combination with doxorubicin with some response and fewer side effects than verapamil. However, as is the case with verapamil, at higher doses of trifluoperazine side effects were dose-limiting (Miller *et al*, 1988). Cyclosporin A has been used in a patient with AML, which resulted in elimination of the *mdr*1 positive cells, and restoration of *in vitro* sensitivity to daunorubicin, although the patient later relapsed with reappearance of the *mdr*1 positive clone (Sonneveld and Nooter, 1990).

Although some success has been achieved with agents such as verapamil and cyclosporin A in clinical trials, P-glycoprotein expressing cells are still able to survive. It is possible that cells may express mechanisms of resistance other than P-glycoprotein which would account for the lack of success. Further studies into the pharmacokinetics of reversal agents in conjunction with chemotherapeutic drugs and investigation of new modifiers with fewer dose-limiting side effects are clearly necessary if reversal agents are to be successfully used in combination with chemotherapy to overcome drug resistance.

1.7 Physiological Role of P-glycoprotein

1.7.1 Tissue Distribution of P-glycoprotein

An understanding of the function of P-glycoprotein in normal tissues is certain to enhance the understanding of P-glyprotein in malignant cells. P-glycoprotein is expressed at the blood-brain barrier (Cordon-Cardo *et al*, 1989), suggesting a protective role for P-glycoprotein, prohibiting the entry of toxins. P-glycoprotein expression is also high in colon, small intestine, liver and brush borders of the kidney (Sugawara *et al*, 1988). P-glycoprotein is thought to act as a protective mechanism against xenobiotic substances in the diet in these tissues. Tumours which develop from these tissues intinsically express P-glycoprotein. P-glycoprotein is also expressed in the adrenal cortex, where it is thought to have a role in the transport of steroid hormones (van Kalken *et al*, 1992). Tissue specific distribution of P-glycoprotein in the human foetus differs from that in adults. Respiratory epithelium of the main bronchi and pharynx express P-glycoprotein in the foetus, while these cells do not express P-glycoprotein in adults. Cells of the adrenal cortex, intestinal epithelium, pancreatic ducts and bile ductules of the liver did not express P-glycoprotein in the foetus, although P-glycoprotein expression is

high in the adrenal cortex of adults (van Kalken *et al,* 1992). The differences in the tissue specific expression between adults and the foetus suggests that the role of P-glycoprotein differs in the function of these tissues as they develop.

P-glycoprotein is found in high levels in the secretory glandular epithelium of endometrium in mice, and the level increases during pregnancy (Arceci *et al*, 1988), suggesting a role for P-glycoprotein in hormone secretion during pregnancy.

P-glycoprotein is also expressed in human haematopoietic bone marrow stem cells, where it is also hypothesised again to be protective to stem cells (Chaudhary and Roninson, 1991). In normal bone marrow precursor cells, *mdr*1 expression has been detected by PCR-analysis in CD34 positive stem cells, immature myeloid cells and B-lymphocytes and weakly in erythroid precursor cells (glycophorin A^+), while monocytic precursor cells were negative. Furthermore, P-glycoprotein function (Rh123 efflux) was found in CD34, CD10 and CD33 positive bone marrow cells (Drach *et al*, 1992). In peripheral blood cells, P-glycoprotein expression and function has been detected in all subpopulations (CD4, CD8, CD14, CD19 and CD56 positive) except granulocytes (CD15) (Drach *et al*, 1992). However, others have found that CD14 positive monocytes do not have P-glycoprotein expression (using the monoclonal antibody UIC-2) or activity (Chaudhary *et al*, 1992), so there is controversey as to whether CD14 positive cells do express functional P-glycoprotein.

1.7.2 Physiological Function of P-glycoprotein

Recent evidence supports the hypothesis that P-glycoprotein is a steroid transporter, in tissues such as the endometrium and adrenal cortex, where it is expressed. Steroid hormones including progesterone have all been shown to be effluxed by drug resistant cells expressing P-glycoprotein (van Kalken *et al*, 1993). Progesterone and its structural analogue megoestrol acetate, as well as testosterone, hydrocortisone and corticosterone have been shown to inhibit photoaffinity labeling of P-glycoprotein in drug resistant cells (Yang *et al*, 1989; Fleming *et al*,1992). [^{3}H]-Progesterone directly photoaffinity labels P-glycoprotein and this can be inhibited by vinblastine, verapamil and testosterone (Qian and Beck, 1990). Progesterone directly regulates the activity of the mouse *mdr*1b gene promoter through the progesterone receptor (Piekarz *et al*, 1993). Expression of P-glycoprotein in the endometrium of the gravid uterus parallels the rise in progesterone during pregnanacy (Arceci *et al*, 1988). P-glycoprotein expression is also induced in uterine secretory epithelium by oestrogen and progesterone (Arceci *et al*, 1990), suggesting hormonal or developmental regulation. P-glycoprotein may therefore be important in maintaining preganacy by keeping levels of progesterone high in the uterus.

P-glycoprotein has also been shown to transport the pepetide N-acetyl-leucyl-leucyl-norleucinal (Sharma *et al*, 1992), suggesting a role for P-glycoprotein in the secretion of peptides. Considering the homology of P-glycoprotein to other transport proteins such as

HlyB, *Ste6* and CFTR, this evidence supports the possibility that P-glycoprotein has a normal physiological role in transport and secretion of peptides, xenobiotics and steroids.

2 P-glycoprotein Expression and Prognosis in Leukaemia

Resistance to chemotherapy continues to be a major problem in the treatment of leukaemia. Expression of P-glycoprotein is often associated with poor response to chemotherapy or short duration of remission, and P-glycoprotein may serve as a marker of residual disease in leukaemia (Haber, 1993). *mdr*1 is expressed in patients with AML, with expression most frequent in cells from patients in which remission was difficult to induce, and shorter remission duration in patients with high *mdr*1 expression (Sato *et al*, 1990a; Sato *et al*, 1990b). P-glycoprotein has been detected in granulocytic cells of CML patients in both chronic and blast phases (Sato *et al*, 1990a; Wiede *et al*, 1990).

Expression of P-glycoprotein has also been correlated with increased resistance to chemotherapy, shorter median survival and higher relapse rates in acute lymphoblastic leukaemia (ALL) (Rothenberg *et al*, 1989; Sato *et al*, 1990b; Goasguen *et al*, 1993). P-glycoprotein is also expressed in lymphocytes from CLL patients (Cumber *et al*, 1990) and T-cell lymphoma (Kuwazuru *et al*, 1990).

P-glycoprotein expression has been correlated with prior drug exposure in cells from myeloma patients, where patients with no prior therapy had a low incidence of P-glycoprotein expression, while those receiving chemotherapy had significantly higher expression, and patients who had received high doses of vincristine and doxorubicin expressed P-glycoprotein in 100% of tumour cells examined (Grogan *et al*, 1993).

The knowledge of the mechanisms of drug resistance in leukaemias is very limited. Although many studies have found that P-glycoprotein expression is associated with failure to respond to chemotherapy, it does not account for all forms of resistance. Pieters *et al* (1992) have found that resistance to anthracyclines and *Vinca* alkaloids in childhood ALL at relapse was not due to P-glycoprotein expression. Goasguen *et al* (1993) found that although 50% of relapses could be predicted by expression of the MDR phenotype in ALL, little was known about the factors that may lead to relapse in the other 50% of patients, suggesting resistance mechanisms other than P-glycoprotein. Furthermore, in childhood ALL, MDR-positive and MDR-negative cases had exactly the same complete response rate (92%) whereas only 56% of MDR-positive adult ALL cases presented with complete relapse.

A recent study of untreated AML samples has shown that a high frequency of cases which expressed the MDR phenotype detected by functional assays (enhancement of both daunorubicin cytotoxicity and accumulation by verapamil and cyclosporin A), did not express P-glycoprotein as detected by C219. Furthermore, there was no correlation between enhancement of daunorubicin uptake by verapamil or cyclosporin A and

response to treatment (Ross *et al*, 1993). Musto *et al* (1991) showed that some AML and ALL patients relapsed without the presence of P-glycoprotein at diagnosis or after relapse, which also indicated that other mechansims of resistance must contribute to relapse in leukaemia after chemotherapy. These mechanisms must also be examined and understood in order to successfully treat leukaemias.

2.1 P-glycoprotein and Cell Surface Antigens as Prognostic Markers in Leukaemia

The CD34 antigen is expressed specifically on 1-5% of human bone marrow stem cells, and expression of CD34 decreases with cellular maturation (Strauss *et al*, 1986). The function of CD34 is as yet unknown, however it is shown to be heavily glycosylated, and it is a substrate for PKC. Together with spectroscopic and electron microscopic studies, data suggests CD34 may be involved in cellular adhesion (Greaves *et al*, 1992). Normal human $CD34^+$ bone marrow stem cells express P-glycoprotein (Chaudhary and Roninson, 1991; Drach *et al*, 1992). Expression of CD34 in leukaemia cells of AML patients has been associated with resistance to chemotherapy (Geller *et al*, 1990) and lower complete response rate (Guinot *et al*, 1991; Thomas *et al*, 1992; te Boekhorst *et al*, 1993). te Boekhorst *et al* (1993) demonstrated that previously untreated AML patients with cells simultaneously expressing both P-glycoprotein and CD34 had a lower complete response rate than those which were negative for both. Further, Tiirikainen *et al* (1992) have also demonstrated a correlation between CD34 and P-glycoprotein expression in AML. Expression of the CD34 stem-cell marker indicates a primitive stem-cell-like phenotype, suggesting that the development of drug resistance and expression of P-glycoprotein may be associated with a less-differentiated phenotype. However, the exact relationship between P-glycoprotein expression and differentiation is yet to be determined.

The CD13 antigen is expressed on committed myeloid progenitor cells and cells of the granulocyte-monocyte lineage (Griffin *et al*, 1981), and is a membrane-anchored metallopeptidase (reviewed by Shipp and Look, 1993). CD13 has also been associated with reduced remission rates in AML (Griffin *et al*, 1986) with decreased CD13 expression at relapse, indicating a loss of differentiation (Thomas *et al*, 1992). Antigens such as CD13 may also be associated with drug resistance and may be useful markers in determining prognosis in some forms of leukaemia.

3 Other Mechanisms of Drug Resistance

MDR is not the only mechanism of drug resistance encountered *in vitro* or in the clinical situation. Other mechanism of drug resistance include increased intracellular glutathione, increased levels or activity of the enzyme glutathione-S-transferase (GST),

changes in intracellular drug distribution, alterations topoisomerase activity, increased DNA repair, expression of the MRP protein, and mechanisms specific to particular drugs such as methotrexate and cisplatinum. Many cell lines display multifactorial drug resistance, involving more than one of these mechanisms of drug resistance, and it is now believed that drug resistance encountered in patients undergoing chemotherapy is likely to be multifactorial.

3.1 Overexpression of Glutathione and GST

Glutathione-S-transferases are a family of proteins which conjugate various electrophiles to the sulphur atom of cysteine on glutathione (Pickett and Lu, 1989). Elevated levels of cellular glutathione, the substrate for GST, have been detected in doxorubicin-resistant HL-60 cells (Raghu *et al*, 1993). Elevated levels of GST together with P-glycoprotein/*mdr*1 have previously been reported as multiple mechanisms of doxorubicin resistance in the doxorubicin-selected ADR^R MCF-7 cells (Fairchild *et al*, 1987). Expression of GST genes has also been correlated with the level of doxorubicin resistance in the human HM-1, G361, FCCM-2, FCCM-9 and NH melanoma cell lines (Ramachandran *et al*, 1993). Expression of the different GST isoforms have been detected in a variety of malignant tissue samples, with increased GSTπ (Moscow *et al*, 1989; Albin *et al*, 1993; Ishikawa *et al*, 1993) and GSTμ expression (Albin *et al*, 1993) compared to matched normal tissue from the same patient. Increased levels of GST are therefore one of several non-P-glycoprotein mechanisms which may contribute to drug resistance in human malignancies.

Toxins are conjugated to glutathione, and the conjugates are transported out of the cell. Recent evidence shows the existence the GS-X pump, which is an ATP-dependent export pump and is thought to transport glutathione-S-conjugates out of the cell. The GS-X pump is thought to have a normal physiological role in the phase III system in xenobiotic metabolism, and could potentially export glutathione-S-conjugates of chemotharapeutic drugs in resistant cells (Ishikawa and Ali-Osman, 1993).

Cisplatinum is an electrophile which is reactive with sulphur containing nucleophiles, and is thought to react with glutathione. Many cisplatinum resistant cell lines have elevated levels of glutathione (Hospers *et al*, 1988; Hrubisko *et al*, 1993; Ogawa *et al*, 1993; Yang *et al*, 1993), and cisplatinum resistance is modified when glutathione is depleted by buthionine sulphoximine (BSO) (Hamilton *et al*, 1985; Yang *et al*, 1993). BSO is an inhibitor of the enzyme γ-glutamyl cysteine synthetase which is necessary for the formation of glutathione (Dusre *et al*, 1989). However, a recent study of human tumour xenografts showed that cellular glutathione content did not correlate with cisplatinum resistance (Pratesi and Zunino, 1993). Cisplatinum is also thought to be effluxed by the GS-X pump (Ishikawa and Ali-Osman, 1993).

Elevated levels of glutathione have also been implicated in resistance to the alkylating agent chlorambucil (Clapper *et al*, 1993). Ethacrynic acid, an inhibitor of GST, has been used clinically, achieving partial reversal of chlorambucil resistance in a B-cell lymphocytic leukaemia patient with elevated GST levels (Petrini *et al*, 1993), indicating that future clinical regimes may also need to include agents which inhibit GST where necessary.

3.2 Altered Intracellular Transport

Many anthracycline resistant cell lines show a decreased intracellular drug accumulation which can be attributed to expression of P-glycoprotein (Hamada *et al*, 1987; Mimnaugh *et al*, 1989). However, anthracycline resistant cells exhibiting the MDR phenotype often show a decreased sensitivity to anthracycline drugs which cannot be accounted for by decreased drug accumulation alone. The high resistance levels have been shown to be due to alterations in the intracellular distribution of the drug, resulting in failure of the drug to reach its target organelle (Schuurhuis *et al*, 1989; Sinha and Politi, 1990).

Furthermore, cells not expressing P-glycoprotein have been shown to have altered intracellular anthracycline transport. The HL-60/AR cell line, selected for resistance to doxorubicin (Bhalla *et al*, 1985) does not express P-glycoprotein and exhibits an altered intracellular distribution of daunorubicin. Daunorubicin is distributed to the Golgi apparatus and later to lysosomes and mitochondria or out of the cell in the resistant HL-60/AR cell line, while daunorubicin is distributed diffusely throughout the cytoplasm and nucleus in the sensitive cells (Hindenburg *et al*, 1989). Another doxorubicin selected HL-60 cell line, HL-60/Adr (Marsh *et al*, 1986), demonstrates altered intracellular daunorubicin distribution, whereby drug which reaches the nucleus is rapidly effluxed out of the cell, whereas the nuclei of sensitive cells retain the drug (Marquardt and Center, 1992). These cells also expressed the MRP protein which is located mainly in the endoplasmic reticulum, but do not express P-glycoprotein (Krishnamachary and Center, 1993).

Doxorubicin-resistant LZ-8 cells (9000-fold more resistant than the parental chinese hamster V79 cells) expressing high amounts of P-glycoprotein have an increased ability to transform doxorubicin to a non-cytotoxic form compared to the parental cells, as well as increased drug efflux resulting from overexpression of P-glycoprotein (Zhang *et al*, 1992). This mode of resistance could operate in other cell lines and contribute to resistance.

3.3 The Multidrug-Resistance associated Protein, MRP.

The *mrp* gene encodes a 190kD membrane bound glycoprotein belonging to the ABC superfamily, suggesting it has a transport function (Cole *et al*, 1992; Krishnamachary and Center, 1993). Overexpression of *mrp* mRNA has been demonstrated in the doxorubicin-selected HT1080/DR4 fibrosarcoma subline (Slovak *et al*, 1993), the doxorubicin-selected HL60/ADR subline (Krishnamachary and Center, 1993), as well as in the doxorubicin-resistant H69AR lung subline from which it was isolated and characterised (Cole *et al*, 1992). Cells which express *mrp* have not as yet been found to co-express P-glycoprotein. Analysis of subcellular fractions has shown that the MRP protein is not only localised in the plasma membrane, but is in fact found in higher levels in the endoplasmic reticulum (Krishnamachary and Center, 1993). Although MRP has been isolated from drug-resistant cell lines, there is at this time no direct evidence that it plays a role in mediating drug resistance, and as yet, MRP has only been detected in anthracycline selected cells.

3.4 Other Membrane Proteins Associated with Drug Resistance

Many other proteins have been observed to be expressed in drug resistant cells, both in the presence and the absence of P-glycoprotein expression. HL60 cells selected with either vincristine (HL60/vinc) express P-glycoprotein as well as a 210kD protein which also reacts with C219, and a 150kD protein which does not react with C219. The 150kD protein is also expressed in HL60 cells selected with doxorubicin (HL60/Adr) in the absence of P-glycoprotein, and is thought to contribute to the reduced drug accumulation observed in these cells (McGrath and Center, 1988).

A 110kD protein (p110) has been shown to be overexpressed in drug resistant SW-1573/2R120 non-small cell lung carcinoma cell line selected with doxorubicin, which is P-glycoprotein-negative and has ATP-dependent decreased drug accumulation compared to the parental cell line. The p110 protein is mainly located in the cytoplasm, and is likely to be associated with vesicles and/or lysosomes (Scheper *et al*, 1993).

3.5 Drug Detoxification Mechanisms

Drugs such as the epipodophyllotoxins and the anthracyclines bind and intercalate with DNA. During the breakage-reunion between topoisomerase II (topo II) and DNA, topo II can form a cleavable complex with DNA. Anthracyclines intercalate with DNA, interfering with the breakage-reunion reaction of topo II by stabilising the cleavable complex (Liu *et al*, 1989). There are two forms of multidrug resistance which confer cross-resistance to topo II poisons such as VP-16. One of these is classical MDR, the other is atypical MDR, whereby susceptibility to topo II poisons is altered, and P-

glycoprotein is not invoved (Baguley *et al*, 1992). Alterations in topo II can include reduction in topo II levels (Cole *et al*, 1991), reduced activity of topo II (de Jong *et al*, 1990), topo II gene rearrangements (Tan *et al*, 1989) and expression of a mutant allele for the topo II gene (Deffie *et al*, 1989).

Topoisomerase I genes have been shown to be overexpressed in leukaemic cells from myeloma patients and may be involved in drug resistance, while topo II expression was not significantly elevated (Ishikawa *et al*, 1993). A recent study of brain tumours found that in 72% of malignant tumours studied, topo II expression could not be detected, which may explain the low effectiveness of the epipodophyllotoxins in treating brain tumours (Mousseau *et al*, 1993).

3.6 Methotrexate Resistance

Methotrexate cytotoxicity results from the generation of high intracellular levels of unbound methotrexate which inhibits dihydrofolate reductase and the folate pathway. Mechanisms of methotrexate resistance include increases in dihydrofolate reductase expression, decreased methotrexate polyglutamylation, decreased uptake and increased efflux of methotrexate, which have been demonstrated both *in vitro* and *in vivo*.

Methotrexate normally enters the cell via the reduced-folate transport system. However, it has been shown that methotrexate resistant cells can inactivate this system. A second system of folate transport is also present in normal cells which transports folate more efficiently than methotrexate, permitting the entry of folate into the cell but not methotrexate (Henderson and Strauss, 1990). Decreased expression of the human membrane-associated folate receptor, which is important in transport of both folate and methotrexate, has also been shown to mediate decreased methotrexate accumulation in resistant cells (Saikawa *et al*, 1993).

Methotrexate is converted to polyglutamyl derivatives within the cell, which exert cytotoxicity through inhibiting dihydrofolate reductase and other enzymes. Human squamous cell carcinomas are intrinsically resistant to methotrexate due to decreased methotrexate polyglutamylation which results in decreased methotrexate cytotoxicity (Pizzorno *et al*, 1989). Increased dihydrofolate reductase activity, the enzyme responsible for detoxification of methotrexate, also mediates methotrexate resistance, and can be accompanied by chromosomal changes due to amplification of the dihydrofolate reductase gene (Mini *et al*, 1985; Ohnuma *et al*, 1985; Saikawa *et al*, 1992). Methotrexate-resistant cells exhibit changes in other membrane proteins which confer changes in methotrexate transport into the cell (Schuetz *et al*, 1989; Matherly *et al*, 1992).

Methotrexate resistance can be also multifactorial, with increased dihydrofolate reductase, decreased methotrexate transport and altered methotrexate polyglutamylation together in one resistant cell line (Assaraf *et al*, 1992). Therefore there are many

mechansism of methotrexate which can operate independently or together in methotrexate-resistant cell lines.

4 Outline of Thesis

In vitro studies of drug resistance have revealed the existence of many mechanisms of drug resistance which a cell can utilise to overcome the effects of cytotoxic drugs. These studies have used cells selected for resistance at drug concentrations far in excess of those which are achieved and maintained in the plasma of a patient receiving chemotherapy. It is therefore more clinically relevant to study drug resistance *in vitro* in cells which have been selected for resistance at drug concentrations comparable to plasma levels maintained during chemotherapy. Furthermore, as many studies have documented drug resistance in leukaemia, leukaemic cell lines are a useful model to study the mechanisms of drug resistance *in vitro*.

The aim of this project was therefore to treat several human leukaemic cell lines with clinically achievable concentrations of the anthracycline epirubicin and the *Vinca* alkaloid vinblastine, and examine their drug resistance phenotype, and any accompanying differentiation. When drug resistant cells were established, the ability of drug-resistant cells to undergo differentiation and the effects of differentiation on drug resistance were examined and compared to sensitive parental cells.

The human leukaemic cell lines used were: the mulitopotent undifferentiated K562 progenitor cells, derived from an erythroid CML (Lozzio and Lozzio, 1975); the monocyte-like U937 cell line derived from a histiocytic lymphoma (Sündstrom and Nillson, 1976); the erythroblastic HEL erythroleukaemia (Martin and Papayannopoulou, 1982) and the undifferentiated blast-like KG-1a cell line derived from an AML (Koeffler *et al*, 1980). These four cell lines are all from leukaemias of different lineages and stages of differentiation along the haematopoietic pathway, and were chosen in order to compare the development of drug resistance in well-differentiated cells as opposed to less-differentiated cells.

Little is known about the ability of drug resistant cells to undergo differentiation. Human leukaemic cell lines provide an ideal model with which to study the ability of cytotoxic drugs to induce both drug resistance and differentiation, as changes in antigenic markers of differentiation are easily quantified. The cells were selected with clinically relevant levels of epirubicin (Tjuljandin *et al*, 1990), and vinblastine (Ratain and Vogelzang, 1987). After stabilisation of cell growth at the selecting drug concentration, cells were assayed for their resistance to the selecting drug and cross-resistance to other chemotherapeutic drugs. P-glycoprotein expression was examined using C219 by Western blotting and MRK16 by flow cytometry, and P-glycoprotein function was also assessed using Rh123 accumulation in the presence of verapamil. Changes in myeloid, erythroid, monocytic and megakaryocytic antigens were also examined in order to

determine any co-expression of P-glycoprotein and antigenic markers of cell differentiation in the drug resistant sublines, which could be used as prognostic indicators in a clinical studies.

The K562 and U937 cells, and drug resistant sublines, were chosen in order to determine the ability of drug resistant sublines lines to undergo differentiation in response to chemical (TPA and sodium butyrate) and a biological inducer (granulocyte-macrophage colony-stimulating factor; GM-CSF) of differentiation in comparison to the parental cell lines. GM-CSF was chosen to determine the success of differentiation therapy in combination with chemotherapy, as this mode of treatment is currently being used clinically. Changes in P-glycoprotein expression and function after induction of differentiation were also characterised, in order to determine any associations between differentiation and the MDR phenotype.

MATERIALS AND METHODS

1 Chemicals

Acrylamide, ammonium persulphate, Coomassie Brilliant Blue, glycine, high molecular weight markers, N-N'-Methylene bis-acrylamide, protein assay dye concentrate, SDS, nitrocellulose membrane, TEMED and Tris were purchased from Bio-Rad (Richmond, CA, USA). 5-bromo,4-chloro,3-indoylphosphate (BCIP), dimethylformamide, DMSO, Fast Red TR salt, levamisole, MTT, Napthol AS-MX phosphate, nitro-blue tetrazolium (NBT), propidium iodide, rhodamine 123, sodium butyrate, staurosporine, TPA, Tween 20, actinomycin D, buthionine sulphoximine, chlorambucil, colchicine, taxol, verapamil and the IgG3 (Y5606) monoclonal antibody were purchased from Sigma Chemical Company (St. Louis, MO, USA). Etoposide (VP-16) was purchased from Delta West (Bentley, WA, Australia). Cisplatinum, daunorubicin, methotrexate, vinblastine and vincristine were purchased from David Bull Laboratories (Melbourne, VIC, Australia). Doxorubicin, epirubicin, and idarubicin were purchased from Farmitalia (Italy). RPMI-1640 medium, Hepes and foetal bovine serum were purchased from Cytosystems (Castle Hill, NSW, Australia). Diethylthiothrietol was purchased from CalBiochem (CA, USA). Alkaline phosphatase conjugated rabbit anti-mouse-immunoglobulins (D314), rabbit anti-mouse immunoglobulins (Z 259), alkaline-phophatase-anti-alkaline phosphatase complex (APAAP; D651) and the monoclonal antibodies WM-47 (anti-human myeloid cell, CD13), TÜK4 (anti-human monocyte, CD14), TÜK3 (anti-human haematopoietic progenitor cell, CD34), Y2/51 (anti-human platelet glycoprotein III, CD61) and JC159 (anti-human glycophorin A) were purchased from Dakopatts (Glostrup, Denmark). C219 monoclonal antibody was from Centocore (Malvern, PA, USA). Fluorescein conjugated sheep anti-mouse immunoglubulins, $F(ab')_2$ fraction was purchased from Silenus Laboratories (Hawthorn, VIC, Australia). Diploma brand skim milk powder was used. Acetic acid, EDTA, ethanol, methanol and sucrose were purchased from BDH chemicals (VIC, Australia). $MgCl_2$, sodium azide and urea were purchased from Ajax Chemicals (Sydney, NSW, Australia). Tissue culture plastic-ware was from Costar. All other chemicals were AR grade.

MRK16 and MRK20 were kind gifts from Dr. T. Tsuruo, University of Tokyo, Japan. GM-CSF was a gift from Schering Plough (Kenilworth, NJ, USA). ID4.5 monoclonal antibody was a gift from R. Thorne, Bill Walsh Cancer Research Laboratory, Royal North Shore Hospital Sydney. The monoclonal antibody K121 was a gift from Prof. R. Raison, Immunobiology Unit, University of Technology, Sydney.

2 Cell Culture

The human leukaemic cell lines K562 (chronic myeloid leukaemia), KG-1a (acute myeloid leukaemia), HEL (erythroleukaemia), U937 (monocyte-like histiocytic lymphoma) and CCRF-CEM (T-cell leukaemia) cells were obtained from the American

Type Culture Collection. The drug resistant VLB_{100} cells were from W. Beck (Beck *et al*, 1979). Cells were grown in RPMI-1640 supplemented with 10% foetal bovine serum, 20 mM Hepes and $NaHCO_3$ (0.85 g/l) at 37°C in a humidified atmosphere with 5% CO_2. The cells were mycoplasma free and were tested regularly.

2.1 Generation of Drug Resistant K562 Sublines

Drug resistance was induced in the K562 cells by intermittent drug exposure for 3 to 14 days over a 4 month period. Epirubicin resistant cells were treated with 5, 10, 15, 20 and 40 ng/ml epirubicin (8.6, 17.2, 25.8, 34.4 and 68.8 nM). These sublines are designated K562/E5, K562/E10, K562/E15, K562/E20 and K562/E40. The K562/E10B subline was derived by exposing cells originally treated at 5 ng/ml to 10 ng/ml epirubicin, and the K562/E15B subline was derived by exposing K562/E10 cells to 15 ng/ml epirubicin. The vinblastine resistant sublines were treated with 0.1, 0.5, 1, 2, 4 and 8 ng/ml vinblastine (0.11, 0.55, 1.1, 2.2, 4.4, 8.8 nM), and are designated K562/V0.1, K562/V0.5, K562/V1, K562/V2, K562/V4 and K562/V8. After cell growth was stabilised at these concentrations, cells were treated with drug for a period of 3 days every month to maintain resistance.

The K562/E10 and K562/E10B sublines were cloned by the limiting dilution method. Cells were plated at a density of 0.9 cells/well. Cloning efficiency was 2 to 9%.

2.2 Generation of Drug Resistant U937 and HEL Sublines

Drug resistant U937 sublines were derived by exposing the cells to either epirubicin or vinblastine for 6 exposures of 2-4 days over a 3 month period. Epirubicin resistant cells were selected by exposure to drug at 10 or 15 ng/ml epirubicin (17.2 or 25.8 nM). These sublines are designated U937/E10 and U937/E15. The vinblastine selected sublines were selected at 1 and 8ng/ml vinblastine (1.1 and 8,8 nM) and are referred to as U937/V1 and U937/V8.

Similarly, drug resistant HEL sublines were also derived by exposing the cells to 10 or 15 ng/ml epirubicin (17.2 or 25.8 nM), and referred to as HEL/E10 and HEL/E15; or 1 and 8 ng/ml vinblastine (1.1 and 8,8 nM), and referred to as HEL/V1 and HEL/V8.

2.3 Generation of Drug Resistant KG-1a Sublines

Drug resistant KG-1a sublines were derived by exposing the cells to 15 and 25 ng/ml epirubicin (25,8 and 43.1 nM), and are referred to as KG-1a/E15 and KG-1a/E25, or 8 and 16 ng/ml vinblastine (8.8 and 17.6 nM), which are referred to as KG-1a/V8 and KG-1a/V16.

3 Cytotoxicity Assays

Exponentially growing cells (3 x 10^4 cells/well) were plated in triplicate into 96-well microtitre plates, in growth medium (200 µl) containing 2-fold serial dilutions of the

drug. Control wells containing cells in 200 µl growth medium without drug were also included on each plate. The K562, KG-1a and HEL cells were incubated for 4 days at 37°C, while the U937 cells were incubated for 3 days, after which cell viability was determined using the MTT assay (Marks *et al*, 1992).

3.1 The MTT Assay

MTT was dissolved in PBS (pH 7.2; 2.5 mg/ml) and added to the cells (20 µl/well). After incubation at 37°C for 2 h, cells were centrifuged at 800 *g* for 5 min and the medium aspirated. The resulting formazan crystals were dissolved in 100 µl DMSO/well. Plates were agitated for 15 min, after which the absorbance was measured using a multiwell plate reader (Oraganon Teknika) with a 540 nm fixed wavelength filter. Results were calculated as: cell viability (%) = average absorbance of triplicate wells/ average absorbance of control wells x 100%.

The 50% inhibitory concentration (IC_{50}) was determined as the drug concentration which resulted in a 50% reduction in cell viability. Relative resistance was calculated by dividing the IC_{50} obtained for the resistant subline by the IC_{50} obtained for the parental cell line.

4 Effect of Verapamil

Reversal of resistance was determined by incubating the cells in the presence or absence of 10 µM verapamil in a cytotoxicity assay. IC_{50}s were determined and fold reversal was calculated by dividing the IC_{50} for cells incubated with the drug alone by the IC_{50} for cells incubated with drug in the presence of verapamil.

5 Effect of BSO

Similarly, the ability of 50 µM buthionine sulfoximine (BSO) to enhance cytotoxicity in the drug resistant U937 cells was also examined, whereby cells were incubated with the drug in the presence or absence of BSO in a cytotoxicity assay. Again, fold reversal of resistance was calculated by division of the IC_{50} for cells incubated with the drug alone by the IC_{50} for cells incubated with drug in the presence of BSO.

6 Detection of P-glycoprotein

6.1 Western Blot Analysis

6.1.1 Isolation of Plasma Membrane Fractions

Between 5 x 10^6 to 10^7 cells were washed twice in PBS by centrifugation for 10 min at 200 *g*, resuspended in 1 ml of lysis buffer (0.01 M tris/HCl, pH 7.4), and stored at -20°C until required. Cells were rapidly thawed, subjected to sonication and checked microscopically to ensure at least 75% of cells were disrupted. Whole cells and nuclei

were removed by centrifugation at 4000 *g* for 10 min. The plasma membrane fraction was then collected from the supernatant by centrifugation at 35000 *g* for 60 min, resuspended in PBS and stored at -20°C. All steps were carried out on ice, and centrifugation was at 4°C.

6.1.2 Electrophoretic Separation of Plasma Membrane Fractions

Protein concentrations were determined using the Biorad protein assay dye concentrate in a protein microassay. The microtitre plate was read at 495nm on a microtitre plate reader. A standard curve was constructed using BSA standards ranging from 0 to 80 µg/ml, from which the protein concentrations of samples were determined. The samples were then diluted and dissolved in sample buffer (0.01 M tris/acetate pH 7.4, 1 mM EDTA, 0.05 M DTT, 5 M urea, 10% sucrose and 1% SDS) and boiled for 5 min to dissociate proteins. 5 µl (containing 2 µg of protein per sample) was loaded onto an SDS-urea polyacrylamide gel (5.6% acrylamide, 0.02 M Tris pH 7.4, 9 M urea and 1% SDS) and electrophoresed for 60 min at 100 V using the Bio-Rad Mini-protein II system in running buffer (0.04 M tris/acetate pH 7.4, 1% SDS). Proteins of known molecular weight were diluted 1:2 in sample buffer and run simultaneously on all gels. Acrylamide gels were then fixed for at least 30 min, preferably overnight, in destain solution (ethanol:acetic acid:water, 3:6:1). In order to ensure equal protein loading in all wells, protein was visualised by staining with Coomassie Brilliant Blue (4% w:v in ethanol:water:acetic acid, 5:4:1) for 30 min, followed by destaining until background staining was minimal.

6.1.3 Detection of P-glycoprotein

The separated proteins from the gels were then electrotransferred to a 0.45µm nitrocellulose membrane for 60 min at 100 V using the Bio-Rad Mini-protein II Trans Blot system in transfer buffer (0.025 M tris/glycine pH 8.2, 20% methanol).

Nitrocellulose membranes (filters) were blocked with 5% skim milk in TBS/0.01% Tween for 60 min at room temperature. Following incubation with each antibody the filter was washed for 3 x 10 min washes with TBS/0.1% Tween.

Filters were then probed for the presence of P-glycoprotein by incubation with 10 µl of C219 monoclonal antibody in 5 ml of 0.5% skim milk/TBS for 2 h. The filter was then incubated with 10 µl of alkaline-phosphatase conjugated anti-mouse-immunoglobulin (D314) in 5 ml of 0.5% skim milk/TBS for 60 min. The filter was then incubated in 5 ml of substrate solution (0.1 M Tris/HCl pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$) containing 0.825 mg 5-bromo,4-chloro-3-indoylphosphate and 1.65 mg nitro-blue tetrazolium until sufficiently stained purple (10-15 minutes). CEM and CEM/VLB_{100} cells were used as negative and positive controls respectively.

6.2 Immunocytochemistry

Reactivity with C219 was assayed by immunocytochemistry according to the method of Frielander *et al*, 1989. Briefly, 50 µl of cells at a density of 5 x 10^5/ml were cytospun and air-dried at room temperature. Slides were fixed in acetone, air-dried then washed. All washes were with TBS pH 7.6 and carried out between the addition of each antibody unless otherwise stated.

100 µl (5 µg) of C219 monoclonal antibody or 5 µg of the control monoclonal antibody 1D4.5 (raised against a Salmonella antigen) was applied overnight at 4°C, followed by rabbit-anti-mouse immunoglobulin (Z 259), at a 1/50 dilution for 30 min at room temperature, then addition of the tertiary APAAP complex at a 1/75 dilution for 30 min at room temperature (Cordell *et al*, 1984). Addition of the secondary and tertiary reagents for 10 min each was then repeated.

The final wash used TBS pH 8.2. The alkaline phosphatase reaction was developed using Fast Red TR salt dissolved alkaline phosphate substrate solution (20 mg napthol AS-MX phosphate, 2 ml dimethylformamide, 98ml 0.1M Tris buffer pH 8.2, 100 µl levamisole) to 1 mg/ml. Cells were counterstained with Harris Haematoxylin. All reagents were diluted in 0.5% TBS/skim milk. Again,CEM and CEM/VLB_{100} cells were used as positive and negative controls respectively.

6.3 Flow Cytometry

10^5 cells were incubated with 2 µg MRK16 or the control mouse IgG_{2a} monoclonal antibody 1D4.5 in 100 µl PBA buffer (phosphate-buffered saline, pH 7.2, containing 0.05% NaN_3 and 0.05% bovine serum albumin) for 30 min. All incubations were carried out on ice. Cells were washed 3 times in PBA and incubated in a 1/50 dilution of fluorescein conjugated sheep anti-mouse immunoglobulins, $F(ab')_2$ fraction for 30 min. Cells were then washed and resuspended in PBS containing 10 µg/ml propidium iodide (PI) and analysed within 1 h of preparation. Cells were analysed using a Hewlett-Packard FACScan flow cytometer using the LYSYS II software. Dead cells which took up PI were gated out and excluded from the analysis.

7 Determination of P-glycoprotein Function Using Rhodamine 123

P-glycoprotein function was measured by the ability of cells to accumulate Rhodamine 123 (Rh123) in the presence or absence of 10 μM verapamil. Rh123 was dissolved in ethanol as a stock solution of 5 mg/ml and stored at 4°C. 5 x 10^5 cells were incubated at 37°C with 10 μM verapamil for 10 min, followed by a further incubation of 45 min after addition of Rh123 to give a final concentration of 150 ng/ml, after which cells were washed once with ice-cold PBS, and resuspended in PBS containing 10 μg/ml PI. Cells were kept on ice and analysed immediately. Dead cells which took up PI were excluded from the analysis by gating.

8 Expression of Cell Surface Antigens

10^5 cells were incubated with DAKO-CD13 (WM-47), DAKO-CD14 (TÜK4), DAKO-CD34 (TÜK3), DAKO-CD61 (Y2/51), DAKO-glycophorin A (JC159) or control mouse IgG_1 (K121, raised against a kappa myeloma antigen) monoclonal antibodies. An irrelevant IgG_3 (Y5606) monoclonal antibody was used as a negative control for CD34. DAKO-CD13 was used at a final IgG concentration of 12.7 μg/ml for K562 cells and 5.08 μg/ml for the other cell lines. DAKO-CD14 was used at a final IgG concentration of 3.6 μg/ml for K562 cells and 1.44 μg/ml for the other cell lines. DAKO-CD34 was used at a final IgG concentration of 13.44 μg/ml for the KG-1a cells and 33.6 μg/ml for the other cell lines. DAKO-CD61 was used at final IgG concentration of 3.15 μg/ml for all cell lines. DAKO-Glycophorin A was used at a final IgG concentration of 2.66 μg/ml for K562 and HEL cells, and 5.32 μg/ml for the other cell lines. Cells were processed for flow cytometry as described in *6.3* then fixed in PBS containing 1% paraformaldehyde and 0.05% NaN_3, and stored at 4°C for no more than 7 days before analysis. Cells were assayed for CD36 expression using the monoclonal antinbody MRK20, as described for MRK16 in *6.3*. CD13 and glycophorin A expression of K562 cells and resistant sublines were also determined by immunocytochemistry as described in *6.2*.

9 Treatment with TPA

TPA was dissolved in DMSO and stored at -20°C, and diluted in RPMI before use. 2 x 10^5 K562 cells or 4 x 10^5 U937 cells were treated with 1, 5 or 16 nM for 18 h or 96 h, after which changes in expression of cell surface antigens were determined according to the method described in *8*. For experiments using staurosporine, an inhibitor of protein kinase C, cells were pretreated with 30 nM staurosporine for 30 min before the addition of TPA and cells were then incubated at 37°C for 18 h.

10 Treatment with Sodium Butyrate

Sodium butyrate was dissolved in RPMI and made fresh as required. 2 x 10^5 cells were incubated with 1.5 and 15 mM sodium butyrate for 18 and 96 h at 37°C, after which changes in cell surface antigen expression were determined according to the method described in *8*.

11 Treatment with GM-CSF

GM-CSF was stored at -20°C, and diluted with RPMI as required. 2 x 10^5 K562 cells were treated with 20 ng/ml GM-CSF for 18 and 42 h at 37°C. 4 x 10^5 U937 cells were treated for 18 hours with 100 ng/ml GM-CSF for 18 and 42 h at 37°C. After each treatment cell surface antigen expression was determined as described in 8.

12 Cell Cycle Analysis

In order to compare the effects of drugs on U937 cells and the U937/E15 subline, 4 x 10^5 cells were treated with 2 μM VP-16 for 18 h, after which drug was removed, and the cell cycle was monitored every 24 h. 10^6 cells were centrifuged and resuspended in PBS containing 50 μg/ml PI and 0.02% nonidet-P40 detergent, and kept on ice until analysis. Cell cycle kinetics and histograms were obtained using the Becton Dickinson program CellFit, using the RFIT model of analysis.

RESULTS AND DISCUSSION

1 DEVELOPMENT AND CHARACTERISATION OF DRUG RESISTANCE IN K562 CELLS

1.1 Introduction

Most *in vitro* studies of drug resistance have used drug concentrations far in excess of the levels maintained in the plasma of patients undergoing chemotherapy. Little is known regarding the ability of malignant cells to develop drug resistance to clinically relevant levels of chemotherapeutic agents *in vitro*.

The K562 cell line was established in 1970 from a pleural effusion in a patient with chronic myeloid leukaemia (CML) and is a highly undifferentiated erythroid cell line (Lozzio *et al*, 1981). The patient was treated with busulfan for 1 year and pipobroman for 3 years (Lozzio and Lozzio, 1975), both of which are alkylating agents and not members of the MDR family of drugs.

Most studies of drug resistance using K562 cells have induced resistance with high drug concentrations. Drug resistant K562 cell lines have been established by treatment with both anthracyclines and *Vinca* alkaloids. Drug resistant K562-R cells treated with 500 nM daunorubicin showed *mdr*1 mRNA expession, but P-glycoprotein expression was not detected (Yanovich *et al*, 1989). The K562/ADR$_{80}$, K562/ADR$_{200}$ and K562/ADR$_{500}$ cell lines were selected with 80, 200 and 500 nM doxorubicin respectively, and all expressed P-glycoprotein (Kato *et al*, 1989). Drug resistant K562 cells have also been selected with 30 nM vincristine which induced P-glycoprotein expression (Tsuruo *et al*, 1983; Tsuruo *et al*, 1986a). The cell line K562/ADM, which was derived from K562/VCR H-1 clone by treatment with 500 nM doxorubicin, also expressed P-glycoprotein (Tsuruo *et al*, 1986b). Therefore both *Vinca* alkaloids and anthracyclines induce MDR and expression of P-glycoprotein at high drug concentrations in K562 cells.

Little is known about lower levels of resistance, more closely paralleling the development of drug resistance in cells from a patient undergoing chemotherapy. K562 sublines resistant to clinically relevant levels of the *Vinca* alkaloid vinblastine (Ratain & Vogelzang, 1987) and the anthracycline epirubicin (Tjuljandin *et al*, 1990) were therefore established.

1.1.1 Cytotoxic Mechanisms and Pharmacokinetics of Epirubicin and Vinblastine

Epirubicin is a derivative of the anthracycline doxorubicin. Anthracyclines are a group of natural product antibiotic drugs, isolated from a species of *Streptomyces* bacteria, which exert their cytotoxicity through their ability to intercalate between DNA bases, leading to inhibition of DNA, RNA and protein synthesis (Sinha and Politi, 1990).

Anthracyclines are thought to enter the cell by passive diffusion or by carrier-mediated passive transport, where they are distributed evenly to both the nucleus and cytoplasm (Hindenburg *et al*, 1989).

The 3'-N of the daunosamine sugar moiety is thought to be positioned along the minor groove of the DNA, allowing intercalation between the base pairs, resulting in interference with DNA synthesis, and subsequently RNA and protein synthesis (Bodley *et al*, 1989). Their cytotoxicity is also exerted through free radical formation, which in turn leads to DNA damage (Keizer *et al*, 1990).

Epirubicin was produced by epimerisation of the aminosugar hydroxyl group at the C-4' position on doxorubicin. Epirubicin produces less cardiac and haematological toxicity than doxorubicin, making it more favourable therapeutically. Epirubicin binds less avidly to DNA and is less alkaline than doxorubicin, which may allow more of the active forms of the drug to enter the tumour cells (Snyder *et al*, 1987; Weiss, 1992).

As epirubicin and doxorubicin are very closely related with regards to structure, it is therefore assumed that the cytotoxic effects of epirubicin are in general similar to those of doxorubicin (Cerosimo and Hong, 1986). Epirubicin, when administered intravenously, reaches a steady state plasma level of 15 to 20 ng/ml within 5 to 10 hours after administration (Tjuljandin *et al*, 1990). Pharmacokinetic studies have shown that epirubicin is more rapidly and extensively metabolised than doxorubicin, with greater formation of its alcohol metabolite epirubicinol (reviewed by Weiss, 1992).

Vinblastine belongs to the group of cytotoxic agents known as the *Vinca* alkaloids, which are derived from plants. The antitumour activity of the *Vinca* alkaloids is assumed to be due to their ability to interact with the tubulin dimer of microtubules, producing depolymerisation and disruption of the cellular microtubular network, including the mitotic spindle, therefore killing dividing cells through inhibition of mitosis. Binding of *Vinca* alkaloids to tubulin has been observed within cells (Safa *et al*, 1987). The cytotoxic effects of *Vinca* alkaloids may also be related to their ability to inhibit RNA, DNA and lipid biosynthesis, and alterations in cellular cAMP and glutathione metabolism (Beck, 1980; Safa *et al*, 1987; Chabner and Horwitz, 1990).

Pharmacokinetic analysis of the serum of patients treated with vinblastine in chemotherapy have shown the maximum concentration achieved is approximately 50 ng/ml (with an interpatient variation of up to 28 ng/ml), with a rapid decrease in serum levels, plateauing at approximately 1 ng/ml after 12 to 24 hours (Ratain and Vogelzang, 1987).

1.1.2 K562 Cells as a Model of Haematopoietic Differentiation

The K562 cell line is also a useful model with which to study haematopoietic differentiation. K562 cells are thought to be a multipotential stem cell (Lozzio *et al*, 1981), and they have the capacity to differentiate along the erythroid, granulocytic, monocytic and megakaryocytic lineages (Sutherland *et al*, 1986). For example, TPA has been shown to induce megakaryocytic differentiation in K562 cells at high doses (8nM) (Alitalo *et al*, 1990), and macrophage-like differentiation at lower doses (0.5nM) (Sutherland *et al*, 1986). Sodium butyrate and hemin induce erythroid differentiation (Sutherland *et al*, 1986). Treatment of K562 cells with antineoplastic drugs including doxorubicin (Toffoli *et al*, 1989), daunorubicin and 1-β-D-arabinofuranosylcytosine (Tonini *et al*, 1987) also causes erythroid differentiation and haemoglobin production, while the topo II inhibitor VP-16 induces granulocyte-like differentiation (Nakaya *et al*, 1991).

1.1.3 Outline

In this chapter results are presented for K562 cells treated with 5 to 40 ng/ml epirubicin and 1 to 8 ng/ml vinblstine. The sublines were examined for drug resistance and changes in expression of antigens associated with haematopoietic differentiation.

1.2 Characterisation of Drug Resistance

K562 cells rapidly adapted to treatment with epirubicin and vinblastine. The epirubicin treated cells showed a correlation between increasing resistance to epirubicin and concentration of epirubicin at which they were treated, with the exception of the K562/E15B subline, which was more resistant to epirubicin than the other sublines (Fig. 1.1). All of the vinblastine treated cells showed resistance to vinblastine, however the IC_{50} for vinblastine was the same for all four sublines, and did not increase with the concentration of vinblastine used to select for resistance (Fig. 1.2).

The cross-resistance to a panel of chemotherapeutic drugs was examined in selected drug resistant K562 sublines and relative resistance to each drug is shown in Table 1.1. As the concentration of selecting drug increased, so too did the level of cross-resistance to other drugs. Relative resistance was only considered to be significant if greater than 2-fold.

Cells treated with epirubicin were more cross-resistant to vinblastine, vincristine, colchicine and actinomycin-D. The K562/E15B subline showed significantly higher cross-resistance than the other epirubicin treated sublines, including the K562/E40 cells. Only the K562/E10B subline showed significant cross-resistance to doxorubicin, and only the K562/E10 subline showed cross-resistance to daunorubicin and was highly

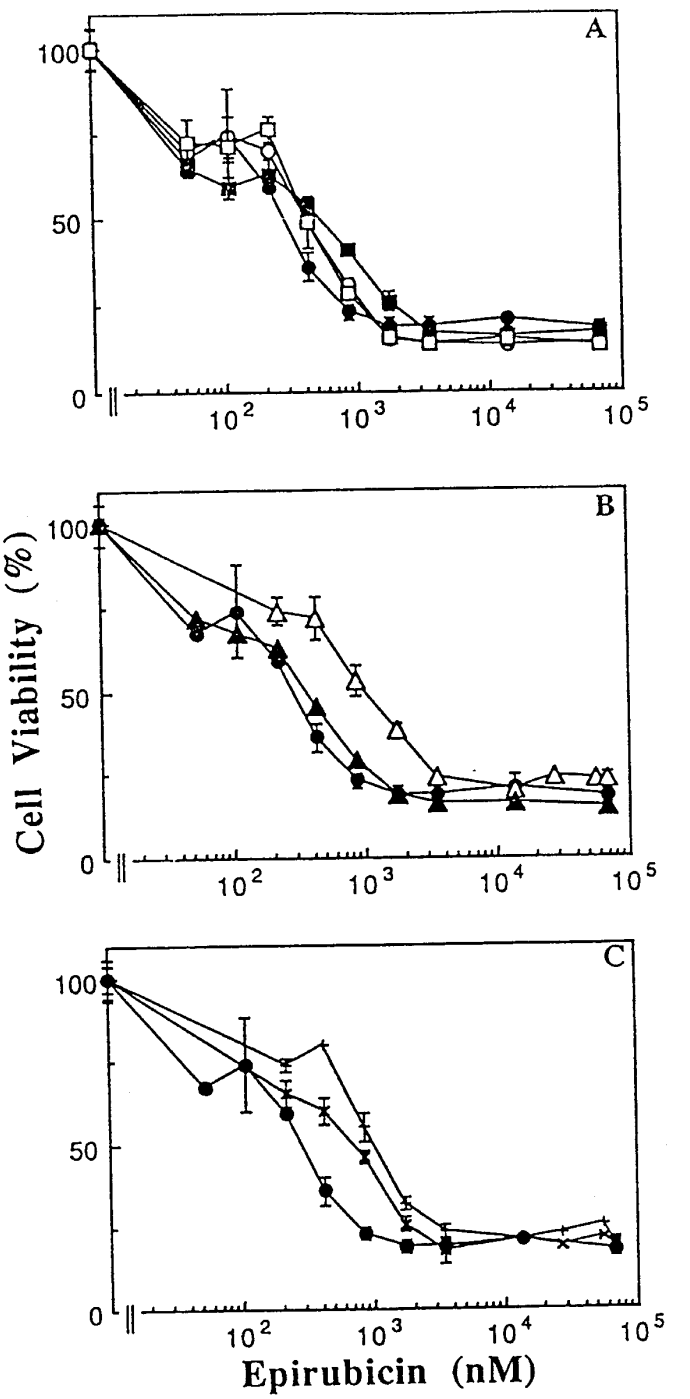

Figure 1.1. Resistance to epirubicin in epirubicin treated K562 sublines. Cells were incubated with serial dilutions of epirubicin for 4 days, after which cell viability was determined using the MTT assay as described in *Materials and Methods 3*. A. K562(●), K562/E5(o), K562/E10(■), K562/E10B(□), B. K562(●), K562/E15(▲), K562/E15B(Δ), C. K562(●), K562/E20(x) and K562/E40(+). *Points*, mean of triplicate determinations; *error bars*, standard deviations. The experiment was repeated at least three times, and a representative result is shown.

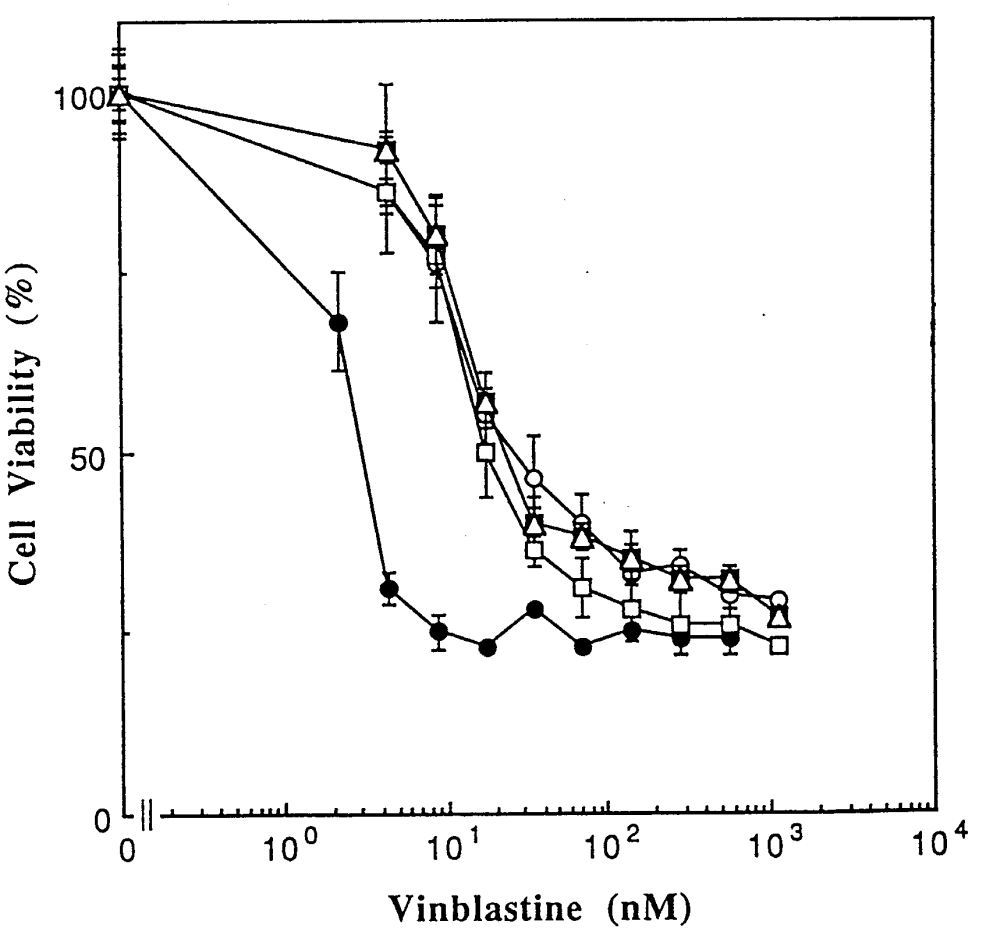

Figure 1.2. Resistance to vinblastine in vinblastine treated K562 sublines. Cells were incubated with serial dilutions of vinblastine for 4 days, after which cell viability was determined using the MTT assay as described in *Materials and Methods 3*. K562(●), K562/V1(o), K562/V2(■), K562/V4(□) and K562/V8(Δ). *Points*, mean of triplicate determinations; *error bars*, standard deviations. The experiment was repeated at least three times, and a representative result is shown.

resistant to the *Vinca* alkaloids. All epirubicin resistant sublines were slightly sensitised to VP-16.

Cells treated with vinblastine were cross-resistant to epirubicin, vincristine and actinomycin, but not to colchicine, doxorubicin, daunorubicin or VP-16. Although the resistance to vinblastine was the same for all of the vinblastine treated sublines, the level of cross-resistance to other drugs increased with the concentration of drug with which the cells were treated (Table 1.1). No sublines showed cross-resistance to the non-MDR drugs methotrexate or cisplatinum (data not shown).

Table 1.1. Relative resistance of representative K562 sublines to MDR drugs.

	Relative Resistance[a]							
Cell Line	EPR	DOX	DNR	VLB	VCR	VP-16	ACT	COL
K562	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
K562/E10	1.6	0.8	**3.0**	**4.6**	**6.7**	0.6	**2.3**	**2.5**
K562/E10B	1.6	**3.2**	1.8	0.6	**2.0**	0.6	0.7	0.7
K562/E15B	**4.1**	**2.1**	**2.1**	**6.3**	**18.0**	0.8	**6.0**	**3.5**
K562/E40	**3.3**	1.9	**2.5**	**4.0**	**8.0**	0.6	**2.0**	1.5
K562/V1	**4.6**	1.2	1.2	**7.8**	**5.3**	0.8	1.7	0.7
K562/V8	**3.3**	1.8	1.4	**8.0**	**25.0**	0.8	**4.7**	1.2

[a] Relative resistance was determined by dividing the IC_{50} of resistant subline by the IC_{50} of the parental K562 cells. Relative resistance greater than or equal to 2.0 is indicated in bold type.
Abbreviations: EPR, epirubicin; DOX, doxorubicin; DNR, daunorubicin; VLB, vinblastine; VCR, vincristine; ACT, actinomycin-D; COL, colchicine.

1.3 P-Glycoprotein Expression

1.3.1. Western Blot Analysis

P-glycoprotein expression was detected as a doublet of approximately 170kD by Western blotting using C219 in all sublines treated with epirubicin concentrations of 10 ng/ml or more, except the K562/E10B subline (Fig. 1.3), and all sublines treated with vinblastine concentrations of 1 ng/ml or more (Fig. 1.4). The K562/E15B and K562/V8 sublines expressed the most P-glycoprotein, while the K562/E40 subline, although treated with a higher concentration of epirubicin, expressed less P-glycoprotein than the K562/E15 and K562/E15B sublines.

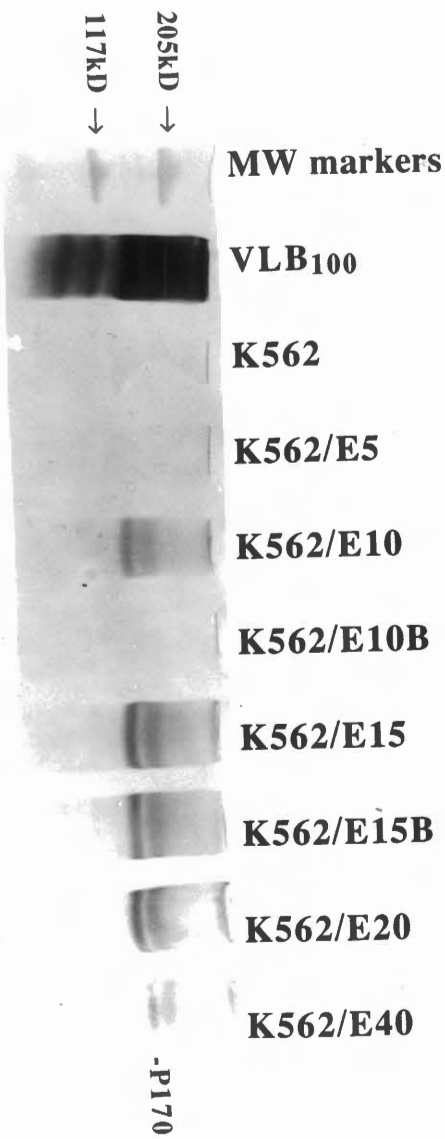

Figure 1.3. Western blot analysis of P-glycoprotein expression in K562 cells and epirubicin treated sublines. Plasma membrane fractions were prepared and analysed as described in *Materials and Methods 6.1*. Positions of molecular weight markers and P-glycoprotein (P170) are indicated.

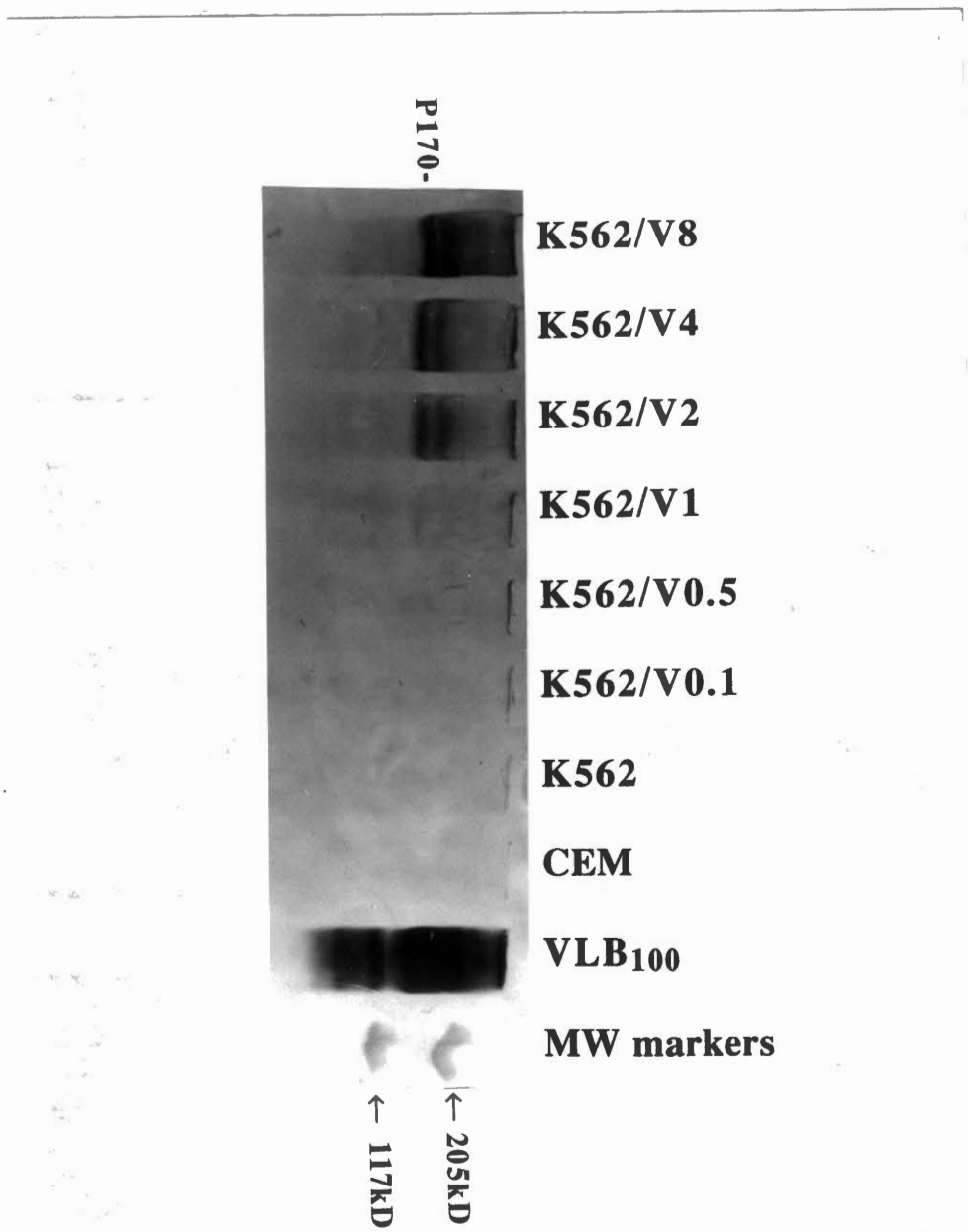

Figure 1.4. Western blot analysis of P-glycoprotein expression in K562 cells and vinblastine treated sublines. Membrane fractions were prepared and analysed as described in *Materials and Methods 6.1*. Positions of molecular weight markers and P-glycoprotein (P170) are indicated.

1.3.2 Immunocytochemistry

Western blot analysis was confirmed by immunocytochemistry with C219 in all of the epirubicin and vinblastine treated sublines, except the K562/E10B subline (Fig. 1.5). Although the K562/E10B cells were negative by Western blot analysis, approximately 5-10% of the cells showed moderate staining when analysed by immunocytochemistry. The other sublines exhibited heterogeneous staining, with some cells staining faintly positive and some staining intensively positive for P-glycoprotein. This heterogeneous staining pattern has remained stable for over 3 years of culture with intermittent drug treatment, and also remained stable without drug treatment for 6 weeks.

1.3.3 Flow Cytometry

Further confirmation of the heterogeneous expression of P-glycoprotein was obtained by flow cytometry with MRK16 monoclonal antibody. The K562/E10 subline, with a proportion of positive cells gave a broad staining profile, confirming the heterogeneous expression detected by immunocytochemistry (Fig. 1.6). Although the K562/E10B subline was negative for P-glycoprotein by Western blot analysis, the cells showed a slight increase in MRK16 binding as compared to the parental K562 cells by flow cytometry. This confirmed the small percentage of positive cells found by immunocytochemistry. The K562/E15, K562/E15B and K562/E20 sublines were strongly positive. The K562/E40 subline however showed similar reactivity with MRK16 to the parental K562 cells. The K562/E40 subline also showed low P-glycoprotein expression by Western blot analysis. The K562/V1 and K562/V8 sublines were both strongly positive with MRK16 (Fig. 1.7). P-glycoprotein expression detected with C219 and MRK16, and resistance to the *Vinca* alkaloids in the drug resistant sublines is summarised in Table 1.2.

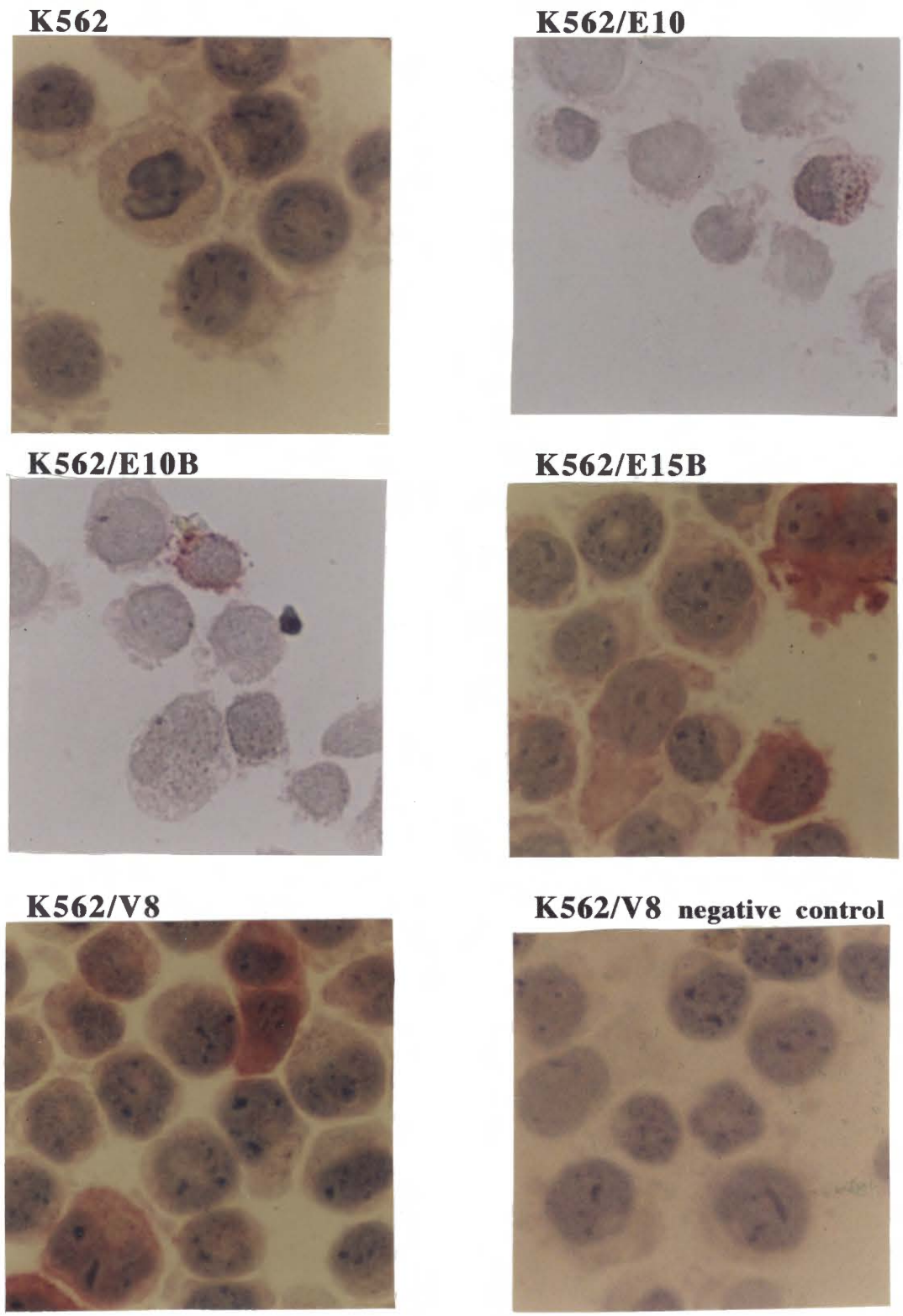

Figure 1.5. Immunocytochemical analysis of P-glycoprotein expression of K562 cells and representative drug resistant sublines. Cells were cytospun and stained as described in *Materials and Methods* 6.2. Magnification 200X.

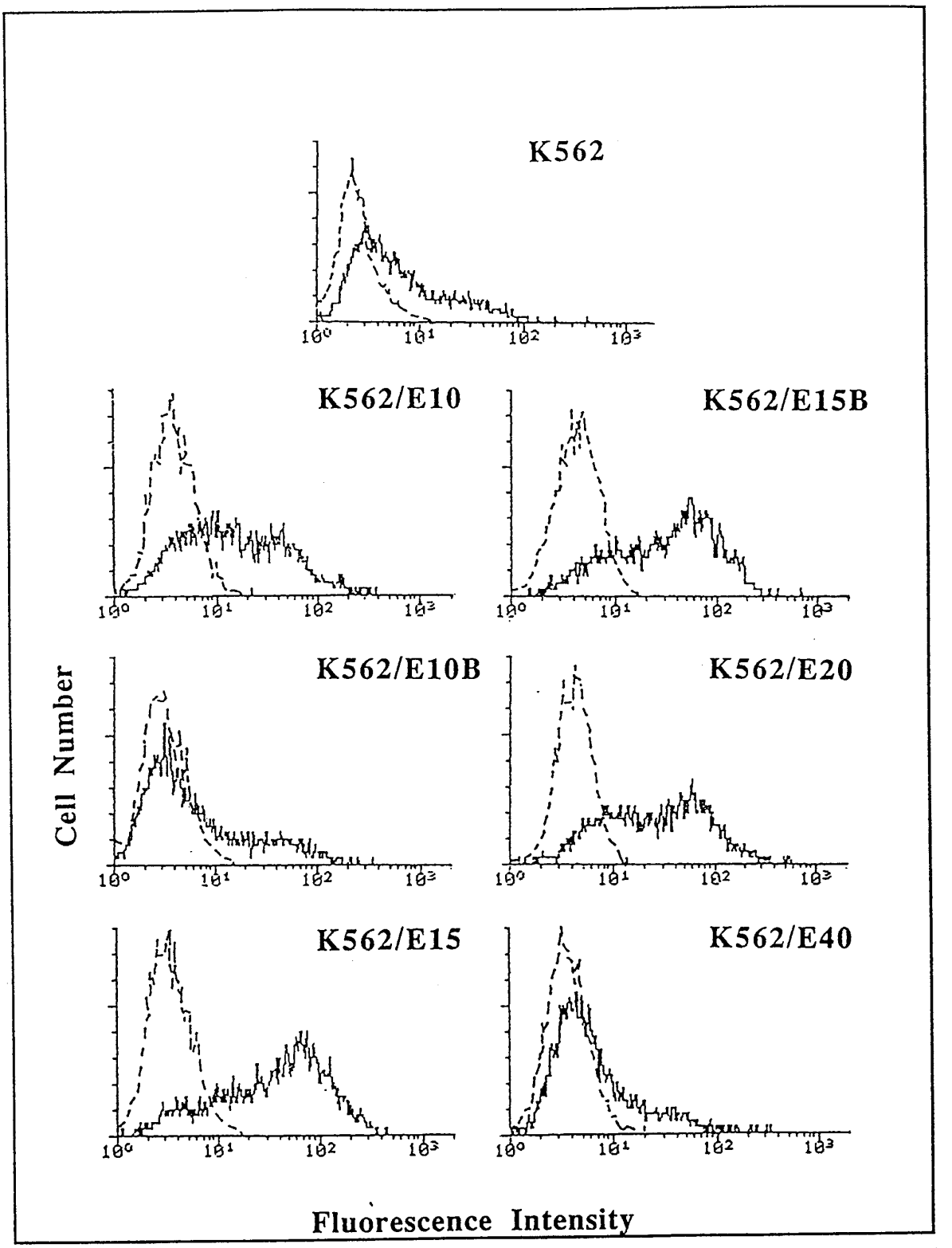

Figure 1.6. Analysis of P-glycoprotein expression in K562 cells and epirubicin treated sublines with MRK16 monoclonal antibody. Cells were incubated with MRK16(—) as described in *Materials and Methods 6.3* and assayed by flow cytometry. Profiles are compared to cells incubated with a negative control antibody(---). The experiment was repeated at least three times and representative results are shown.

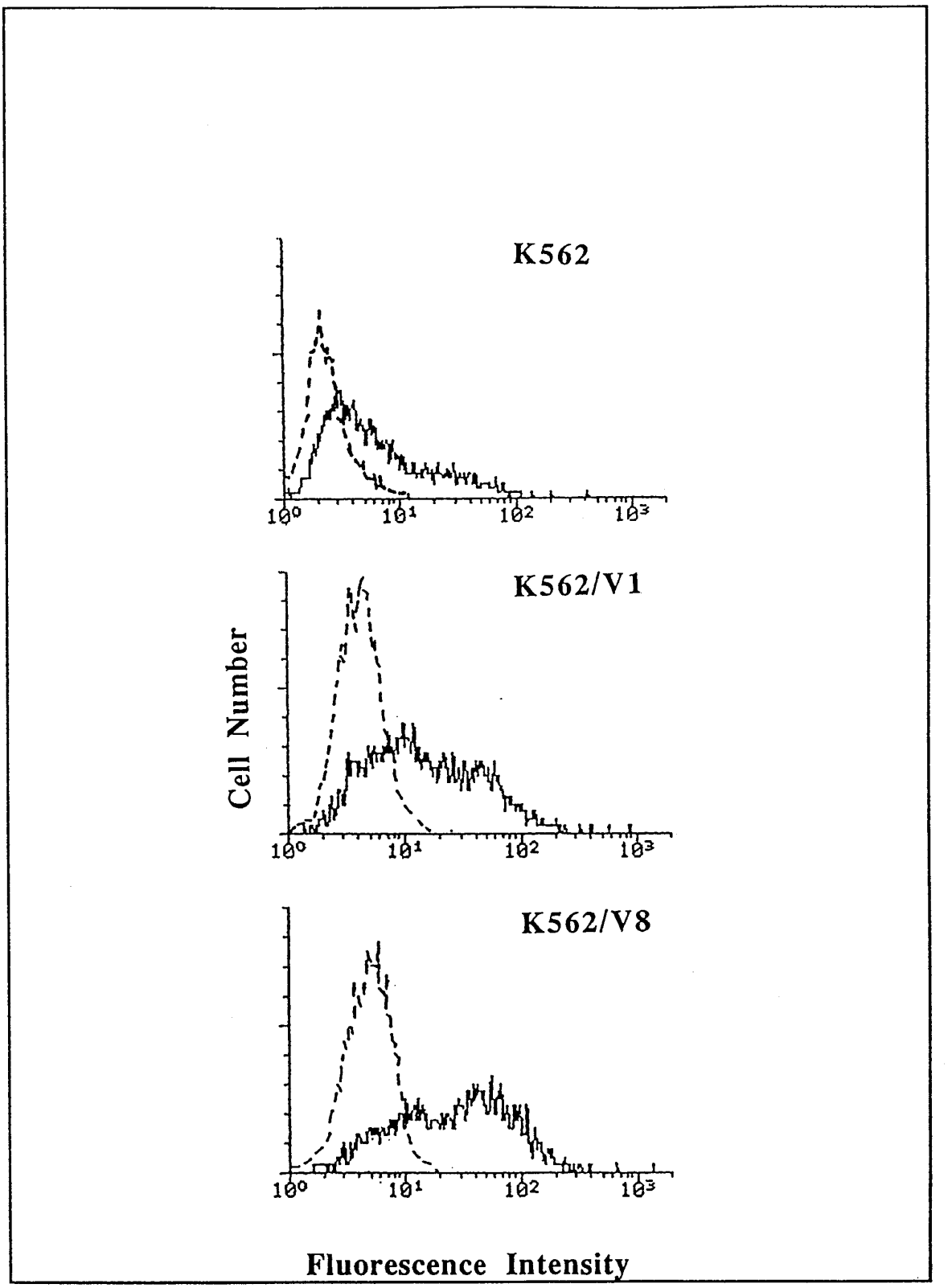

Figure 1.7. Analysis of P-glycoprotein expression in K562 cells and vinblastine treated sublines with MRK16 monoclonal antibody. Cells were incubated with MRK16(—) as described in *Materials and Methods 6.3* and assayed by flow cytometry. Profiles are compared to cells incubated with a negative control antibody(---). The experiment was repeated at least three times and representative results are shown.

Table 1.2. Expression of P-glycoprotein and resistance to *Vinca* alkaloids in drug resistant K562 sublines.

Cell Line	*Vinca* Alkaloid Resistance	P-glycoprotein Expression		
		C219: Western blot	C219: Immunocytochemistry	MRK16
K562	-[a]	-	-	-/±
K562/E10	+	+	+	+
K562/E10B	<K562	-	+	±
K562/E15	ND	+	+	+
K562/E15B	+++	+	+	+
K562/E20	ND	+	+	+
K562/E40	++	+	+	-/±
K562/V1	++	±/+	+	+
K562/V8	+++	+	+	+

a + Denotes detection, - denotes not detected, ND denotes not done.

1.4 Cloning

The K562/E10 and K562/E10B sublines were cloned in order to examine the stability of P-glycoprotein expression. The clones derived from the K562/E10B subline were all negative for P-glycoprotein expression by Western blot analysis and demostrated a similar MDR phenotype to the K562/E10B subline with 2-3 fold resistance to epirubicin and no cross-resistance to vinblastine. Of the 17 clones obtained from the K562/E10 subline, 12 (70%) showed P-glycoprotein expression by Western blot analysis and immunocytochemistry, while 5 clones (30%) showed no P-glycoprotein expression by either method. Western blot analysis of representative clones is shown in Fig. 1.8. Two representative clones were further studied, clone K562/E10_4 which expressed P-glycoprotein, and K562/E10_{15}, which did not express P-glycoprotein.

The K562/E10_4 clone, which had high, homogeneous P-glycoprotein expression was more resistant to epirubicin and vinblastine than the parental K562/E10 subline (Table 1.3). Clone K562/E10_{15}, which did not express P-glycoprotein, was not resistant to epirubicin or vinblastine (Table 1.3).

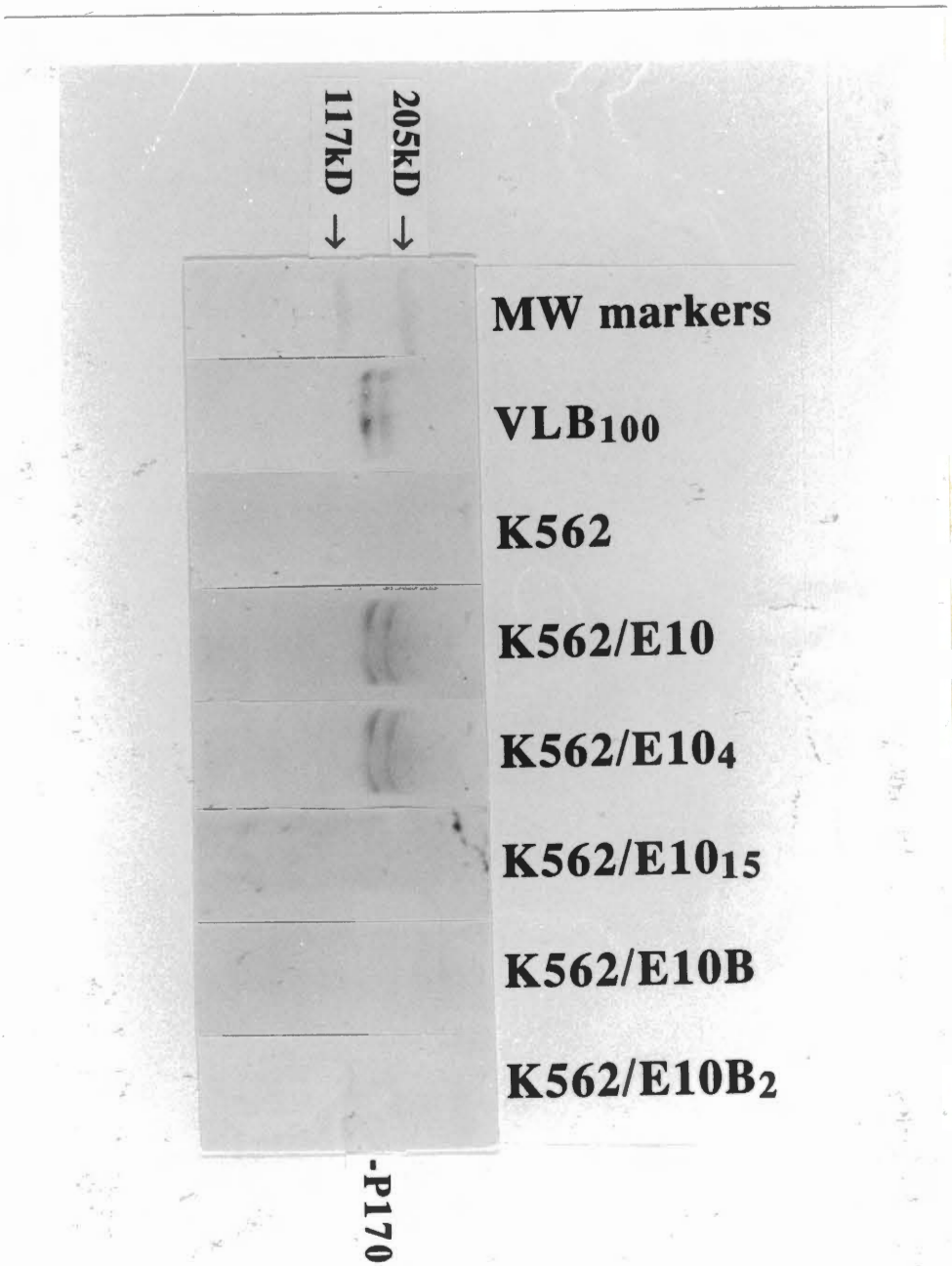

Figure 1.8. Western blot analysis of P-glycoprotein expression in K562/E10 and K562/E10B clones. Plasma membrane fractions were prepared and analysed as described in *Materials and Methods 6.1*. Positions of molecular weight markers and P-glycoprotein (P170) are indicated.

Table 1.3. Reversal of resistance to epirubicin and vinblastine by verapamil (VER) in K562 sublines and clones.

		IC_{50} (nM)	
Cell Line	VER	Epirubicin	Vinblastine
K562	-	290	3.5
	+	200 (1.5)	2.0 (1.8)
K562/E10	-	450	16.0
	+	150 (3.0)	1.8 (8.9)
K562/E10B	-	450	2.0
	+	260 (1.7)	1.3 (1.5)
K562/E15B	-	1200	22.0
	+	250 (4.8)	1.9 (11.6)
K562/V8	-	1000	28.0
	+	260 (3.9)	1.5 (18.6)
Clones			
$K562/E10_4$	-	1500	90.0
	+	260 (5.8)	8.0 (11.3)
$K562/E10_{15}$	-	260	1.3
	+	120 (2.2)	0.6 (2.2)

a Numeral in parenthesis indicates fold-reversal which is calculated by dividing the IC_{50} of the cells in the absence of verapamil by the IC_{50} in the presence of verapamil.

1.5 Reversal of Resistance

The ability of verapamil to sensitise the drug resistant sublines to epirubicin and vinblastine was examined in representative sublines (Table 1.3; Fig. 1.9). All of the drug resistant sublines were sensitised to both epirubicin and vinblastine by verapamil, with the exception of the K562/E10B subline. The P-glycoprotein expressing $K562/E10_4$ clone was also sensitised to both epirubicin and vinblastine (Fig. 1.10). Verapamil slightly sensitised the K562 cells and the drug senstive $K562/E10_{15}$ clone (Fig. 1.9 and Fig. 1.10).

1.6 Rhodamine 123 Accumulation

Accumulation of the fluorescent dye rhodamine 123 (Rh123) was examined in the K562 cells and the resistant sublines. Rh123 accumulation was decreased in all sublines in comparison to the parental K562 cells, with the exception of the K562/E10B subline (Fig. 1.11), which had very low P-glycoprotein expression. This suggests that the P-glycoprotein expressed in the K562/E10B subline was not functional, or alternatively that this method was not sufficiently sensitive to detect any changes due to the low level of P-

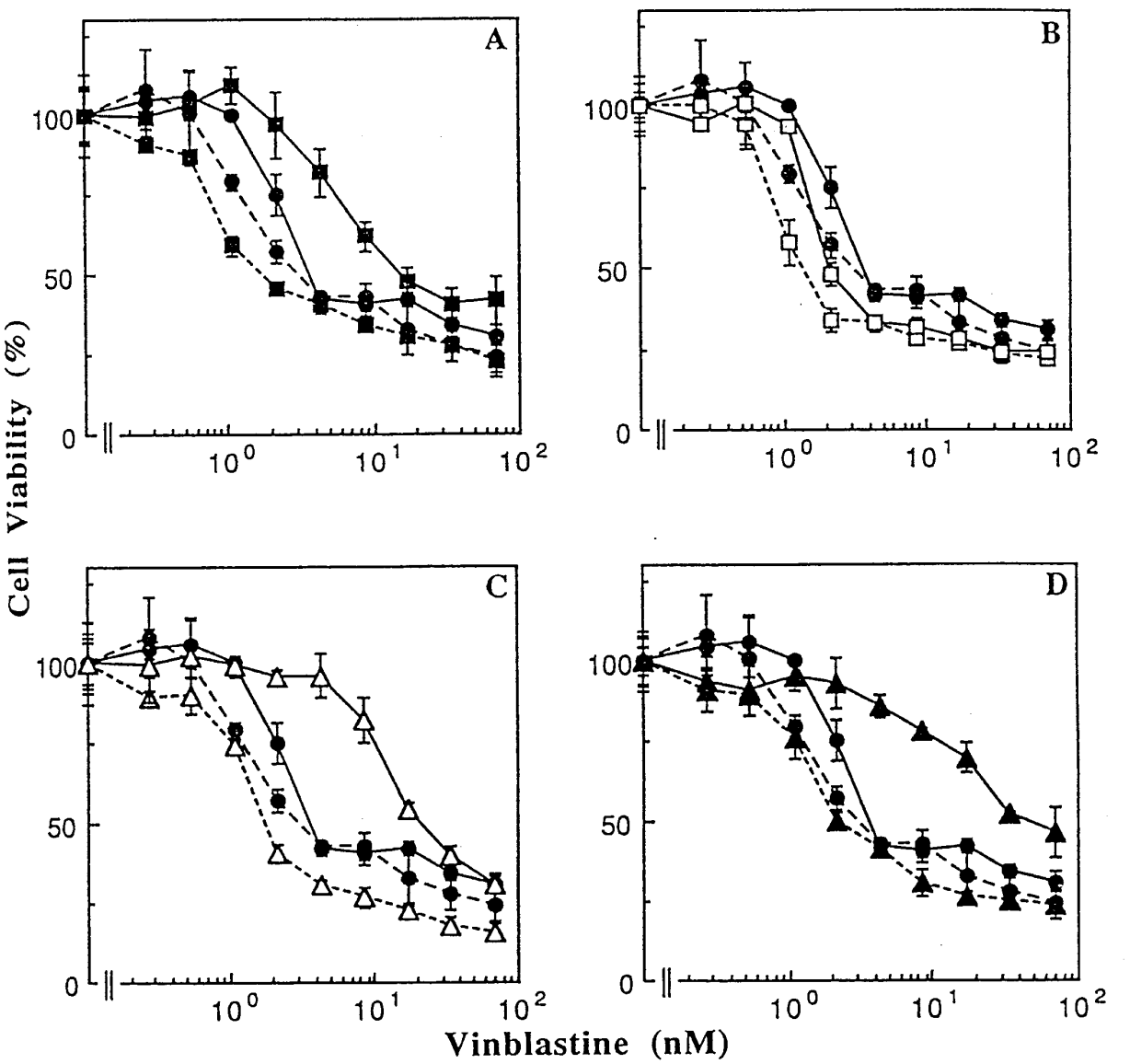

Figure 1.9. The effect of verapamil on vinblastine cytotoxicity in K562 cells and drug resistant sublines. Cells were incubated with serial dilutions of vinblastine in the presence(---) and absence(—) of 10 μM verapamil for 4 days, after which cell viability was measured using the MTT assay as described in *Materials and Methods 3*. A. K562(●), K562/E10(■), B. K562(●), K562/E10B(□) and C. K562(●), K562/E15B(Δ) and D. K562(●), K562/V8(▲). *Points*, mean of triplicate determinations; *error bars*, standard deviations. The experiment was repeated at least three times, and a representative result is shown.

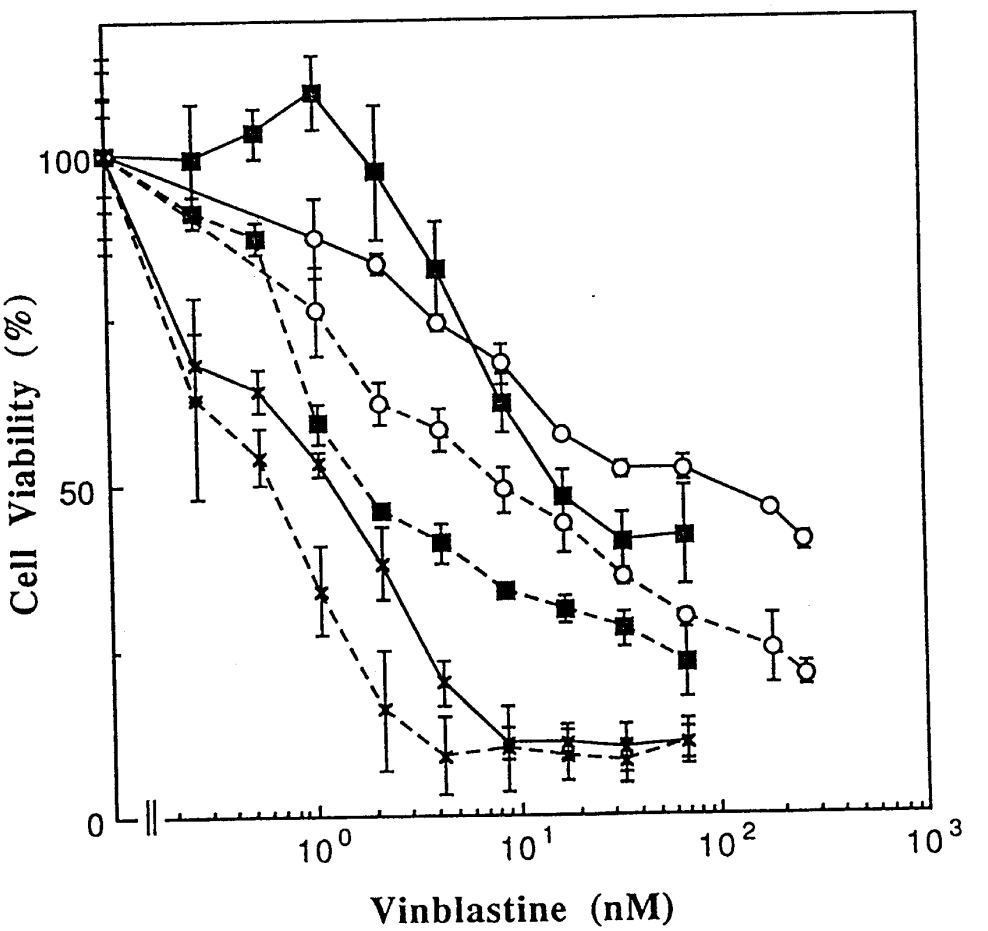

Figure 1.10. The effect of verapamil on vinblastine cytotoxicity in K562/E10 clones. Cells were incubated with vinblastine in the presence(---) and absence(—) of 10 μM verapamil for 4 days, after which cell viability was measured using the MTT assay as described in *Materials and Methods 3*. K562/E10(■), K562/E10_4(o) and K562/E10_{15}(x). *Points*, mean of triplicate determinations; *error bars*, standard deviations. The experiment was repeated at least three times, and a representative result is shown.

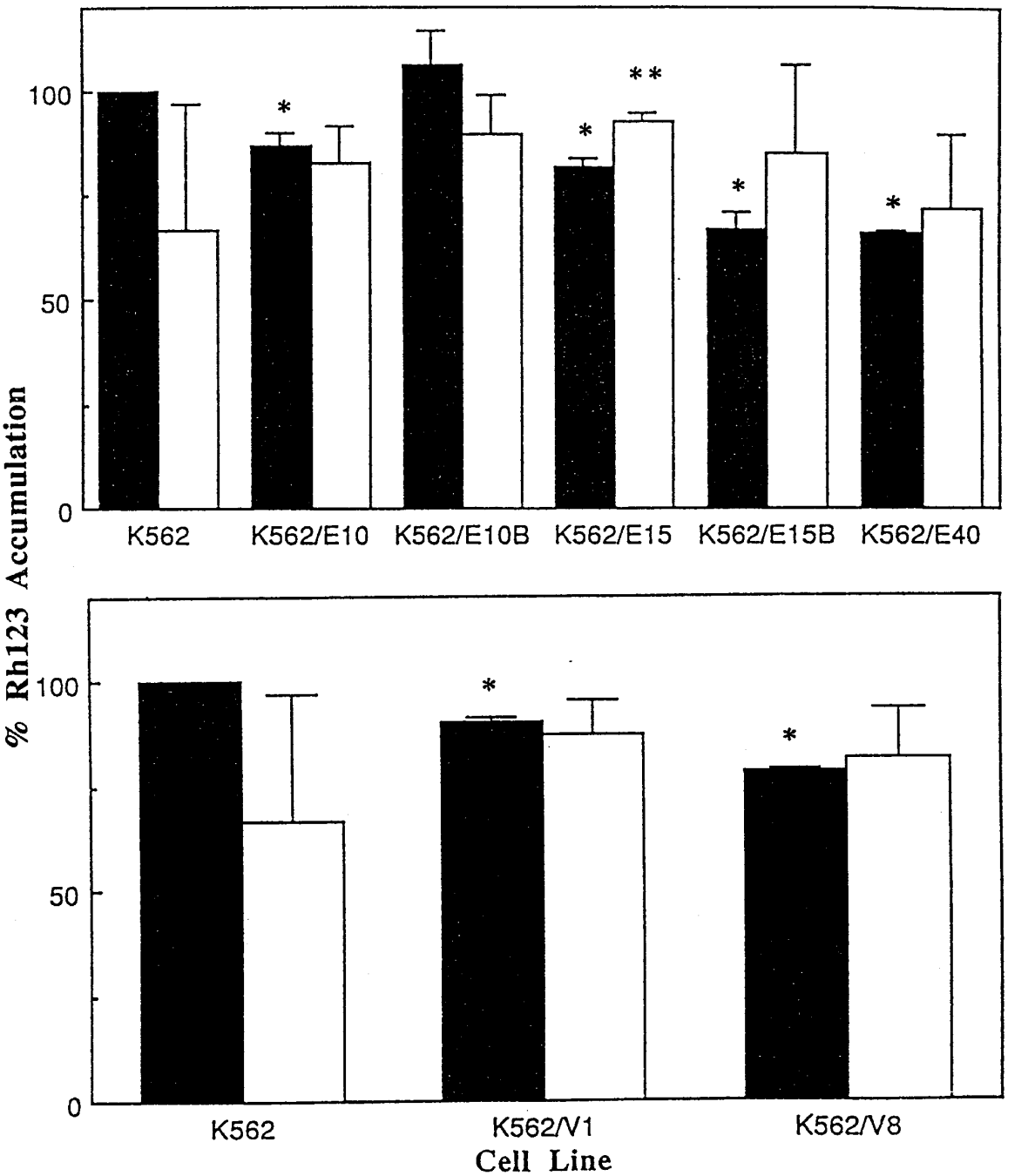

Figure 1.11. Rhodamine 123 accumulation in K562 cells and drug resistant sublines. Cells were incubated with Rh123 in the presence(□) or absence(■) of 10 μM verapamil, after which cell fluorescence was measured by flow cytometry as described in *Materials and Methods 7*. Results are the mean of two experiments and calculated as a percentage of Rh123 accumulation in the K562 cells. *Error bars*, standard deviations; * indicates significant decrease ($p<0.05$) compared to the parental K562 cells and ** indicates a significant increase ($p<0.05$) in accumulation in the presence of verapamil using the student's t-test.

glycoprotein expressed in these cells. In the epirubicin resistant sublines, Rh123 accumulation decreased with increasing selecting concentration of epirubicin.

The effect of verapamil on Rh123 accumulation was also examined. Verapamil increased Rh123 accumulation in the K562/E15, K562/E15B, and K562/E40 sublines, with no effect in the K562/E10, K562/E10B sublines, and only a small increase in the K562/E40 subline (Fig. 1.11). Although verapamil increased Rh123 accumulation, the increase was only statistically significant in the K562/E15 subline. Furthermore, verapamil did not increase Rh123 accumulation in either the K562/V1 or the K562/V8 sublines, even though they expressed P-glycoprotein and were drug resistant (Fig. 1.11). Rh123 accumulation in the resistant sublines and the effect of verapamil is summarised in Table 1.4.

Table 1.4. P-glycoprotein function in drug resistant sublines.

		Effect of Verapamil	
Cell Line	Decrease in Rh123 Accumulation	Rh123 Accumulation	Drug Cytotoxicity
K562	-[a]	-	±
K562/E10	+	-	+
K562/E10B	-	-	-
K562/E15	++	+	ND
K562/E15B	++	+	+++
K562/E20	ND	ND	ND
K562/E40	+++	+	ND
K562/V1	+	-	ND
K562/V8	+	-	+++

a + denotes an effect, - denotes no effect and ND denotes experiment was not done

1.7 Expression of Antigens Associated with Differentiation

1.7.1 Expression of CD13

K562 cells expressed CD13 and the epirubicin resistant sublines showed slight decreases in expression of CD13, with the exception of the K562/E15B subline, in which CD13 expression was increased (Fig. 1.12). The vinblastine treated K562 sublines all showed more homogeneous CD13 expression than the parental K562 cells. However, unlike the epirubicin treated sublines, expression increased with the concentration of vinblastine at which the cells were treated (Fig. 1.13).

The P-glycoprotein expressing K562/E10_4 clone (P-glycoprotein positive) showed no expression of CD13 by immunocytochemistry, while there was a marked increase in

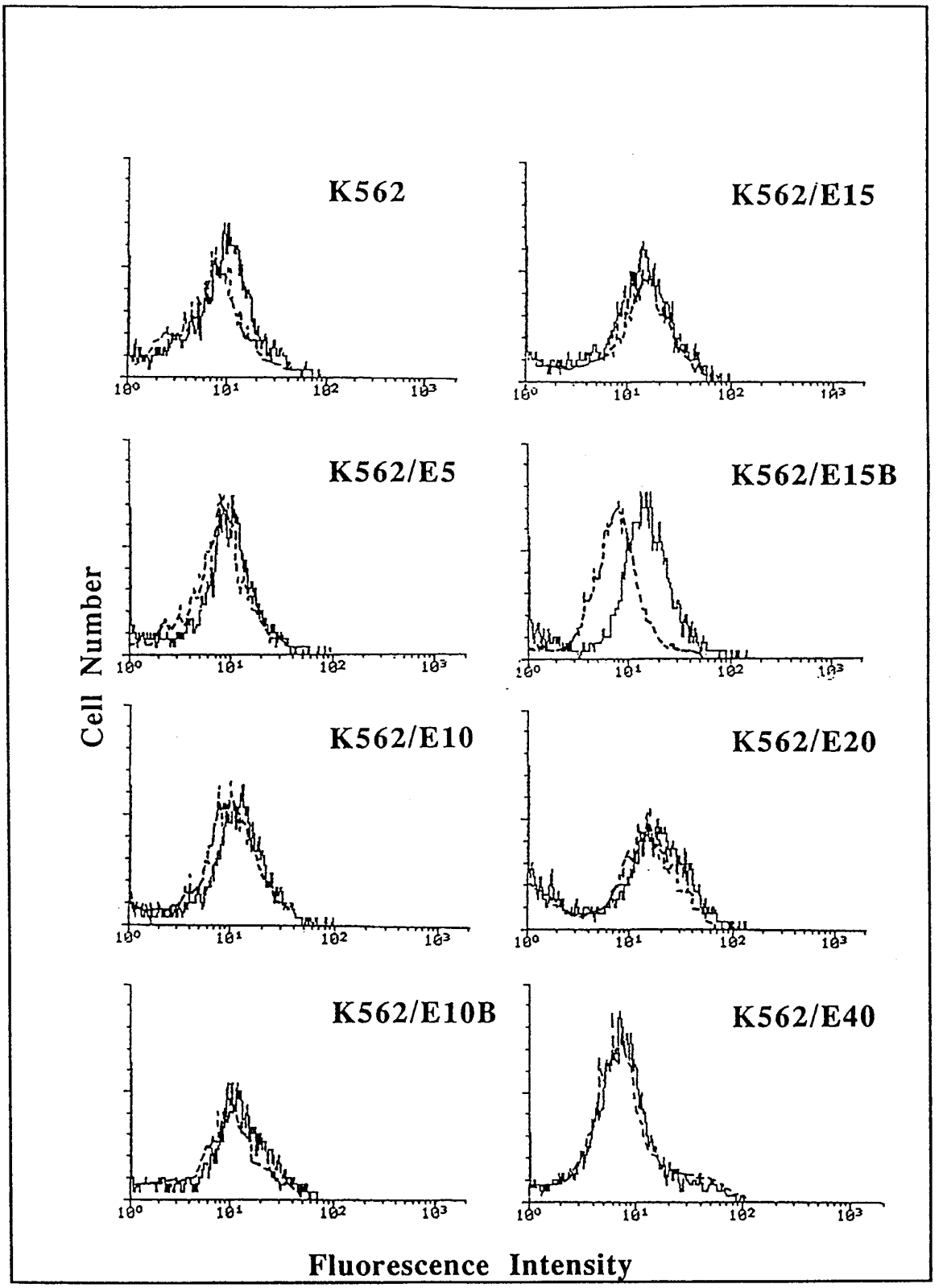

Figure 1.12. CD13 expression of K562 cells and epirubicin treated sublines. Cells were analysed for CD13 expression(—) by flow cytometry as described in *Materials and Methods 8* and the fluorescence profiles are compared to cells incubated with a negative control antibody(---). The experiment was repeated at least three times, and a representative result is shown.

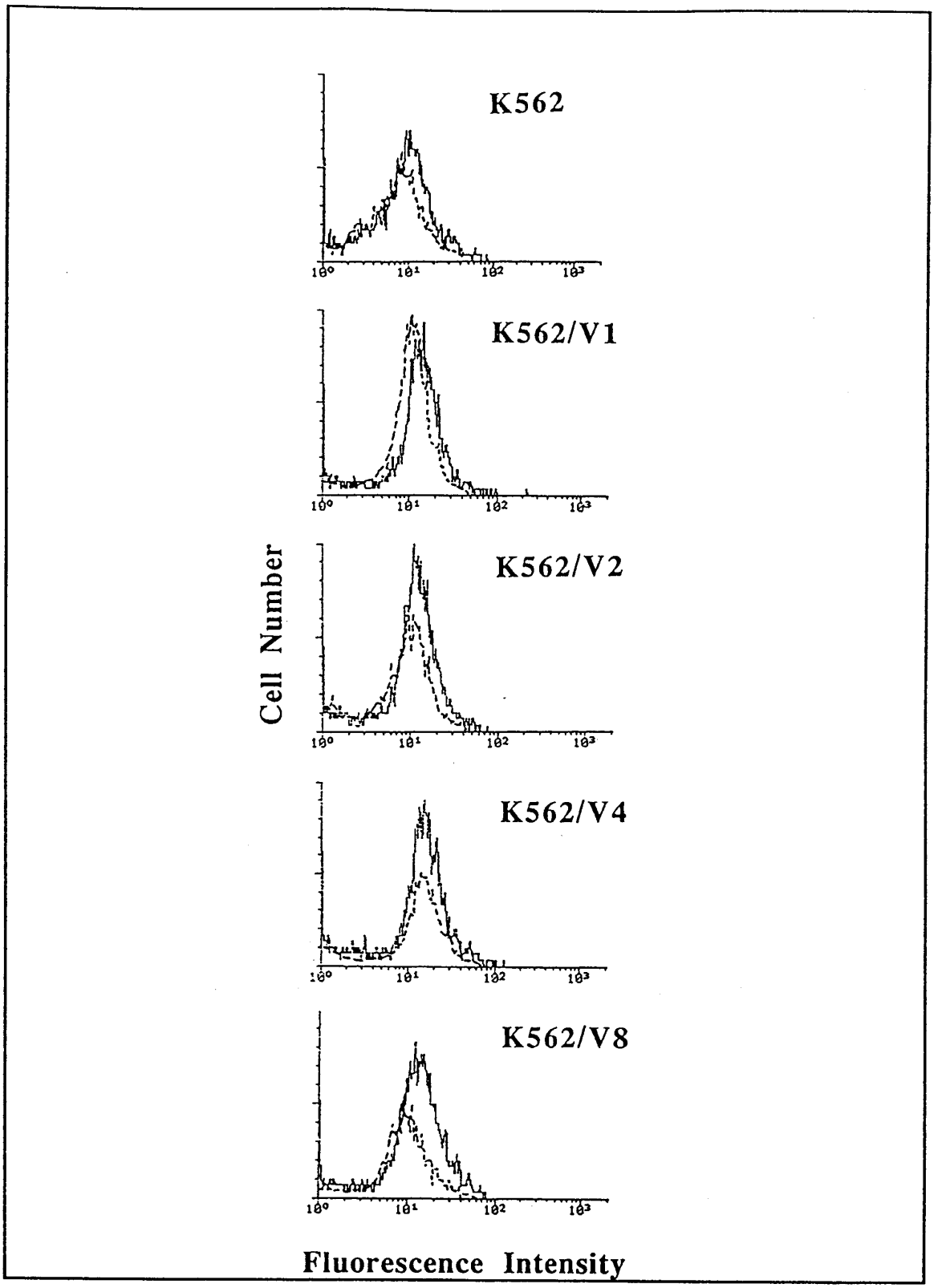

Figure 1.13. CD13 expression of K562 cells and vinblastine treated sublines. Cells were analysed for CD13 expression(—) by flow cytometry as described in *Materials and Methods 8* and the fluorescence profiles are compared to cells incubated with a negative control antibody(---). The experiment was repeated at least three times, and a representative result is shown.

CD13 expression in the K562/E10$_{15}$ clone (P-glycoprotein negative) (Fig. 1.14), indicating that CD13 expression was not linked to P-glycoprotein expression.

1.7.2 Glycophorin A

K562 cells expressed glycophorin A and expression was heterogeneous (Fig 1.15). Although most of the epirubicin resistant sublines had higher glycophorin A expression than the K562 cells, there was no correlation between P-glycoprotein and glycophorin A expression. Glycophorin A expression of the K562/E10$_4$ and K562/E10$_{15}$ clones was higher than the parental K562 cells (Fig. 1.17).

Glycophorin A expression showed a dose-dependent increase in the vinblastine resistant sublines, with expression increasing as the selecting concentration of vinblastine increased. In the K562/V8 subline, almost 100% of the cells expressed glycophorin A (Fig. 1.16), and this was confirmed by immunocytochemistry (data not shown).

1.7.3 Others Antigens

Expression of other antigens associated with differentiation were examined more extensively in the K562 cells and the K562/E15B and K562/V8 sublines. All of the cells examined, including the parental K562 cells, were negative for CD14 (Fig. 1.18) and CD34 expression (Fig. 1.19), and little change in CD61 expression (Fig. 1.20).

1.8 Cell Morphology

The drug resistant sublines did not increase in size or alter in shape compared to the parental K562 cells. This is represented in dot-plots obtained by flow cytometry, measuring forward-angle light scatter and side-angle light scatter of the K562, K562/E15B and K562/V8 cells (Fig. 1.21).

1.9 Discussion

The Western blot and immunocytochemical analysis with C219, the flow cytometry with MRK16, the drug cross-resistance profiles and the increases in drug sensitivity in the presence of verapamil are consistent with drug resistance in the K562 sublines being due to P-glycoprotein expression. The K562 cell line expressed the MDR phenotype in response to clinically achievable levels of epirubicin and vinblastine. This reflects the clinical situation where many leukaemias show increased *mdr*1 mRNA and P-glycoprotein following chemotherapy (Kuwazuru *et al*, 1990; Holmes *et al*, 1990; Shustik *et al*, 1991).

The ease with which cells became resistant and the heterogeneous expression of P-glycoprotein are two notable features with important clinical implications. Firstly

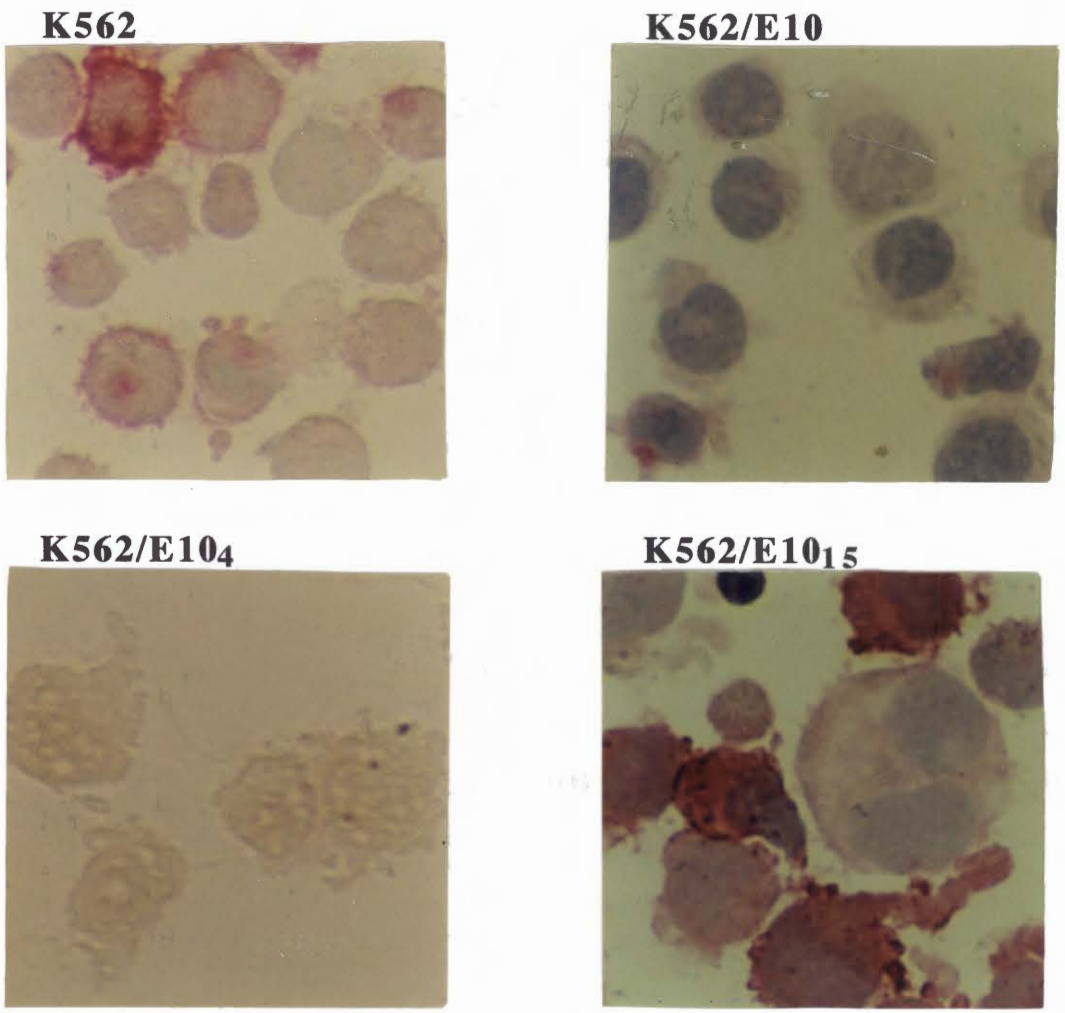

Figure 1.14. CD13 expression of E10 clones detected by immunocytochemistry. Cells were analysed for CD13 expression using immunocytochemistry as described in *Materials and Methods 8*. Magnification 200X.

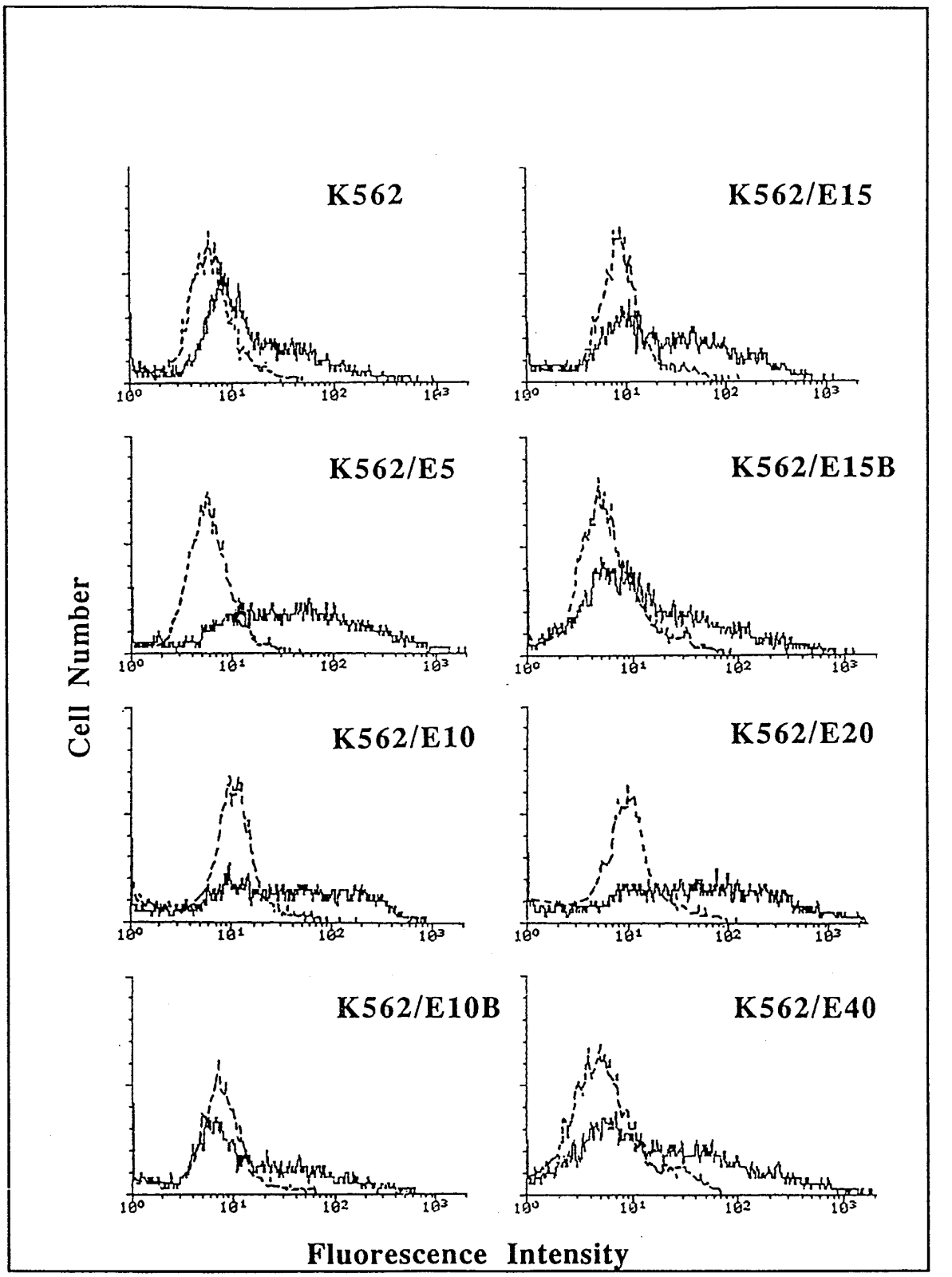

Figure 1.15. Glycophorin A expression of K562 cells and epirubicin treated sublines. Cells were analysed for glycophorin A expression(—) by flow cytometry as described in *Materials and Methods 8* and the fluorescence profiles are compared to cells incubated with a negative control antibody(---). The experiment was repeated at least three times, and a representative result is shown.

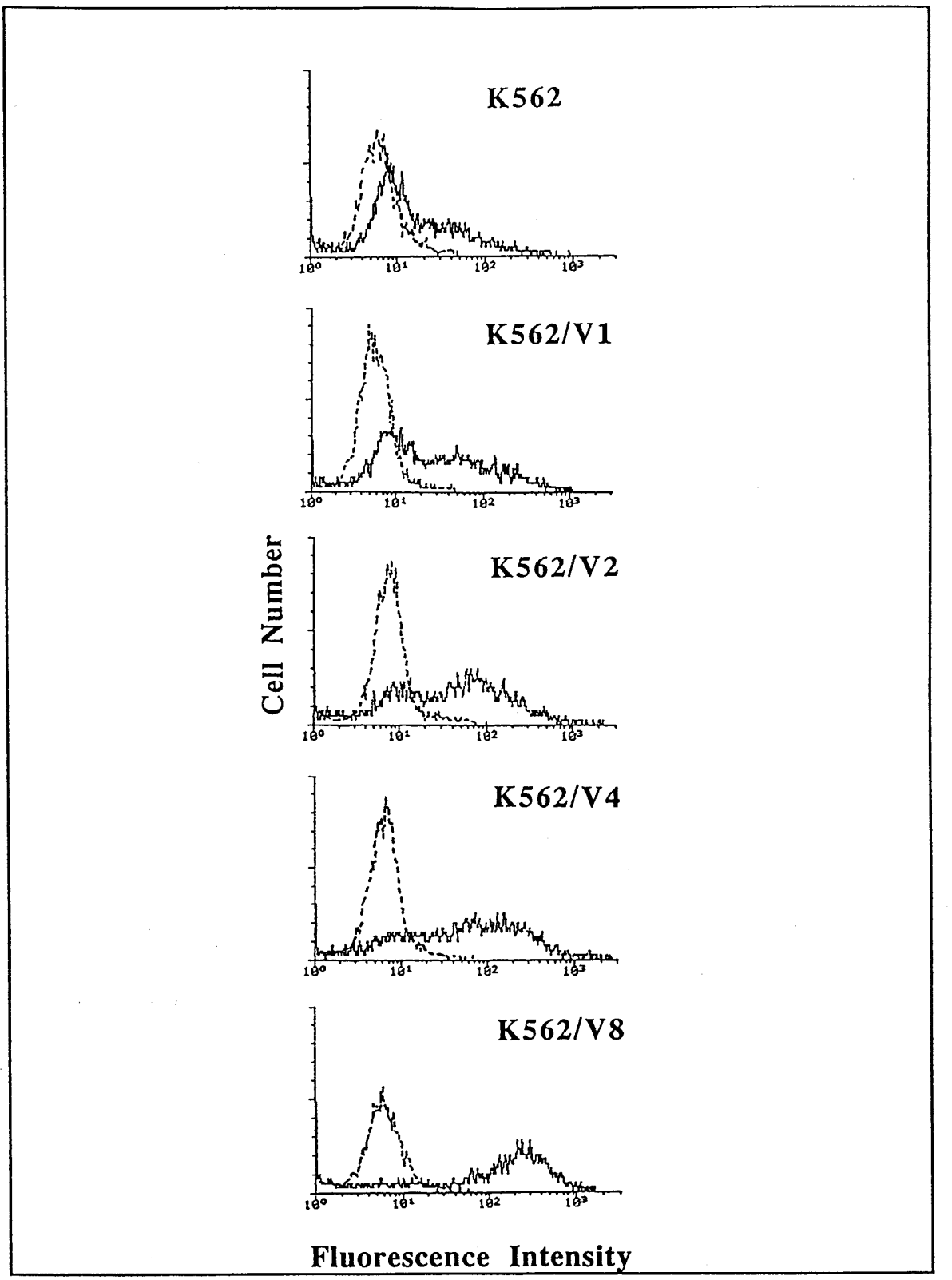

Figure 1.16. Glycophorin A expression of K562 cells and vinblastine treated sublines. Cells were analysed for glycophorin A expression(—) by flow cytometry as described in *Materials and Methods 8* and the fluorescence profiles are compared to cells incubated with a negative control antibody(---). The experiment was repeated at least three times, and a representative result is shown.

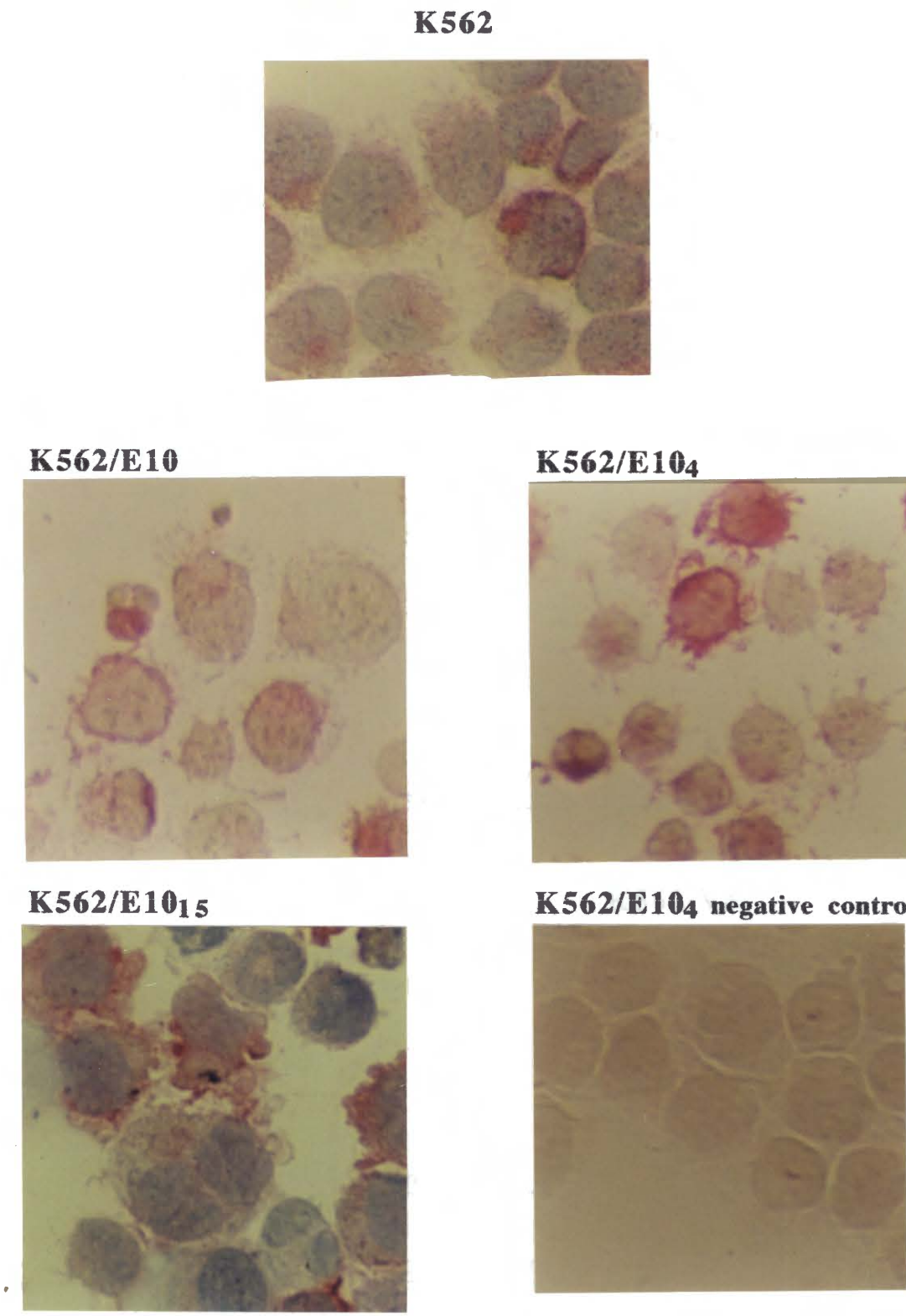

Figure 1.17. Glycophorin A expression of K562/E10 clones. Cells were cytospun and glycophorin A expression was determined using immunocytochemistry as described in *Materials and Methods 8*. Magnification 200X.

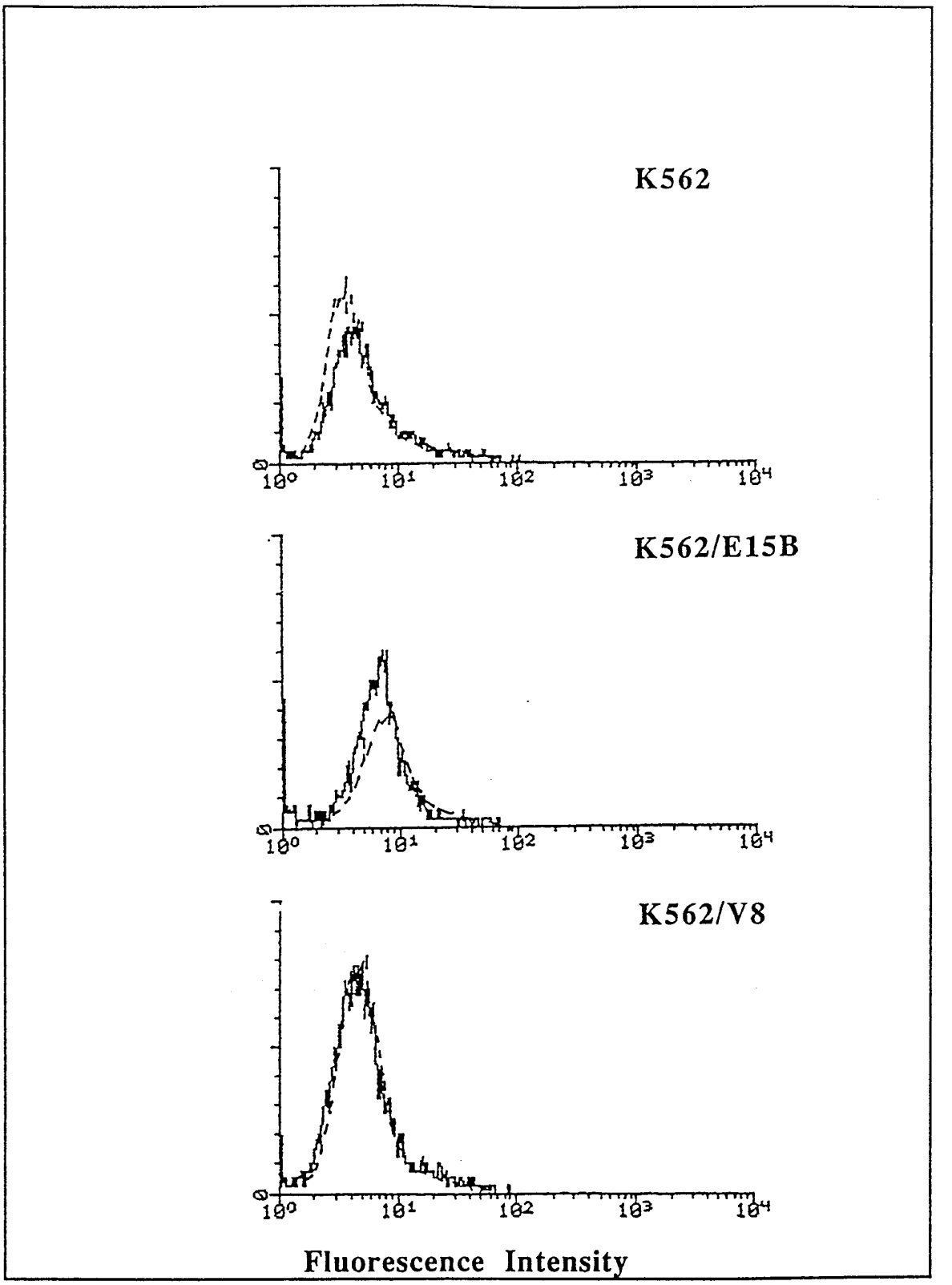

Figure 1.18. CD14 expression of K562 cells and drug resistant sublines. Cells were analysed for CD14 expression(–) by flow cytometry as described in *Materials and Methods 8* and the fluorescence profiles are compared to cells incubated with a negative control antibody(---). The experiment was repeated at least three times and a representative result is shown.

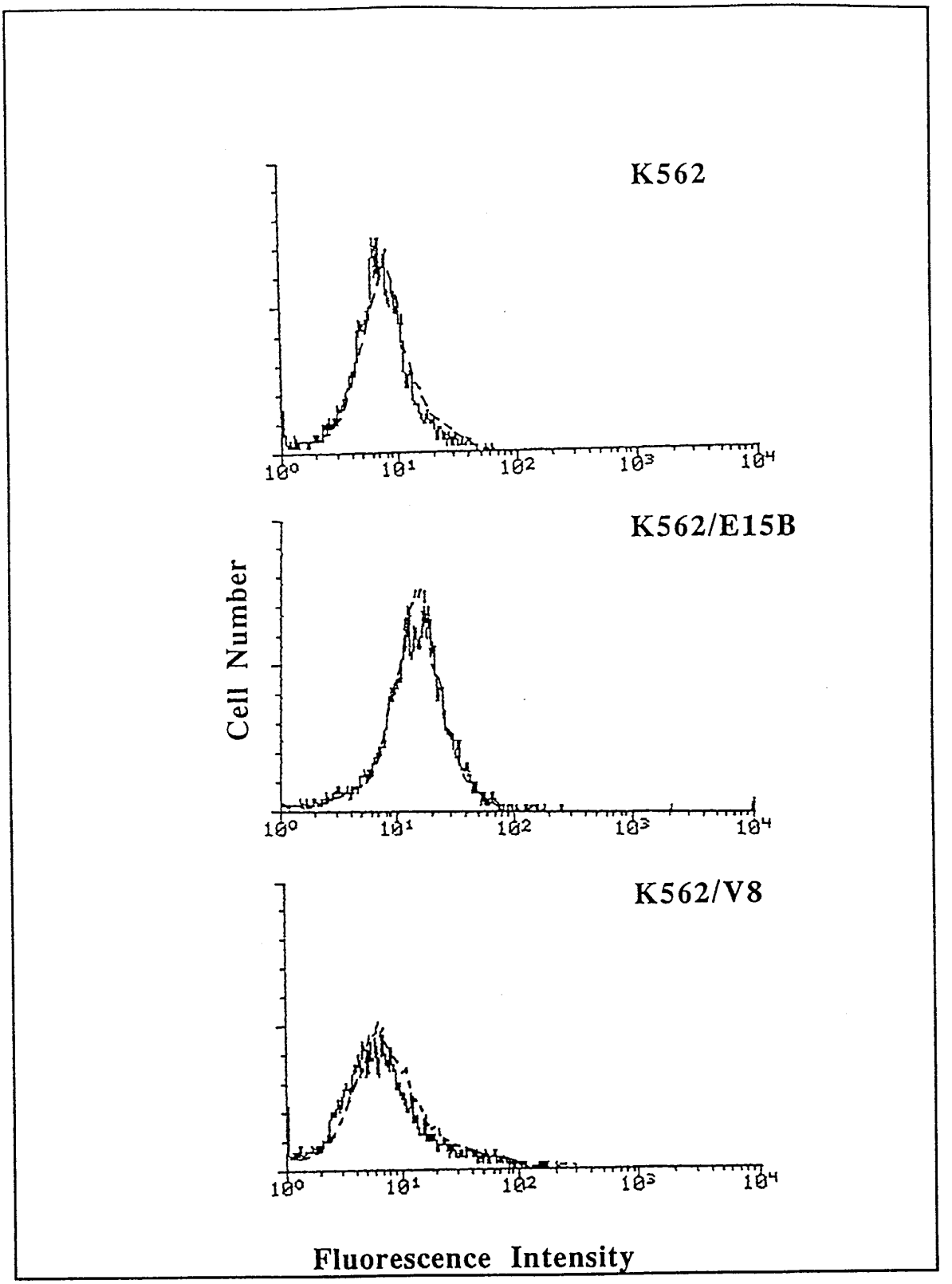

Figure 1.19. CD34 expression of K562 cells and drug resistant sublines. Cells were analysed for CD34 expression(–) by flow cytometry as described in *Materials and Methods 8* and the fluorescence profiles are compared to cells incubated with a negative control antibody(---). The experiment was repeated at least three times and a representative result is shown.

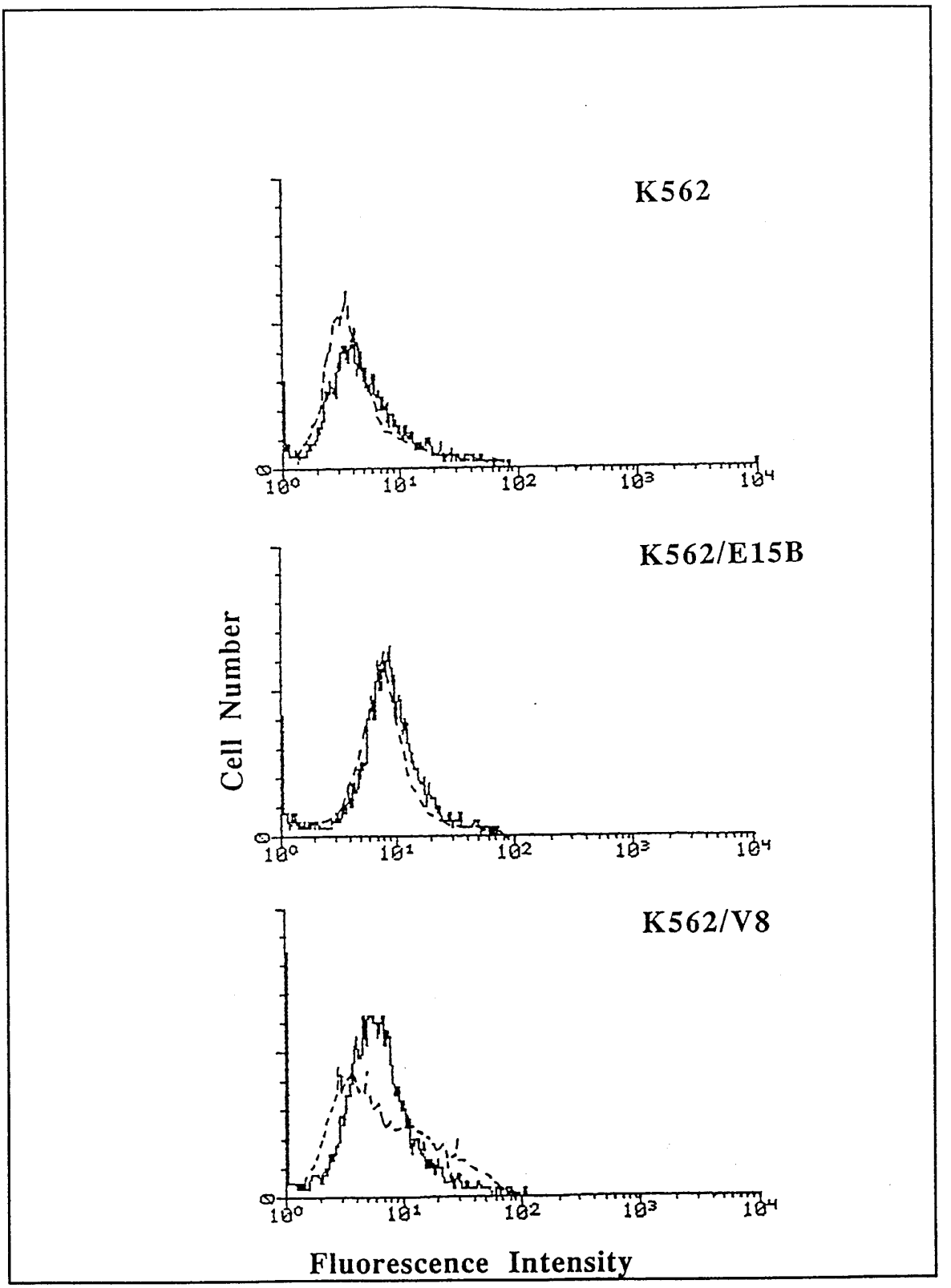

Figure 1.20. CD61 expression of K562 cells and representative drug resistant sublines. Cells were analysed for CD61 expression(—) by flow cytometry as described in *Materials and Methods 8* and the fluorescence profiles are compared to cells incubated with a negative control antibody(---). The experiment was repeated at least three times and a representative result is shown.

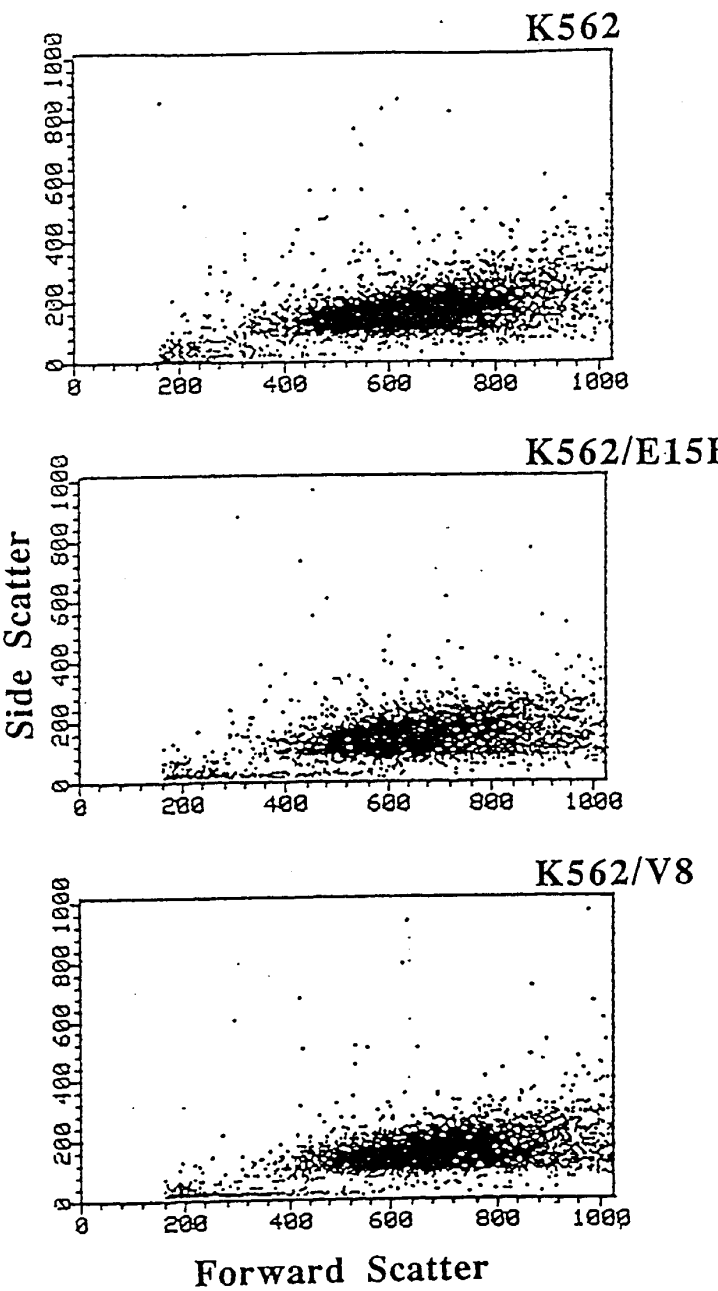

Figure 1.21. Flow cytometric analysis of cell size and granularity of K562 cells and drug resistant sublines. Forward and side scatter of light were plotted in a dot-plot format using the LYSYS II software.

exposure to low, but clinically relevant levels of drug (1 ng/ml vinblastine and 10 ng/ml epirubicin) resulted in some cells expressing high levels of P-glycoprotein which were highly cross resistant. The most resistant subline, K562/E15B, showed the highest expression of P-glycoprotein, the most cross-resistance to other drugs, and the most sensitisation to drugs by verapamil, while being treated with only 15 ng/ml epirubicin, which is a clinically achievable concentration.

The question must be addressed as to whether expression of P-glycoprotein was due to selection of a P-glycoprotein-expressing sub-population of K562 cells or induction of P-glycoprotein in non-expressing cells. K562 cells have been reported to express low levels of P-glycoprotein (Hamada and Tsuruo, 1988b). Further low levels of *mdr*1 mRNA have been detected in K562 cells although without detectable P-glycoprotein on their cell surface (Chaudhary and Roninson, 1993). However, Chaudhary and Roninson (1993) demonstrated an increase in the Rh123 "dull" population of K562 cells after treatment with drugs for 12 to 72 hours, which was accompanied by a 2 to 3-fold increase in resistance to vinblastine. Furthermore, the P-glycoprotein induced was stable for up to 6 weeks. The authors concluded that induction of P-glycoprotein was associated with visible cell damage, as the levels of drug used to treat the cells were approximately IC_{90}s. However, the drug concentrations used in the development of these drug resistant K562 sublines were far lower (the IC_{50} for epirubicin is 290 nM and the IC_{50} for vinblastine is 3.5 nM). The drug resistant K562 cells were treated for approximately 12 weeks with multiple drug exposures before resistance was examined, and it is likely that low level stable induction of resistance remained after one drug treatment, and was increased during further treatments. These results therefore support the induction of P-glycoprotein. However, the low concentrations of drugs used in these treatments together with stable expression of P-glycoprotein at 10 ng/ml (17 nM) epirubicin and 1 ng/ml (1.1 nM) vinblastine do not support induction of resistance being associated with cellular damage. Rather, a more likely hypothesis is that the chemotherapeutic drugs induced P-glycoprotein expression by acting on elements of the promoter region of the mdr1 gene, such as the heat shock response element, as demonstrated by Chin et al, 1990a.

The exception to this is the K562/E40 subline. Treatment of the K562 cells with 40 ng/ml epirubicin was much more cytotoxic to the cells than treatment at the lower doses (20 ng/ml or less) While after treatment at lower doses, cells recovered rapidly upon removal of drug (<1 week), after treatment at 40 ng/ml, few cells survived, and these were grown for retreatment (approximately 4 weeks for recovery). Development of this subline took considerably longer than at the lower doses, and was therefore significantly different from development of the other sublines in which P-glycoprotein may be induced by drug treatment. The K562/E40 subline may represent selection rather than induction

of drug resistance, and this may be the reason for the lower P-glycoprotein expression in this subline.

The resistance induced was stable, as demonstrated by the K562/E10$_4$ clone which was 5-fold more resistant to epirubicin than the parental K562/E10 subline (Table 1.3). Furthermore, such drug exposure can produce heterogeneous expression of P-glycoprotein, as demonstrated by the K562/E10 and K562/E10B sublines in which 5-10% of the cells expressed P-glycoprotein as detected by immunocytochemistry. Again, the stability of this phenotype was confirmed by cloning, which produced both P-glycoprotein expressing and non-expressing clones. Such heterogeneity would be difficult to interpret in blood samples, and could explain some of the variation in reports of a correlation between P-glycoprotein detection and response to chemotherapy (Ma *et al*, 1987; Weide *et al*, 1990).

MRK16 labelling is frequently used clinically for determination of P-glycoprotein expression. Initial results with this antibody were difficult to interpret, and neuraminadase, which has been used to increase reactivity with MRK16 (Cumber *et al*, 1990), had no effect (data not presented). Chaudhary and Roninson (1991) reported that high concentrations of MRK16 were required to obtain sufficient sensitivity in analysis. Similarly, it was found that higher levels of MRK16 (2 $\mu g/10^5$ cells) than most monoclonal antibodies (eg glycophorin A: 0.46 $ng/10^5$ cells) were needed to obtain adequate results. This could again lead to problems in the detection of P-glycoprotein in clinical samples if lower amounts of MRK16 were used. Furthermore, the K562/E40 subline showed no increase in P-glycoprotein expression with MRK16 although it was positive with C219, which also highlights the problems which may be encountered when detecting low-level P-glycoprotein expression in clinical samples.

The decreases in Rh123 accumulation in the epirubicin and vinblastine treated sublines confirm that P-glycoprotein was functional in the drug resistant sublines (Fig.1.11). However, Rh123 accumulation was not decreased in the K562/E10B subline, suggesting that the P-glycoprotein expression detected by immunocytochemistry was not functional or that the method was not sufficiently sensitive. Further, Rh123 accumulation was not significantly increased by verapamil, except in the K562/E15 subline, even though the drug resistant sublines were sensitised to epirubicin and vinblastine by verapamil in cytotoxicity assays, suggesting that cytotoxicity assays are more sensitive. The K562/E40 subline, which expressed very little P-glycoprotein showed the greatest decrease in Rh123 accumulation. These results therefore highlight that problems may be encountered when using Rh123 accumulation to detect drug resistance clinically, as has been proposed by Ludescher *et al* (1991).

It still remains to be determined whether or not low amounts of P-glycoprotein contribute to the resistance phenotype in leukaemic cells of patients, therefore it was

chosen to look at P-glycoprotein expression itself and not *mdr*1 mRNA. More than one method of detection was chosen as there has been controversy as to which detection method is most reliable (Cumber *et al*, 1990; Ludescher *et al*, 1992). The use of immunocytochemistry and flow cytometry allowed the heterogeneity of expression to be detected. This heterogeneity would not have been detected if only Western blot analysis was used, which involves bulk preparation of cells and does not facilitate examination of individual cells in a population. Detection of P-glycoprotein in patient samples involves detection of low levels of expression, in which not every cell expresses detectable levels of P-glycoprotein. These sublines are also very similar to cells analysed clinically, and this study also highlights the need to use several methods of P-glycoprotein detection.

Many reports have linked expression of differentiation antigens in leukaemia with prognosis (Pilkington *et al*, 1989; Hernandez *et al*, 1991), and P-glycoprotein expression has been linked to a stem cell phenotype, as defined by the human progenitor cell antigen CD34 (Chaudhary and Roninson, 1991; List *et al*, 1991). CD34 expression in AML has been found to be significantly associated with resistance to remission induction treatment (Guinot *et al*, 1991) and is also related to poor prognosis in ALL (Vaughan *et al*, 1988).

Multidirectional differentiation and expression of CD34 has previously been demonstrated in the HEL erythroleukaemia cell line after treatment with differentiating agents (Hooper *et al*, 1991). However, the K562 cells do not express CD34, nor did the drug resistant sublines in response to vinblastine or epirubicin treatment, although they did express P-glycoprotein. This suggests that coordinate expression of CD34 and P-glycoprotein may be limited to more primitive cells.

The K562 cells were however induced to express a more differentiated phenotype in response to drug treatment. The antigen CD13, expressed on committed myeloid progenitor cells of the granulocyte-monocyte lineage has been associated with reduced remission rates in AML (Griffin *et al*, 1986), and decreased expression at first relapse (Thomas *et al*, 1992). While expression of CD13 was generally lower in most of the epirubicin treated sublines, the K562/E15B subline showed some cells which were highly reactive with CD13, indicating myeloid differentiation. The K562/V2, K562/V4 and K562/V8 sublines all expressed more CD13 than the parental K562 cells, which increased with the concentration of vinblastine used to treat the cells, suggesting that the development of MDR was paralleled by an increase in CD13. However, the K562/E10_4 clone, did not express CD13, although exhibiting intense and homogeneous P-glycoprotein expression, and the K562/E10_{15} clone expressed high levels of CD13 but no P-glycoprotein. Thus CD13 expression is not linked to P-glycoprotein expression in these cells, and the poor prognosis associated with expression of CD13 is probable not due to co-expression of P-glycoprotein.

Glycophorin A expression also changed with drug treatment. As with CD13, there were no correlations between P-glycoprotein expression and glycophorin A expression in the epirubicin treated cells. The vinblastine treated cells clearly showed dose-dependent increases in expression of glycophorin A, correlating with increases in P-glycoprotein expression.

Overall, these results suggest that vinblastine induces aberrant and multidirectional differentiation, with increased expression of both myeloid and erythroid antigens, which is paralleled by the development of drug resistance.

Thus the MDR phenotype and haematopoietic differentiation were both induced by treatment with low, clinically relevant levels of epirubicin and vinblastine *in vitro*. This study provides a model for gaining further understanding of the induction mechanisms of MDR and cell differentiation, and highlights the problems encountered clinically when attempting to detect P-glycoprotein and low-level drug resistance.

2 DEVELOPMENT AND CHARACTERISATION OF DRUG RESISTANCE IN U937 CELLS.

2.1 Introduction

U937 cells are a monocyte-like cell line derived from a histiocytic lymphoma (Sündstrom and Nillson, 1976). In order to compare the emergence of drug resistance in the K562 cells with another human leukaemia cell line, U937 cells were treated with low, clincally relevant levels of epirubicin (10 and 15 ng/ml) and vinblastine (1 and 8 ng/ml). To date there are no reports of drug resistant U937 cells.

U937 cells are reported to be a committed monocyte progenitor. U937 cells express F_c receptors, and are capable of phagocytosis and other normal monocyte functions (Harris and Ralph, 1985). U937 cells express CD13 (granulocyte antigen) and CD14 (monocyte-macrophage antigen). They do not express glycophorin A (erythroid antigen), CD34 (stem cell antigen) or CD61 (megakaryocyte antigen). As such, they serve as a leukemic model of monocytic development, and provide a useful comparison to the K562 cells, which are an uncommitted progenitor cell.

2.2 Characterisation of Drug Resistance

After 6 drug treatments, growth of the drug resistant U937 sublines was stable at the concentration of drug at which the cells were treated. Resistance to the selecting drug was then assessed. The U937/E10 subline was 5.6-fold resistant to epirubicin and the U937/E15 subline was 7.5-fold resistant to epirubicin (Fig. 2.1A). The U937/V1 subline was not resistant to vinblastine, while the U937/V8 subline was 22-fold resistant to vinblastine (Fig. 2.1B). As the U937/V1 subline was not resistant to vinblastine they were not further assessed for cross-resistance to other drugs.

Cross-resistance to other drugs was then examined, and is summarised in Table 2.1. The U937/E10 subline was 3.5- to 6.5-fold resistant to doxorubicin, idarubicin, vinblastine, vincristine and taxol but were 22.0-fold resistant to VP-16. The U937/E15 subline was 4.9- to 15.0-fold resistant to doxorubicin, idarubicin, vinblastine vincristine and taxol, but were 19.4-fold resistance to VP-16. In general the U937/V8 subline was more resistant than the epirubicin resistant sublines. The U937/V8 subline was 16-fold resistant to epirubicin, 17.6-fold resistant to doxorubicin, 5.2-fold to idarubicin, 41.8-fold resistant to taxol, 13.4-fold resistant to VP-16, and was very resistant to the *Vinca* alkaloids, being 20-fold resistant to vinblastine and 78-fold resistant to vincristine.

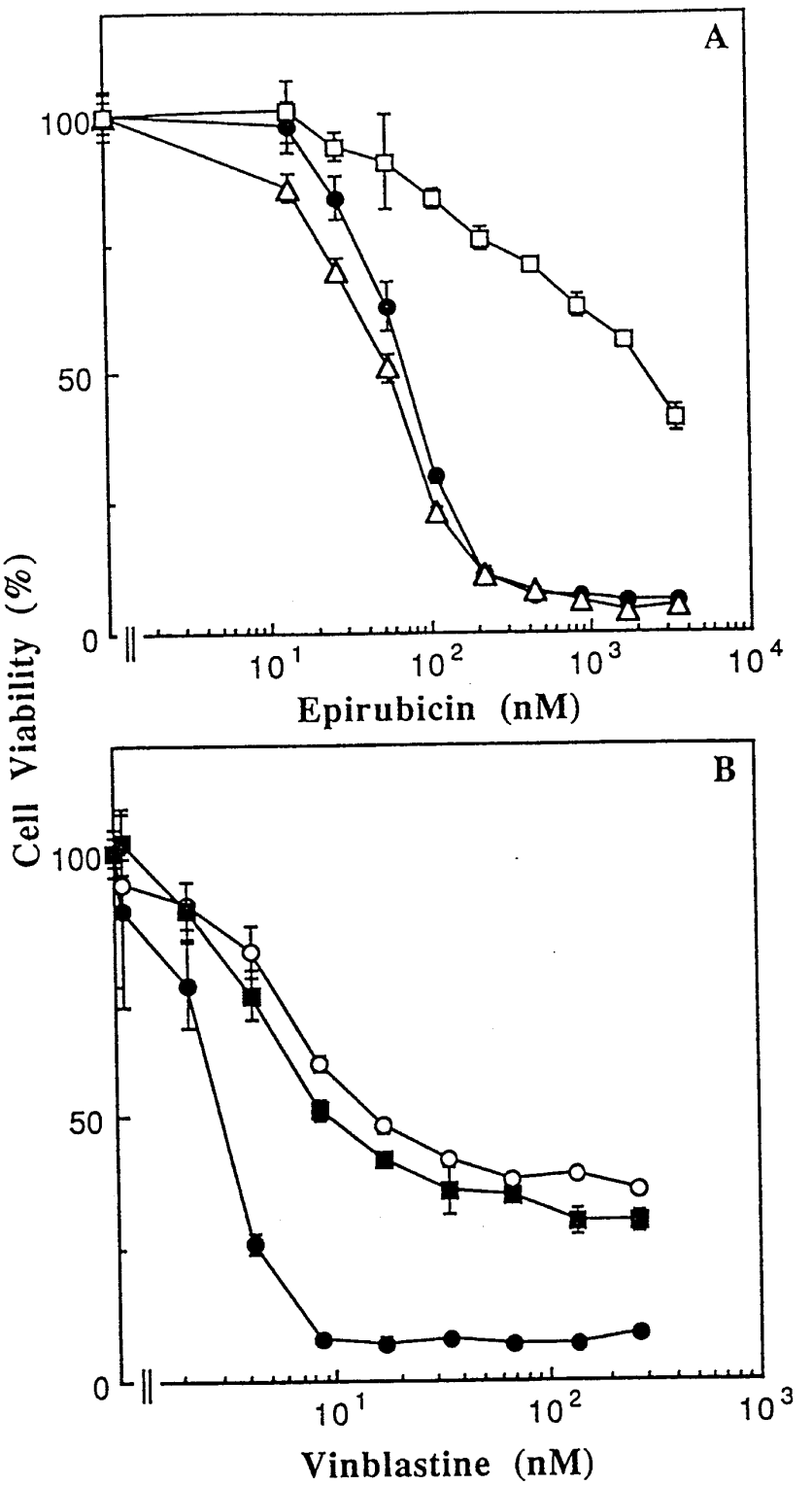

Figure 2.1. Resistance to the selecting drug in U937 cells and drug resistant sublines. Cells were incubated with serial dilutions of A. epirubicin or B. vinblastine for 3 days, after which cell viability was determined using the MTT assay as described in *Materials and Methods 3*. U937(●), U937/E10(o), U937/E15(■), U937/V1(Δ) and U937/V8(□). *Points*, mean of triplicate determinations; *error bars*, standard deviations. The experiment was repeated at least three times, and a representative result is shown.

Table 2.1 Relative resistance of drug resistant U937 sublines to MDR drugs .

	Relative Resistance[a]			
DRUG	U937	U937/E10	U937/E15	U937/V8
epirubicin	1.0	5.6	7.5	16.0
doxorubicin	1.0	4.9	11.1	17.6
idarubicin	1.0	6.5	7.1	5.2
vinblastine	1.0	3.5	4.9	20.0
vincristine	1.0	4.4	8.8	78.0
taxol	1.0	3.8	15.0	41.8
VP-16	1.0	22.0	19.4	13.4

a The results are calculated from IC_{50}s taken from at least three experiments. Relative resistance is calculated from the IC_{50} of the resistant subline divided by the IC_{50} of the parental U937 cells.

The drug resistant sublines also showed cross-resistance to drugs not normally associated with MDR. They were cross-resistant to cisplatinum, the alkylating agent chlorambucil and were slightly resistant to the antifolate methotrexate, as summarised in Table 2.2. Levels of resistance to cisplatinum and chlorambucil were similar in all 3 cell lines, ranging from 4.3-fold to 6.4-fold. The U937/E10 subline was only 1.8-fold resistant to methotrexate, while the U937/E15 and U937/V8 subline were both 2.5-fold resistant to methotrexate.

Table 2.2. Relative resistance of drug resistant U937 sublines to non-MDR drugs.

	Relative Resistance[a]			
DRUG	U937	U937/E10	U937/E15	U937/V8
cisplatinum	1.0	4.3	3.7	3.3
chlorambucil	1.0	5.7	4.3	5.4
methotrexate	1.0	1.8	2.5	2.5

a The results are calculated from IC_{50}s taken from at least three experiments. Relative resistance is calculated from the IC_{50} of the resistant subline divided by the IC_{50} of the parental U937 cells.

2.3 P-glycoprotein Expression

P-glycoprotein expression was determined by Western blot analysis using the monoclonal antibody C219. The parental U937 cells did not express P-glycoprotein. P-glycoprotein expression was detected in all resistant sublines, including the U937/V1 subline, which was not resistant to vinblastine. However, P-glycoprotein expression was lower in the U937/V1 subline than the other three sublines (Fig. 2.2).

2.4 Reversal of Resistance

The ability of verapamil to sensitise drug resistant sublines to cytotoxic drugs was examined, and results are summarised in Table 2.3. Reversal was considered to be significant if greater than 2-fold and only if greater in the resistant subline than in the parental U937 cells. Verapamil partially sensitised the resistant sublines to epirubicin

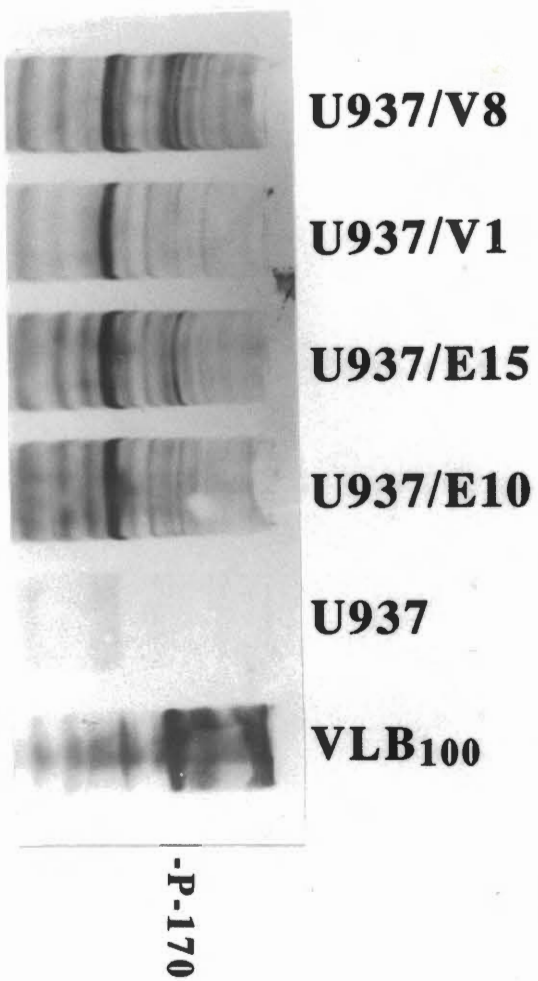

Figure 2.2. Western blot analysis of P-glycoprotein expression in U937 cells and drug resistant sublines. Plasma membrane fractions were prepared and analysed as described in *Materials and Methods 6.1*. The position of P-glycoprotein (P170) is indicated.

(Fig. 2.3), and to doxorubicin (Table 3.2) but did not affect resistance to the anthracycline idarubicin in any of the resistant sublines (Table 3.2).

Verapamil sensitised all three resistant sublines to vinblastine, increasing vinblastine cytotoxicty 6.0-fold in the U937/E10 subline, 11.5-fold in the U937/E15 subline, and 20-fold in the U937/V8 subline, fully returning them all to the level of the parental U937 cells (Fig. 2.4). The parental U937 cells were also sensitised to vinblastine by verapamil (2.2-fold). Verapamil also sensitised the U937/E10 (2.8-fold), U937/E15 subline (6.0-fold) and the U937/V8 subline (3.7-fold) to taxol (Fig. 2.5). Verapamil had no effect on resistance to VP-16 (Fig. 2.6). Resistance to cisplatinum was not reversed by verapamil (data not shown).

Table 2.3. The effect of verapamil (VER) on drug resistance in U937 cells and drug treated sublines.

		IC_{50} (μM)			
DRUG	VER	U937	U937/E10	U937/E15	U937/V8
epirubicin	-	0.106±0.052	0.618±0.278	0.983±0.197	1.783±0.526
	+	0.048±0.002	0.261±0.078	0.162±0.073*	0.228±0.069*
		(2.2)[b]	(2.4)	(6.1)	(7.8)
doxorubicin	-	0.105±0.050	0.518±0.284	1.167±0.605	1.850±0.360
	+	0.063±0.021	0.350±0.391	0.650±0.568	0.583±0.625
		(1.7)	(1.5)	(1.8)	(3.2)
idarubicin	-	0.050, 0.090	0.650, 0.330	0.450, 0.360	0.450, 0.180
	+	0.113, 0.080	0.900, 0.330	0.600, 0.360	0.450, 0.190
vinblastine	-	0.002±0.0001	0.009±0.005	0.011±0.010	0.047±0.027
	+	0.001±0.0006*	0.002±0.0003	0.001±0.0008	0.003±0.002
		(2.0)	(4.5)	(11.)	(15.7)
taxol	-	0.005±0.002	0.018±0.003	0.071±0.008	0.198±0.023
	+	0.003± 0.000	0.007± 0.001*	0.008± 0.001*	0.021± 0.001*
		(1.7)	(2.8)	(8.9)	(9.4)
VP-16	-	0.860± 0.272	19.100± 5.197	16.700± 9.311	11.500± 2.549
	+	0.485± 0.190	13.000± 8.485	15.084± 16.381	13.000± 7.071
		(1.8)	(1.5)	(1.1)	(0.9)

a Results are the mean of ≥two separate experiments± standard deviation. Where only 2 results are available, individual results are given.
b Numbers in parentheses indicate fold-reversal which was calculated by dividing the IC_{50} of the cells in the absence of verapamil by the IC_{50} of the cells in the presence of verapamil.
* Indicates $p \leq 0.05$ using the student's t-test.

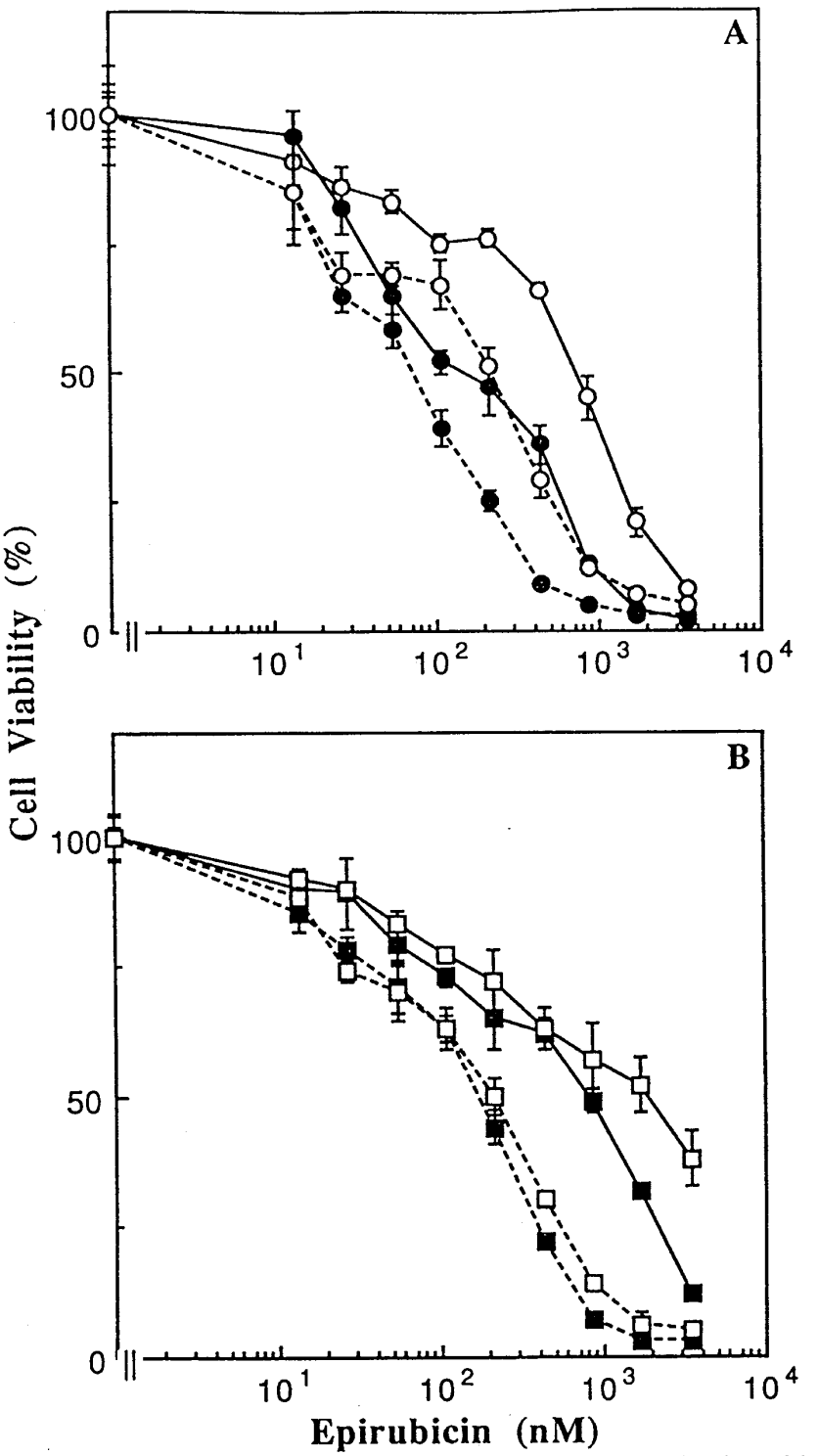

Figure 2.3. The effects of verapamil on epirubicin cytotoxicity in U937 cells and drug resistant sublines. Cells were incubated with serial dilutions of epirubicin in the presence(---) and absence(—) of 10 μM verapamil for 3 days, after which cell viability was determined using the MTT assay as described in *Materials and Methods 3*. A. U937(●), U937/E10(o), B. U937/E15(■) and U937/V8(□). *Points*, mean of triplicate determinations; *error bars*, standard deviations. The experiment was repeated at least three times, and a representative result is shown.

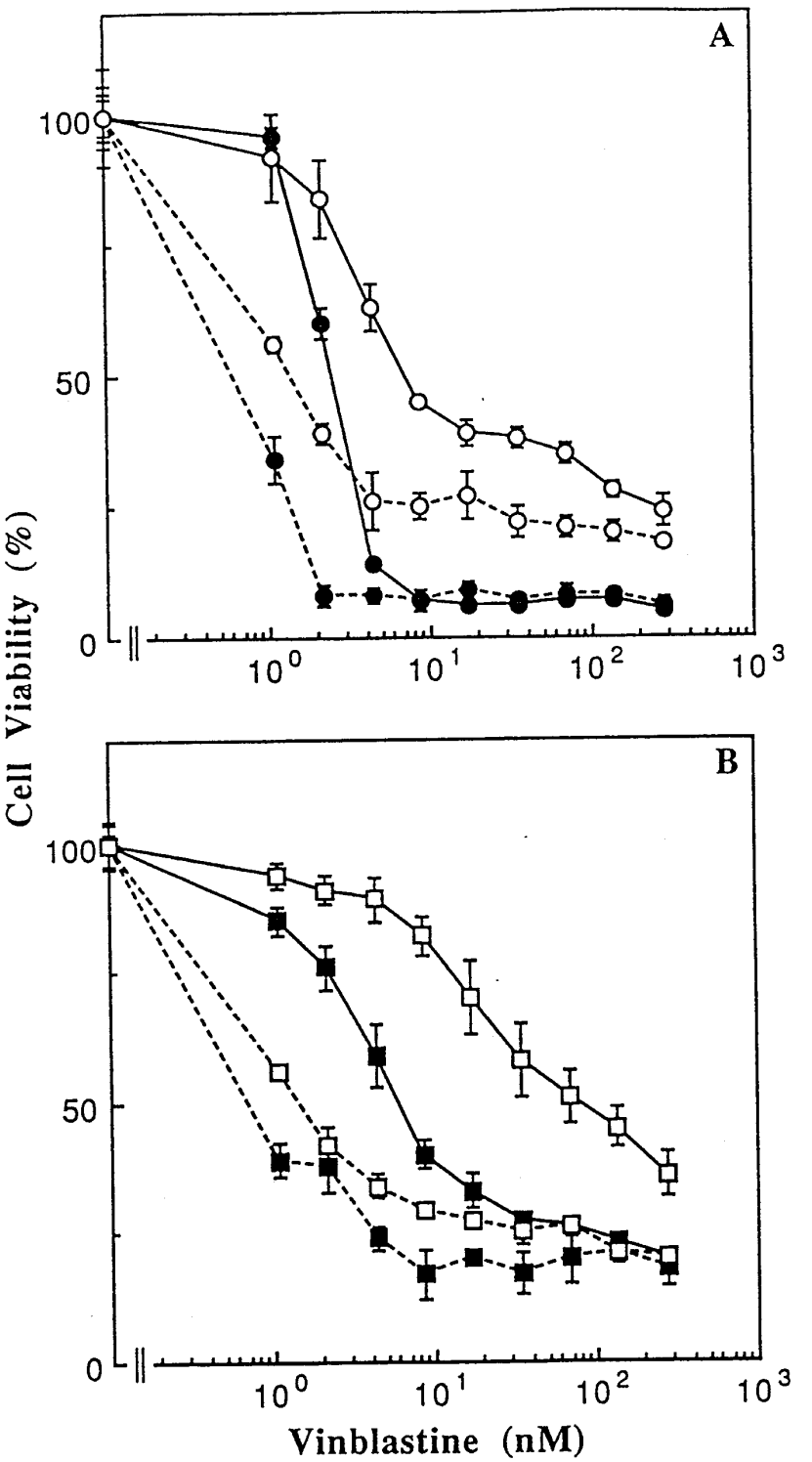

Figure 2.4. The effects of verapamil on vinblastine cytotoxicity in U937 cells and drug resistant sublines. Cells were incubated with serial dilutions of vinblastine in the presence(---) and absence(—) of 10 μM verapamil for 3 days, after which cell viability was determined using the MTT assay as described in *Materials and Methods 3*. A. U937(●), U937/E10(o), B. U937/E15(■) and U937/V8(□) cells. *Points*, mean of triplicate determinations; *error bars*, standard deviations. The experiment was repeated at least three times, and a representative result is shown.

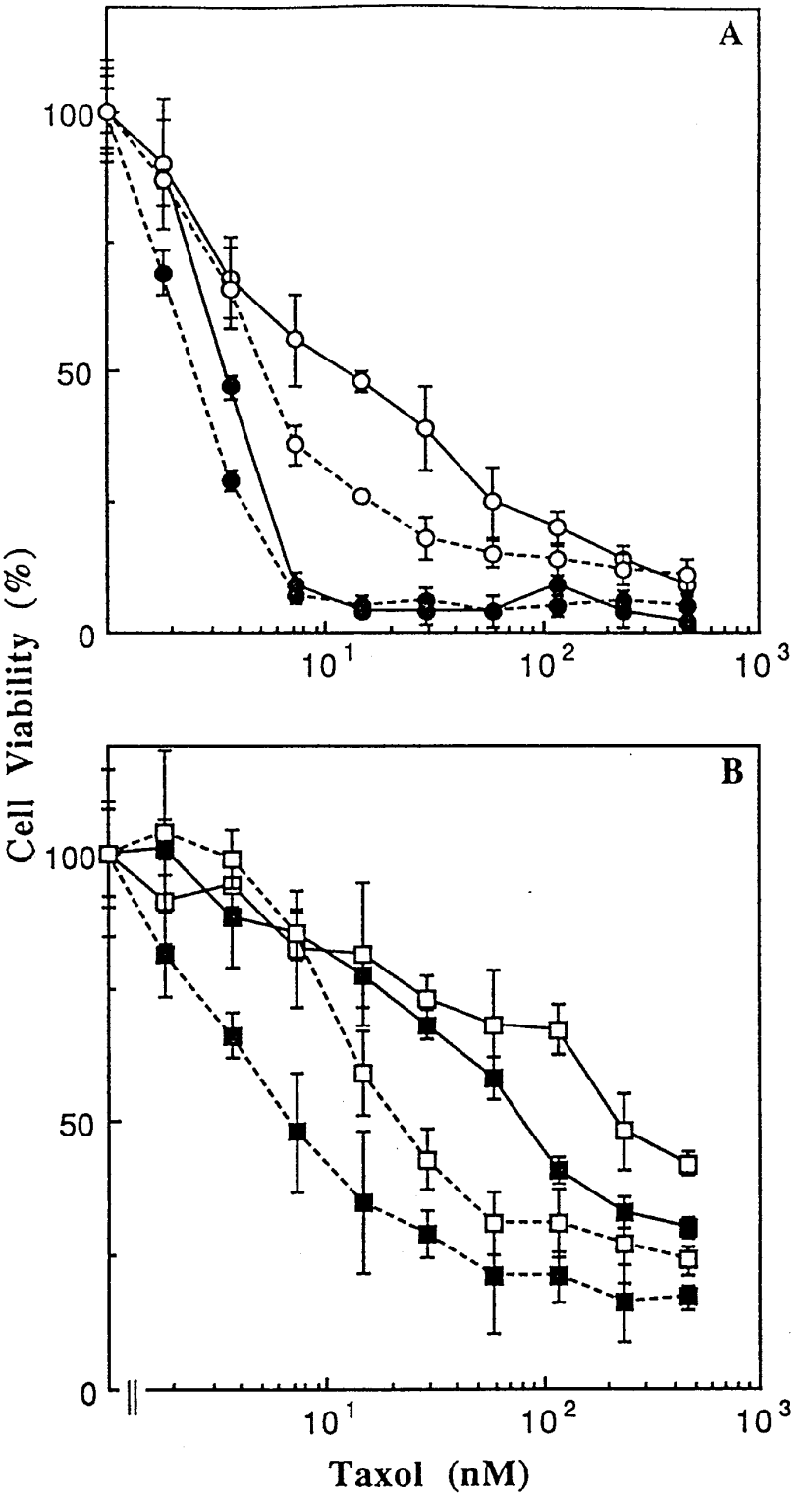

Figure 2.5. The effects of verapamil on taxol cytotoxicity in U937 cells and drug resistant sublines. Cells were incubated with serial dilutions of taxol in the presence(---) and absence(—) of 10 μM verapamil for 3 days, after which cell viability was determined using the MTT assay as described in *Materials and Methods 3*. A. U937(●), U937/E10(o), B. U937/E15(■) and U937/V8(□). *Points*, mean of triplicate determinations; *error bars*, standard deviations. The experiment was repeated at least three times, and a representative result is shown.

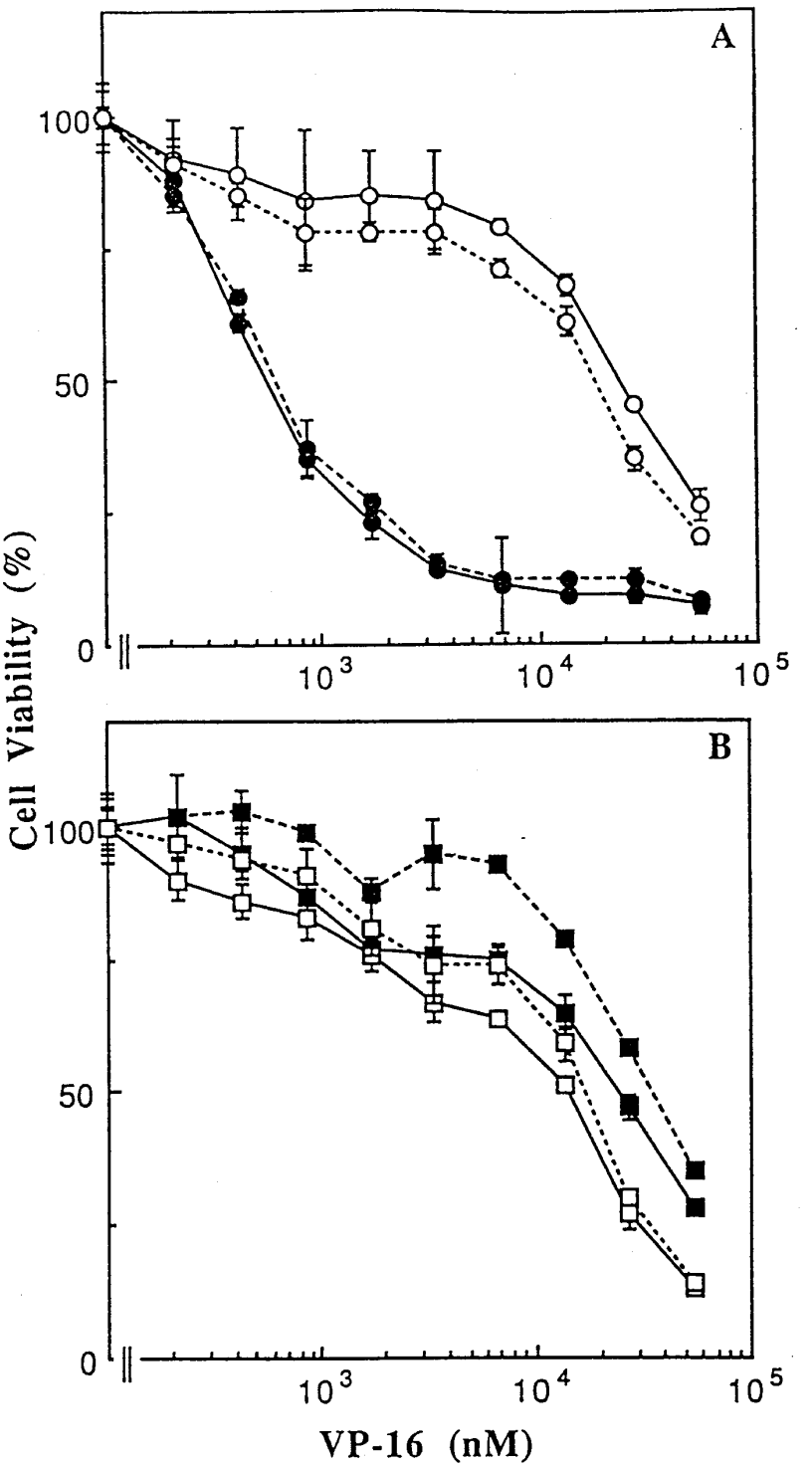

Figure 2.6. The effects of verapamil on VP-16 cytotoxicity U937 cells and drug resistant sublines. Cells were incubated with serial dilutions of VP-16 in the presence(---) and absence(—) of 10 μM verapamil for 3 days, after which cell viability was determined using the MTT assay as described in *Materials and Methods*. A. U937(●), U937/E10(o), B. U937/E15(■) and U937/V8(□) cells. *Points*, mean of triplicate determinations; *error bars*, standard deviations. The experiment was repeated at least three times, and a representative result is shown.

2.5 Effect of Buthionine Sulphoximine

Alterations in glutathione metabolism have been demonstrated in many anthracyline resistant cells. To determine the role of glutathione metabolism in drug resistance, the effect of BSO, an inhibitor of glutathione synthesis, was examined. The cytotoxicity of BSO in the U937 cells and the three drug resistant sublines was first examined. BSO had little effect on cell viability at concentrations up to 500 μM BSO (Fig. 2.7). 50μM BSO was chosen for treatment as this concentration has previously been demonstrated to deplete glutathione (Neumann *et al*, 1992). Cells were considered to be sensitised by BSO only if sensitisation was greater than 2-fold, and as long as sensitisation in the resistant subline exceeded that of the parental U937 cells. The results are summarised in Table 2.4.

BSO partially sensitised all three resistant sublines to doxorubicin (Fig. 2.8). However, the drug resistant sublines were not sensitised to epirubicin, as BSO produced a greater increase in epirubicin cytotoxicity in the parental U937 cells (3.5-fold) than in the drug resistant sublines, which were sensitised between 1.8 and 2.7-fold. BSO did not alter resistance to idarubicin (data not shown).

The drug resistant sublines were sensitised to vinblastine, with a 4.2-, 3.4- and 2.7-fold decrease in resistance to vinblastine in the U937/E10, U937/E15 and U937/V8 sublines respectively, although the decreases were not significantly significant (Table 2.4). Only the U937/E10 and the U937/E15 sublines were partially sensitised to VP-16 by BSO.

BSO partially sensitised all three resistant sublines to chlorambucil. Only the U937/E10 and U937/E15 cells were sensitised to cisplatinum by BSO (2.3- and 2.6-fold respectively). BSO therefore partially sensitised the resistant sublines to doxorubicin, vinblastine and chlorambucil, and sensitised the epirubicin treated sublines to cisplatinum.

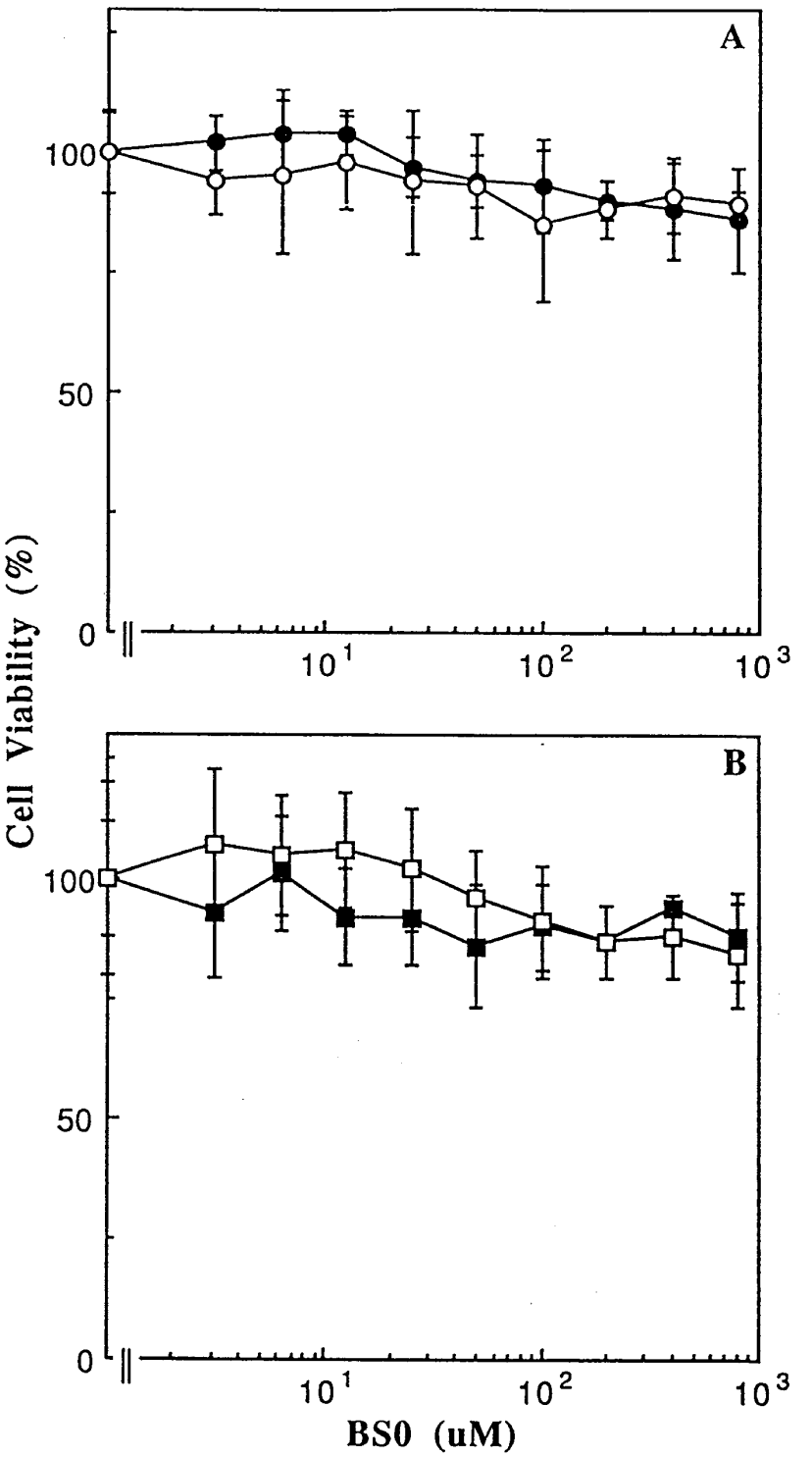

Figure 2.7. The effect of BSO on cell viability in U937 cells and the drug resistant U937 sublines. Cells were incubated with serial dilutions of BSO for 3 days, after which cell viability was determined using the MTT assay as described in *Materials and Methods.* A. U937(●), U937/E10(o), B. U937/E15(■) and U937/V8(□). *Points*, mean of triplicate determinations; *error bars*, standard deviations.

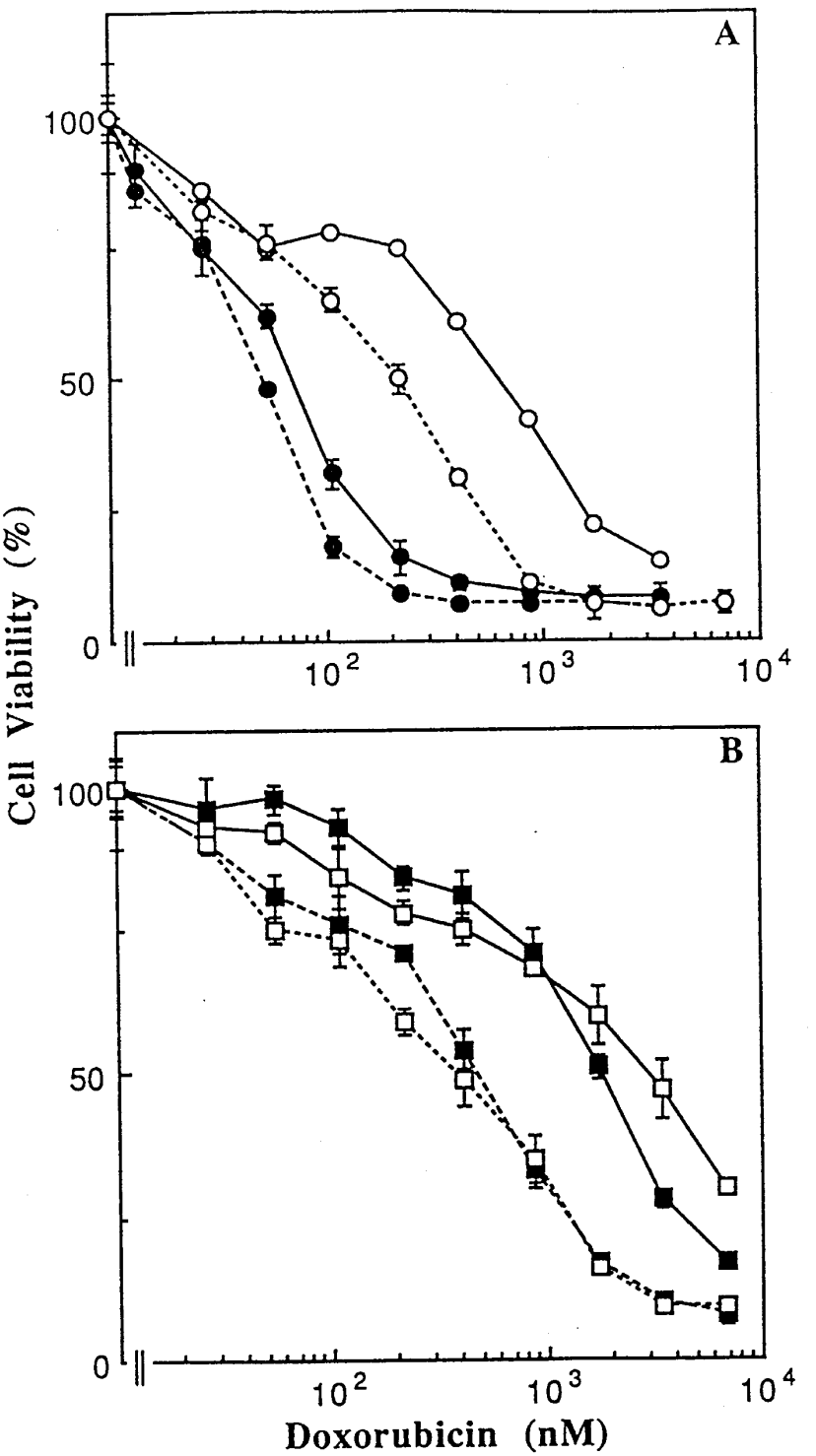

Figure 2.8. The effects of BSO on doxorubicin cytotoxicity in U937 cells and drug resistant sublines. Cells were incubated with serial dilutions of vinblastine in the presence(---) and absence(—) of 50 μM BSO for 3 days, after which cell viability was determined using the MTT assay as described in *Materials and Methods 3*. A. U937(●), U937/E10(o), B. U937/E15(■) and U937/V8(□). *Points*, mean of triplicate determinations; *error bars*, standard deviations. The experiment was repeated at least three times, and a representative result is shown.

Table 2.4. The effect of BSO on drug resistance in U937 cells and drug resistant sublines.

DRUG	BSO	IC_{50} (μM) U937[a]	U937/E10	U937/E15	U937/V8
epirubicin	-	0.106±0.052	0.618±0.278	0.983±0.196	1.783±0.525
	+	0.030±0.007	0.229±0.065	0.465±0.049*	0.900±0.141
		(3.5)[b]	(2.7)	(1.8)	(2.0)
doxorubicin	-	0.105± 0.050	0.518± 0.284	1.167± 0.196	1.850± 1.007
	+	0.055± 0.007	0.150± 0.099	0.362±0.194	0.338± 0.088
		(1.7)	(3.5)	(3.2)	(5.4)
vinblastine	-	0.003±0.0002	0.010±0.002	0.015±0.005	0.059±0.012
	+	0.002±0.0001	0.002±0.0000	0.004±0.0002	0.020±0.011
		(1.3)	(4.2)	(3.4)	(2.7)
VP-16	-	0.860±0.272	19.109±5.198	16.700±9.311	11.500±2.550
	+	0.500±0.282	6.150±1.202*	3.150±2.616	8.500±5.657
		(1.8)	(3.6)	(5.3)	(1.4)
chlorambucil	-	7.833±3.014	45.000±7.071	33.667±8.172	42.667±11.015
	+	4.167±1.258	27.667±12.503	10.25±3.072*	13.267±9.981*
		(1.9)	(2.1)	(3.3)	(3.2)
cisplatinum	-	12.375±4.608	54.430±4.179	46.040±7.178	41.165±16.076
	+	6.500±2.179	23.750±5.303*	17.500±4.330*	21.667±1.443*
		(1.9)	(2.3)	(2.6)	(1.9)

a Results are the mean of ≥3 experiments ± standard deviations.
b Numbers in parenthesis indicate fold-reversal which was calculated by dividing the IC_{50} of the cells in the absence of BSO by the IC_{50} of the cells in the presence of BSO.
* Indicates $p \leq 0.05$ using the student's t-test.

2.6 Rhodamine 123 Accumulation

Accumulation of the fluorescent dye Rh123 was examined in the U937 cells and drug resistant sublines. The U937/E10 subline accumulated 83%, the U937/E15 subline accumulated 75% and the U937/V8 subline accumulated 55% of the level of Rh123 compared to the parental U937 cells (100%; Fig. 2.9). The ability of verapamil to enhance Rh123 accumulation was also examined. Verapamil significantly increased Rh123 accumulation only in the U937/V8 subline, with no effect in the epirubicin treated sublines or the parental U937 cells (Fig. 2.9). The effects of verapamil on drug resistance and Rh123 accumulation in the U937 sublines are summarised in Table 2.5.

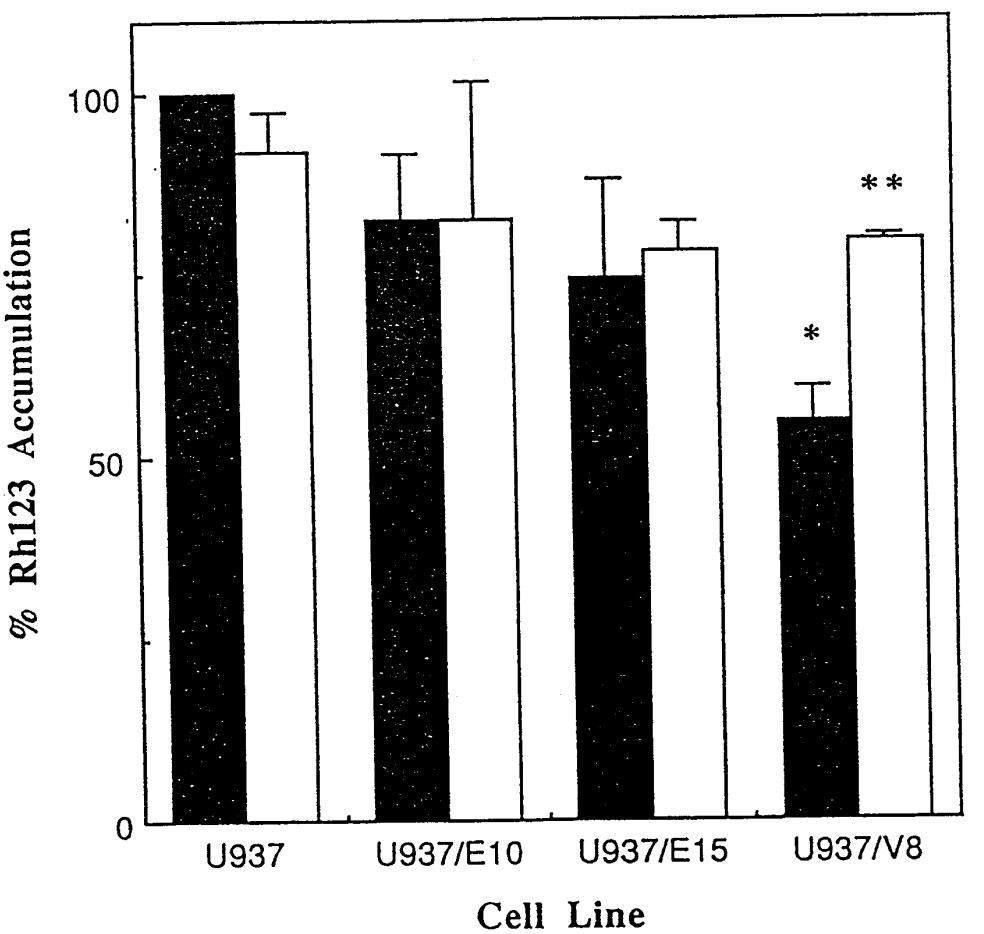

Figure 2.9. Rh123 accumulation in U937 cells and drug resistant sublines. Cells were incubated with Rh123 in the presence(□) or absence(■) of 10 μM verapamil, after which cell fluorescence was measured by flow cytometry as described in *Materials and Methods 7*. Results are the mean of two experiments and calculated as a percentage of Rh123 accumulation in the U937 cells. *Error bars*; standard deviations; *indicates a significant decrease compared to the parental U937 cells and ** indicates a significant increase ($p<0.05$) in accumulation in the presence of verapamil using the student's t-test.

Table 2.5. P-glycoprotein expression and function in drug resistant U937 sublines.

		Vinca alkaloid resistance		Rhodamine 123 accumulation	
Cell Line	P-glycoprotein expression	Alone	Verapamil reversal	Alone	Effect of verapamil
U937	-[a]	-	-	+	-
U937/E10	+	+	+	decreased	-
U937/E15	+	+	+	decreased	-
U937/V1	+	-	ND	ND	ND
U937/V8	+	+	+	decreased	+

a - denotes negative or no change, + denotes positive or a change, ND denotes not done

2.7 Cell Morphology

Selection for resistance resulted in an increase in cell size in all three drug resistant sublines. The cells were noticably larger by normal light microscopy. Assessment of forward scatter and side scatter of light by flow cytometry in the drug resistant sublines showed that U937/E10, U937/E15 and U927/V8 sublines were larger than the parental U937 cells, having increased forward light scatter, and were also more granular, with increased side scatter (Fig. 2.10).

2.8 Effects of VP-16 on Cell Cycle

The drug resistant sublines were highly resistant to VP-16 and P-glycoprotein and glutathione-related mechanisms did not fully account for their resistance. The effects of VP-16 on cell cycle kinetics were therefore compared in the U937 cells and the U937/E15 subline in order to elucidate a mechanism of resistance to VP-16 in the drug-resistant sublines

After treatment for 18 h with 2 μM VP-16, the U937 cells underwent apoptosis, demonstrated by an extra peak with lower DNA content at the G_0/G_1 peak (Fig. 2.11). 24 h after removal of VP-16, no cells were in S-phase, indicating no DNA synthesis was occurring, and 48 h after removal of VP-16 cell death had occured.

The untreated U937/E15 subline had a significantly higher DNA content than the parental U937 cells, at both the G_0/G_1 and the G_2+M peaks (Table 2.6), demonstrating that the development of drug resistance in these cells was accompanied by an increase in DNA content. The U937/E15 subline responded very differently to VP-16 treatment. When the U937/E15 cells were treated for 18 h with 2 μM VP-16, the cells accumulated in G_2/M phase, with only a small proportion of cells in G_0/G_1 and S-phase, suggesting that the cells were undergoing some repair process and not continuing through the cell cycle. 24 h after removal of VP-16, approximately 50% of the cells had undergone

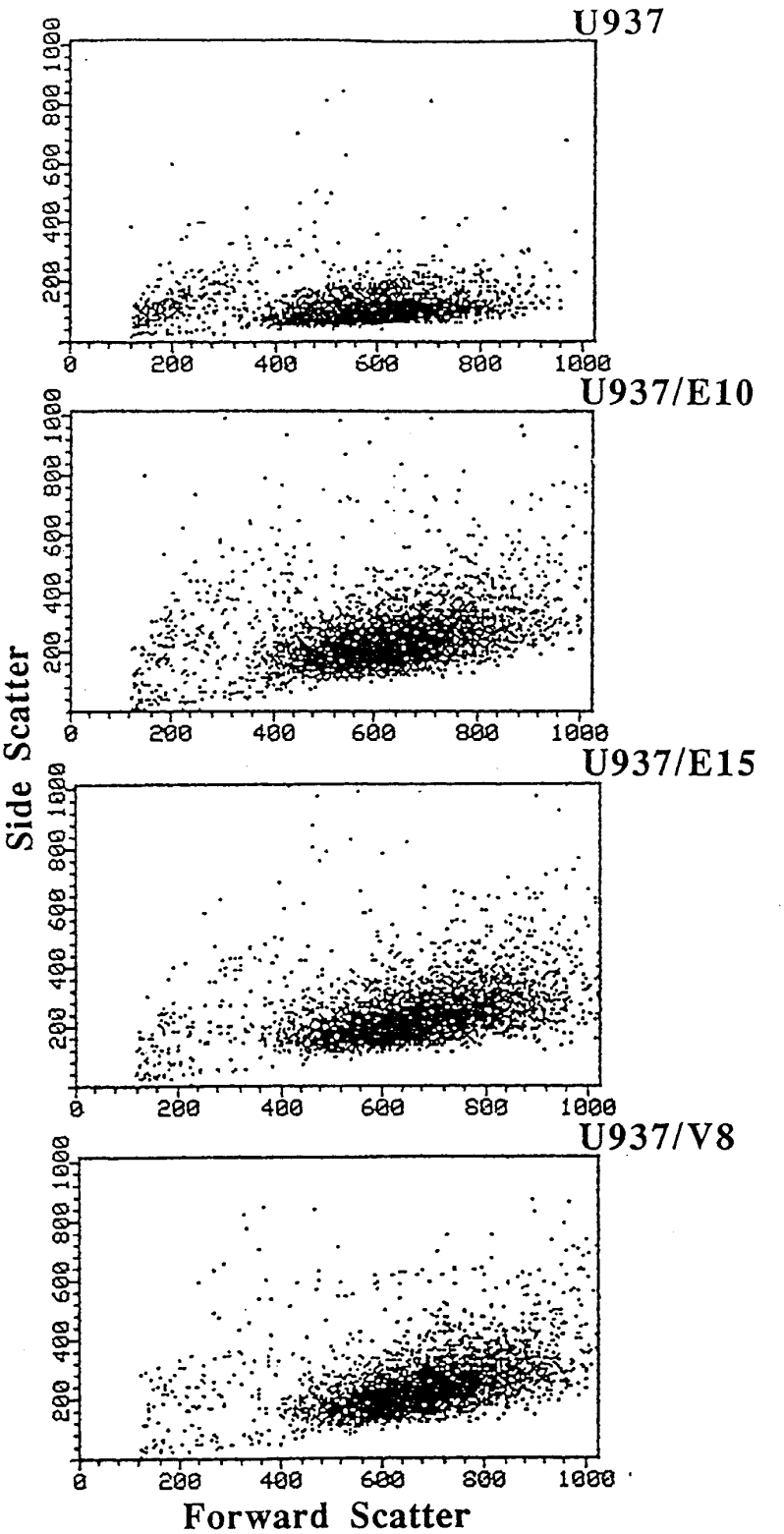

Figure 2.10. Flow cytometric analysis of cell size and granularity in drug resistant U937 sublines. Forward and side scatter of light were plottted in a dot-plot format using the LYSYS II software. The experiment was repeated at least three times and representative results are shown.

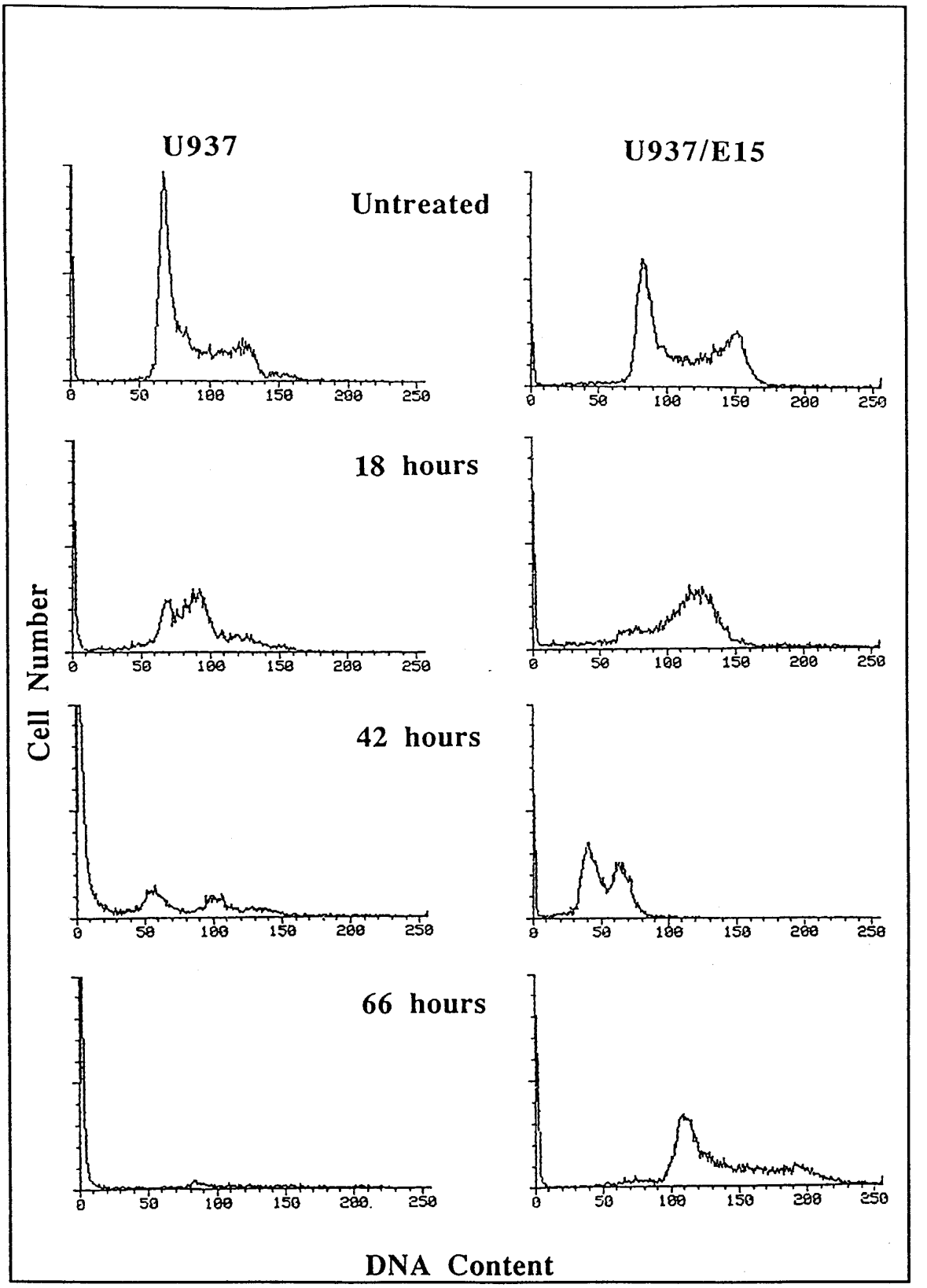

Figure 2.11. The effects of VP-16 on the cell cycle of U937 and U937/E15 cells. U937 and U937/E15 cells were incubated with 2 μM VP-16 for 18 h, after which cell cycle kinetics were determined 24 and 48 h after removal of the drug as described in *Materials and Methods 12.*

apoptosis, while approximately 50% remained in G_0/G_1 phase. However, 48 h after removal of the VP-16, DNA analysis demonstrated cells in all phases of the cell cycle, and cells counts indicated the U937/E15 cells had began to grow again at 66 h. The DNA content of the remaining cells had also increased compared to the untreated U937/E15 cells, indicated by the position of the peaks (Fig. 2.11).

Table 2.6 DNA content of the U937 cells and the U937/E15 subline.

Cell Line	DNA Content	
	G_0/G_1 peak[a]	G_2+M peak
U937	80 ± 9.407	145.4±16.32
U937/E15	101 ± 12.3, p=0.014[b]	180.6 ±18.42, p=0.013

a Mean ± standard deviation, n=5

b p-values comparing the U937 cells and U937/E15 subline were obtained using the student's t-test.

2.9 Expression of Antigens Associated with Differentiation

Treatment with 10 and 15 ng/ml epirubicin resulted in sublines which no longer expressed CD13 (Fig. 2.12) or CD14 (Fig. 2.13), while expression of glycophorin A was induced by epirubicin in the U937/E15 subline (Fig. 2.14). Selection with 8 ng/ml vinblastine induced glycophorin A expression in the U937/V8 subline, while CD14 expression was decreased in the U937/V8 subline, with no expression of CD13, as shown in Fig. 2.12. Control antibody binding in the drug resistant sublines was also higher due to increased F_c receptor expression, and this partly masked glycophorin A expression. CD34 expression was not altered in any of the drug resistant sublines examined (data not shown). Thus expression of P-glycoprotein was accompanied by aberrant expression of glycophorin A.

2.10 Discussion

Drug resistance developed rapidly in the U937 sublines. The drug resistant sublines were cross-resistant to MDR drugs, expressed P-glycoprotein and were sensitised to some MDR drugs by verapamil. This is consistent with drug resistance in the U937 sublines being due to P-glycoprotein. The U937 cells, like the K562 cells, therefore expressed the MDR phenotype in response to clinically achievable levels of epirubicin and vinblastine. Furthermore, these sublines not only expressed P-glycoprotein and the MDR phenotype, but were also resistant to chlorambucil, cisplatinum and were slightly resistant to methotrexate. Therefore these sublines displayed the broad type of resistance exhibited by patients who fail chemotherapy. While cross resistance to both anthracyclines and alkylating agents has often been demonstrated (Hamilton *et al*, 1985), such a broad cross

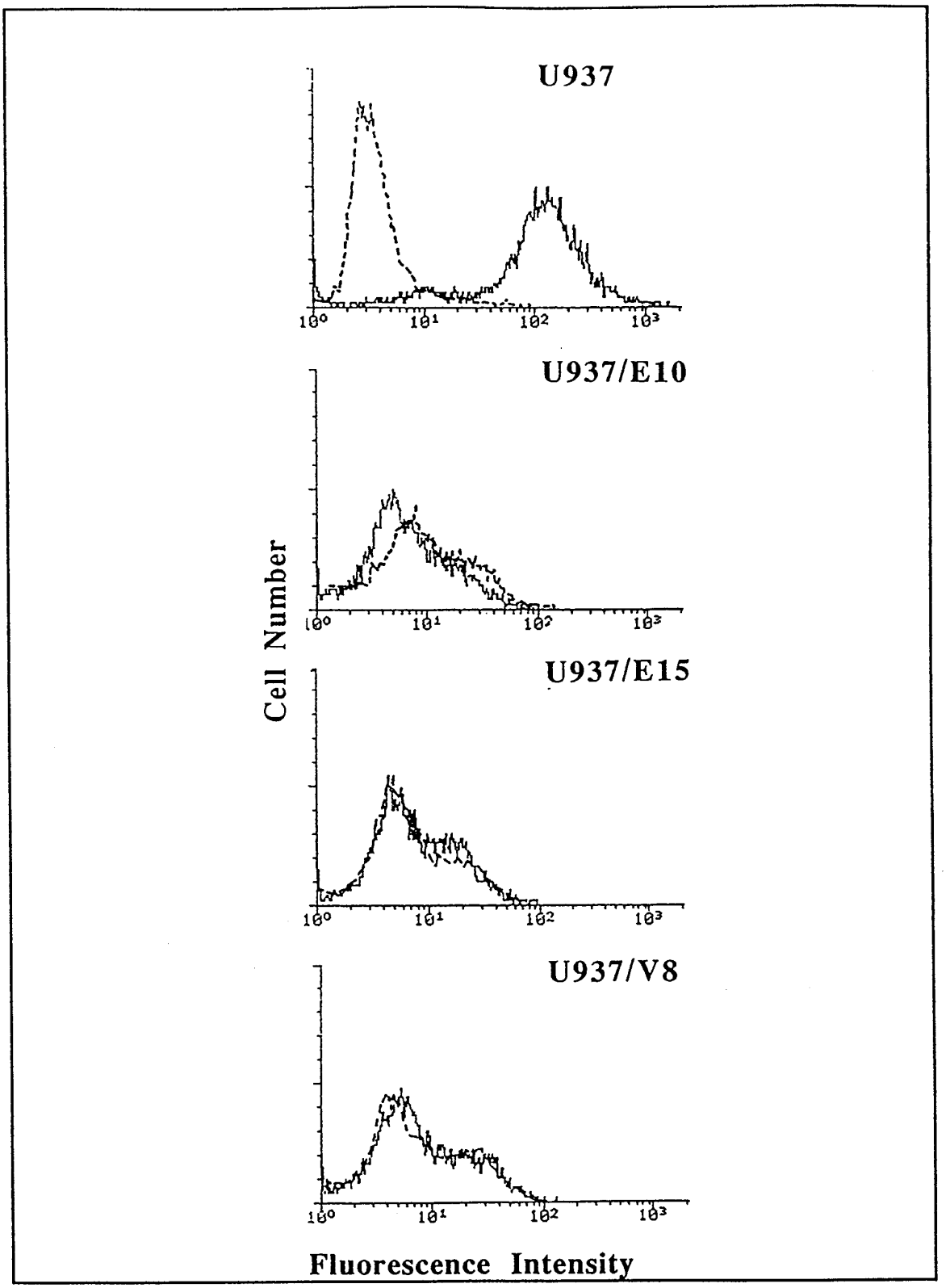

Figure 2.12. CD13 expression in U937 cells and drug resistant sublines. Cells were analysed for CD13 expression(—) by flow cytometry as described in *Materials and Methods 8*, and fluorescence profiles are compared to cells incubated with a negative control antibody(---). The experiment was repeated at least three times and a representative result is shown.

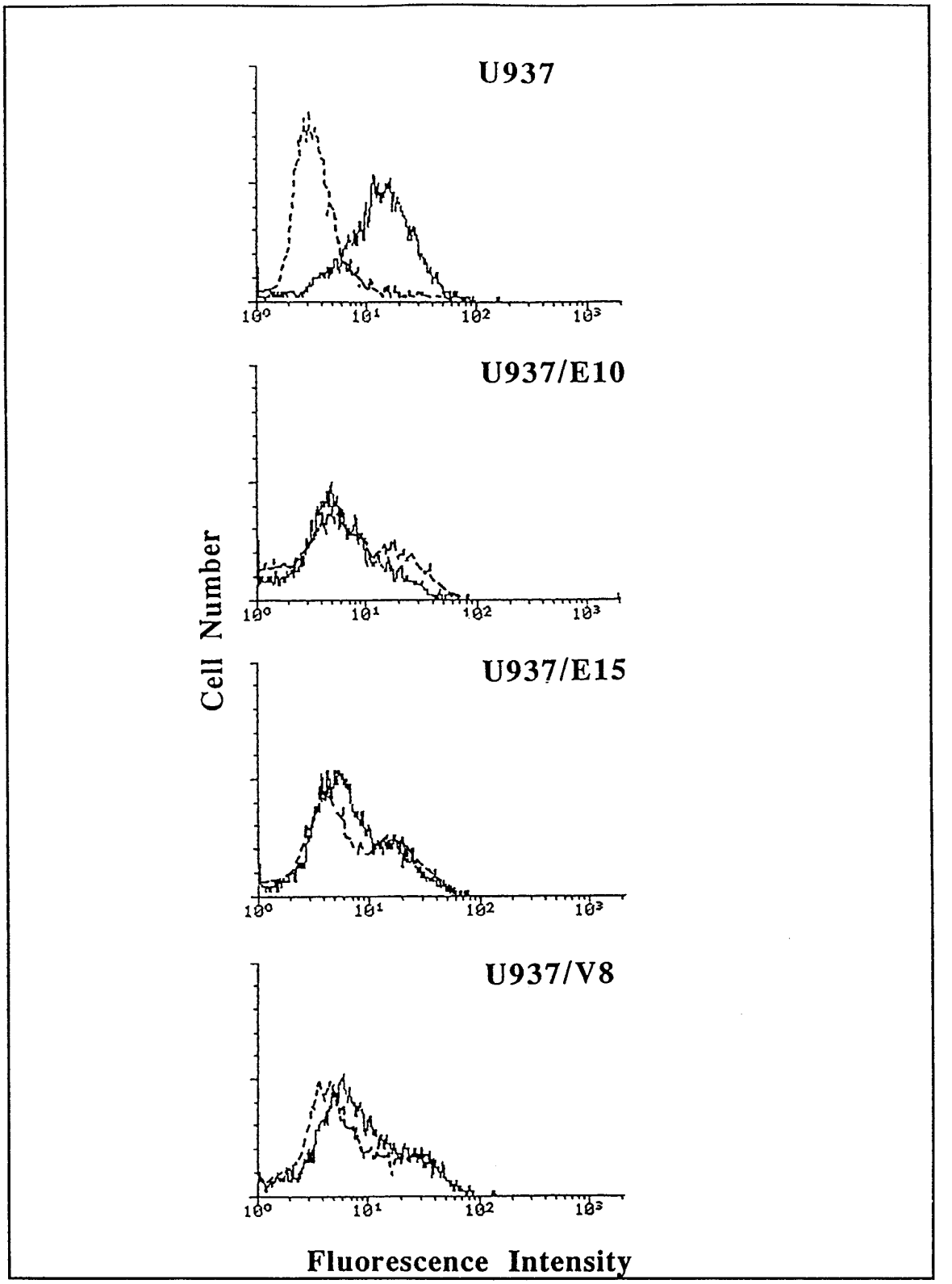

Figure 2.13. CD14 expression of U937 cells and drug resistant sublines. Cells were analysed for CD14 expression(—) by flow cytometry as described in *Materials and Methods 8*, and fluorescence profiles are compared cells incubated with a negative control antibody(---). The experiment was repeated at least three times and a representative result is shown.

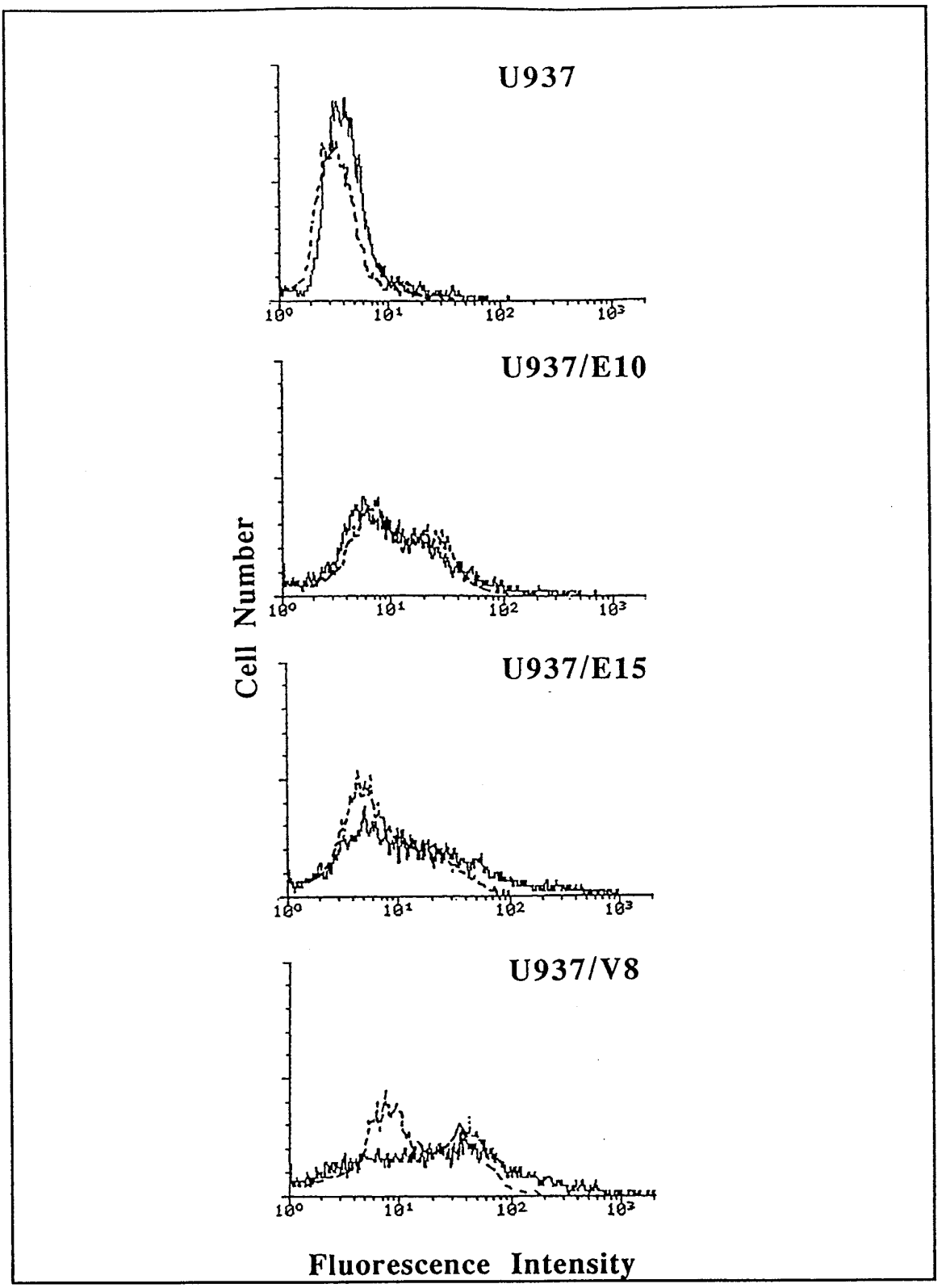

Figure 2.14. Glycophorin A expression of U937 cells and drug resistant sublines. Cells were analysed for glycophorin A expression(—) by flow cytometry as described in *Materials and Methods 8*, and fluorescence profiles are compared to cells incubated with a negative control antibody(---). The experiment was repeated at least three times and a representative result is shown.

resistance, especially to the *Vinca* alkaloids, has not been reported. The drug resistant sublines were developed by treatment with low doses of drug, far less than the IC_{50} (Fig. 2.1). As little cell destruction occurred as a result of drug treatment, it is unlikely that drug resistance developed by selection of a subclone of resistant U937 cells from the initial population. Therefore treatment with low levels of drug have probably induced this phenotype rather than selected it. As demonstrated for K562 cells, these results suggest that increased P-glycoprotein expression demonstrated in patients after treatment may be due to induction of P-glycoprotein during treatment rather than selection of a pre-existing clone of resistant cells.

P-glycoprotein expression was induced at all drug concentrations used. P-glycoprotein expression was much lower in the U937/V1 subline, and these cells were not drug resistant, suggesting that this low level of expression was not sufficient to mediate drug resistance. This is difficult to explain as this is the only case where drug-induced P-glycoprotein expression did not result in drug resistance. Even in the K562/V1 subline, where expression of P-glycoprotein was very low, cells were 7.8-fold resistant to vinblastine. The Western blot (Fig. 2.2) shows multiple banding with C219 for all drug resistant sublines which was not present in the parental U937 cells. Inclusion of protease inhibitors during membrane preparation had no effect on this banding pattern. As each cell line resulted in different patterns, this must reflect the cellular response to drug treatment. C219 recognises an ATP-binding site and so the pattern may be a result of the high sensitivity of the detection assay, with C219 detecting similar membrane proteins with ATP-binding domains.

The decreases in Rh123 accumulation in the three resistant sublines confirmed that P-glycoprotein was functional. However, the P-glycoprotein expressed in the resistant sublines conferred a different phenotype on the cells, as in the epirubicin treated sublines, the decrease in Rh123 accumulation was not changed by verapamil, while Rh123 accumulation was significantly increased by verapamil in the U937/V8 subline (summarised in Table 2.5). Therefore, while all the drug resistant sublines expressed P-glycoprotein (Fig. 2.2), and cytotoxicity to vinblastine was reversible by verapamil (Fig. 2.4), Rh123 accumulation was not increased by verapamil in all sublines.

This has important clinical implications as Rh123 accumulation in the presence of verapamil is used to assess P-glycoprotein function (Ross *et al*, 1993). These results, as for the K562 sublines, suggest that P-glycoprotein function may not always be accurately assessed by Rh123 and verapamil. The variable response to verapamil in these sublines is in accordance with clinical observations, which have demonstrated that *in vitro* uptake of drugs in leukaemic cells may not always be increased by verapamil, even when P-glycoprotein is expressed (Marie *et al*, 1992). These sublines therefore provide a clinically relevant model to examine the phenotype of drug resistance in leukaemia.

Epirubicin resistance was reversed almost completely by verapamil (Fig. 2.3), while the resistance to doxorubicin was only partially reversed by verapamil (Table 2.3), demonstrating that P-glycoprotein was at least one of the mechanisms conferring resistance to epirubicin and doxorubicin in these cells.

Resistance to idarubicin is of particular interest in these cells. Resistance to idarubicin was not reversed by verapamil in any of the drug resistant U937 sublines. This is in agreement with the results of Berman and McBride (1992), which showed that verapamil did not increase idarubicin cytotoxicity or intracellular accumulation in P-glycoprotein expressing CEM/VLB$_{100}$ cells. There are, however, conflicting reports in the literature as Damiani *et al* (1993) reported that verapamil increased the cytotoxicity of idarubicin, even at clinically tolerable levels of verapamil, in the CEM/VLB$_{100}$ and also in LOVO DX cells.

The mechanisms of sensitisation to anthracyclines by verapamil in P-glycoprotein expressing cells is not clear. While verapamil binds to P-glycoprotein and inhibits drug efflux (Qian and Beck, 1990), it has also been suggested that verapamil may cause changes in the intracellular distribution of anthracyclines (Hindenburg *et al*, 1989). Idarubicin is an analogue of daunorubicin, while epirubicin is an analogue of doxorubicin (reviewed by Weiss, 1992). Idarubicin differs from daunorubicin as the methoxyl group at position C-4 on ring D of the molecule is absent. This makes it more lipohilic than daunorubicin (Ganzina *et al*, 1986). A study of the distribution of daunorubicin and doxorubicin in a hydrophobic/hydrophilic environment demonstrated that verapamil can alter the solubility of anthracyclines, redistributing them from the aqueous phase into the hydrophobic phase (Hindenburg *et al*, 1987). Thus idarubicin may normally distribute to hydrophobic intracellular compartments, such as the cell membrane, and would therefore not be affected by verapamil. Alternatively, idarubicin may bind more strongly to P-glycoprotein than verapamil or it may bind to a different site on P-glycoprotein, hence explaining why verapamil had no effect on idarubicin cytotoxicity.

In agreement with the results for the U937 sublines, a study of AML patients also showed no correlation between P-glycoprotein expression and sensitivity to idarubicin, and verapamil did not increase the sensitivity of the AML cells to idarubicin (Müller *et al*, 1992).

Resistance to anthracyclines and alkylating agents has also been associated with detoxification mechanisms especially the glutathione redox cycle (Kuzmich and Tew, 1992). It has previously been shown that drug resistance may be reversed by lowering the glutathione levels in the cells with BSO (Hamilton *et al*, 1985, Neumann *et al*, 1992). BSO also significantly sensitised the three drug resistant sublines to cisplatinum, while only the U937/E15 and U937/V8 sublines were significantly sensitised to chlorambucil (Table 2.4), suggesting that changes in gluthathione metabolism are responsible for

increased resistance to these cells. The sublines also showed a slight sensitisation to the anthracyclines in the presence of BSO, suggesting that changes in glutathione metabolism may be partly responsible for anthracycline resistance in these cells, which has been reported by others (Raghu *et al*, 1993).

Interestingly BSO treatment also sensitised the drug resistant sublines to vinblastine, although the decrease was not significantly different. Vinblastine is a mitotic spindle inhibitor, and does not generate free radicals within the cell. However, increases in GST have been observed in MCF-7 cells selected for vincristine resistance (Whelan *et al*, 1989), indicating that *Vinca* alkaloids as well as anthracyclines are able to induce drug resistance which is mediated through modifications of glutathione metabolism.

It has been proposed that *Vinca* alkaloids interfere with microtubule dynamics by perturbing the equilibrium between reduced and oxidised glutathione, and consequently the cells uses its glutathione-reducing capacity in an attempt to maintain the assembly of tubulin (Beck, 1980). As *Vinca* alkaloids can alter cellular glutathione levels (Beck, 1980), it is therefore proposed that levels of GST or glutathione would increase to compensate for this. If glutathione is depleted by BSO, the drug resistant cells are therefore unable to overcome the effect of vinblastine on microtubules, and are sensitised to vinblastine.

Of particular interest is the high cross-resistance to VP-16 exhibited by the drug resistant U937 cells, in contrast to the K562 cells which were not cross-resistant to VP-16. VP-16 cytotoxicity in the drug resistant U937 sublines was not enhanced by verapamil, suggesting P-glycoprotein was not mediating resistance. While VP-16 is a member of the MDR family of drugs (Gottesman and Pastan, 1993), other investigators have shown that VP-16 resistance was not modulated by verapamil, such as in daunorubicin resistant Ehrlich ascites cells (Sehested *et al*, 1992), and drug resistant MCF-7 and DC3F cell lines (Politi *et al*, 1990). However, VP-16 did not bind to the plasma membrane of drug resistant cells (Sehested *et al*, 1992), and was shown to be 100 to 500-fold less effective in competing with P-glycoprotein photoaffinity labeling than vinblastine (Politi *et al*, 1990). The lack of reversal of VP-16 resistance by verapamil in the U937 cells, and the lack of resistance to VP-16 in the K562 cells in the presence of P-glycoprotein expression, along with evidence from other investigators brings into question the ability of P-glycoprotein to mediate resistance to VP-16 in these two cell lines.

Although verapamil had no effect on VP-16 cytotoxicity, BSO significantly increased VP-16 cytotoxicity in the U937/E10 and U937/E15 sublines. It is likely that glutathione/GST may therefore be involved in resistance to VP-16 in these two sublines. Other studies have shown that cytotoxicity of VM-26, which is related to VP-16, can be modified by BSO (Neumann *et al*, 1992). However, the sensitisation by BSO did not fully account for the resistance to VP-16 in the U937/E10 sublines. Further, BSO had no

effect on BSO cytotoxicity in the U937/V8 subline. As P-glycoprotein was not the mechanism for resistance to VP-16 in the U937 sublines, other possibilities were considered.

In an attempt to understand the mechanisms of resistance to VP-16 operating in the drug resistant U937 sublines, the effects of 2 μM VP-16 on cell cycle kinetics of the U937 cells were compared to the U937/E15 subline (Fig. 2.11). When U937 cells were treated with 2 μM VP-16, they underwent apoptosis, indicated by the "sub-G_0/G_1"peak (Darzynkiewicz *et al*, 1992). However, the U937/E15 subline became blocked in the G_2+M phase. Some of the cells subsequently underwent apoptosis and many cells died. However after 66 hours some cells recovered and began to progress through the cell cycle. It appears that the drug resistant U937/E15 cells were able to overcome the cytotoxic effects of VP-16 by arresting at the G_2+M phase of the cell cycle, suggesting that the resistant sublines have mechanisms allowing them to recover from VP-16 induced DNA damage.

Cole *et al* (1991) showed reduced levels of topo II and decreased DNA damage in doxorubicin-selected H69AR cells, which express the MRP protein. These cells also exhibited cross-resistance to VP-16 and vinblastine, but did not express P-glycoprotein. The U937 sublines have been shown to express *mrp* mRNA, while the parental U937 cells did not (personal communication from Dr. T. Longhurst, Department of Clinical Oncology, Royal North Shore Hospital, Sydney). These U937 sublines are therefore the first report of drug resistant cells expressing both P-glycoprotein and *mrp*. Expression of *mrp* mRNA has been found in high levels in normal blood peripheral mononuclear cells (Cole *et al*, 1992) and P-glycoprotein has also been detected in CD14-positive peripheral blood monocytes (Drach *et al*, 1992). U937 cells are monocyte-like (Sündstrom and Nilsson, 1976) and induction of both P-glycoprotein and *mrp* could be due to monocytic properties of these cells. Further, the resistant sublines may have reduced levels of topo II which could also account for their increased resistance to VP-16.

The increased cell volume and DNA content displayed by the drug resistant sublines is of particular interest. Increased DNA content suggests an increase in ploidy, which has been associated with poor prognosis in many tumours including AML (Raza *et al*, 1990). The increase in DNA content in the drug resistant sublines therefore emphasises the clincal relevance of the resistance mechanisms operating in these cells.

The U937 sublines were only slightly resistant to methotrexate. However even this small increase in resistance is clinically relevant. Methotrexate induces apoptosis (Barry *et al*, 1990) and the mechanisms of resistance which enabled the U937/E15 subline to overcome DNA damage after VP-16 treatment, together with the increase in DNA content may also mediate resistance to methotrexate.

Analysis of antigen expression showed that the drug resistant sublines had become less differentiated, loosing CD13 and CD14 and expressing glycophorin A, which is normally associated with erythroid differentiation. As the U937 cell line is monocyte-like, it is very unusual to observe expression of antigens associated with erythroid differentiation in these cells. These results are clinically significant as AML has been shown to relapse with a less differentiated phenotype, and a less differentiated phenotype is associated with poor prognosis at relapse in AML (Thomas *et al*, 1992).

These cells were selected with clinically relevant levels of a single chemotherapeutic agent, yet they exhibit a broad cross-resistance to almost all types of drugs regularly used in chemotherapy, closely reflecting drug resistance observed in patients. The response to drug treatment in these sublines suggests the co-induction of multiple mechanisms, including P-glycoprotein, similar to the heat shock response where multiple changes are coordinately induced (Lindquist and Craig, 1988). This system therefore provides a clinically relevant model for the study of initial events in the development of drug resistance.

Further investigation is necessary to elucidate other mechanisms of resistance operating in these cells, including measurements of glutathione-S-transferase levels and activity, and measurement of topoisomerase II in order to further explain the differences in cell cycle kinetics in the drug resistant cells.

3 DEVELOPMENT AND CHARACTERISATION OF DRUG RESISTANCE IN KG-1a AND HEL CELLS

3.1 Introduction

Drug resistant KG-1a and HEL sublines were established to further examine the development of drug resistance in leukaemic cells. The HEL cells are erythroblastic and came from a patient with erythroeukaemia (Martin and Papayonnopoulou, 1982) and are capable of differentiating towards megakaryocytes (Ylänne *et al*, 1988). KG-1a cells are undifferentiated myeloblasts and were derived from the KG-1 cell line, which came from a patient with acute myeloid leukaemia (Koeffler *et al*, 1980), and are capable of differentiating towards macrophages (Satterthwaite *et al*, 1990).

Both the KG-1a and HEL cell lines both intrinsically express P-glycoprotein, unlike the K562 and U937 cells. Therefore these cell lines were used to assess the effect of P-glycoprotein expression on selection for resistance and the ability of cells to undergo differentiation in reponse to drug treatment.

HEL cells were treated with 10 and 15 ng/ml epirubicin and 1 and 8 ng/ml vinblastine. However, higher concentrations were used to develop drug resistant KG-1a sublines, as the KG-1a cells were intrinsically more resistant to both drugs than the other three cell lines. KG-1a cells were therefore treated with 15 and 25 ng/ml epirubicin and 8 and 16 ng/ml vinblastine.

3.2 The KG-1a Sublines

3.2.1 Development of Drug Resistance

KG-1a cells were treated for 3-6 days with drug over a 3 month period. However, the KG-1a/E15 and KG-1a/E25 sublines did not demonstrate increased resistance to epirubicin (Fig. 3.1) nor increased cross-resistance to vinblastine (Fig. 3.2). Similar results were observed for the KG-1a/V8 and KG-1a/V16 sublines (Fig. 3.1 and Fig. 3.2).

3.2.2 P-glycoprotein Expression and Function

The parental KG-1a cells express P-glycoprotein (Fig. 3.3), and P-glycoprotein expression did not increase in any of the drug treated sublines. The ability of P-glycoprotein in the drug treated sublines to accumulate Rh123 was determined, in the presence and absence of 10 μM verapamil. Compared to the parental KG-1a cells, the drug treated sublines showed little change in Rh123 accumulation (Fig. 3.4). Rh123

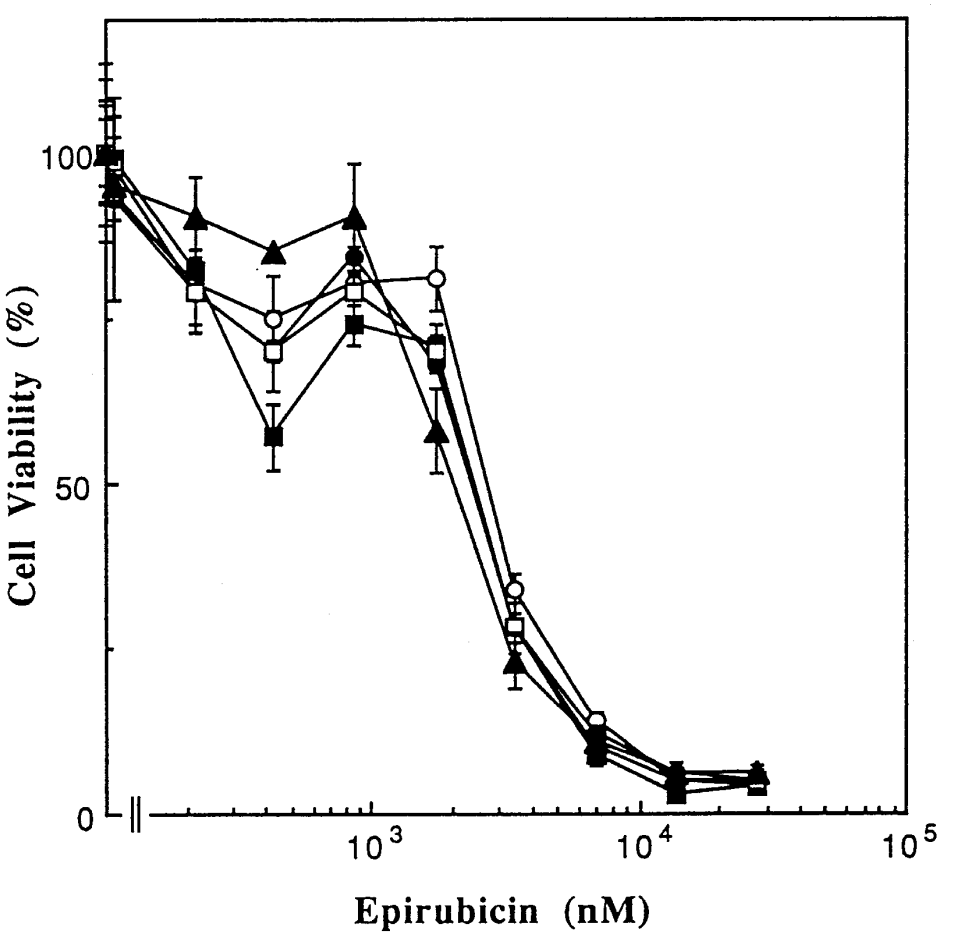

Figure 3.1. Resistance to epirubicin in the KG-1a cells and drug resistant sublines. Cells were exposed to serial dilutions of epirubicin for 4 days, after which cell viability was determined using the MTT assay as described in *Materials and Methods 3*. KG-1a (●), KG-1a/E15(o), KG-1a/E25(■), KG-1a/V8(□) and KG-1a/V16(▲). *Points*, mean of triplicate determinations; *error bars*; standard deviations. Experiments were repeated at least three times and a representative result is shown.

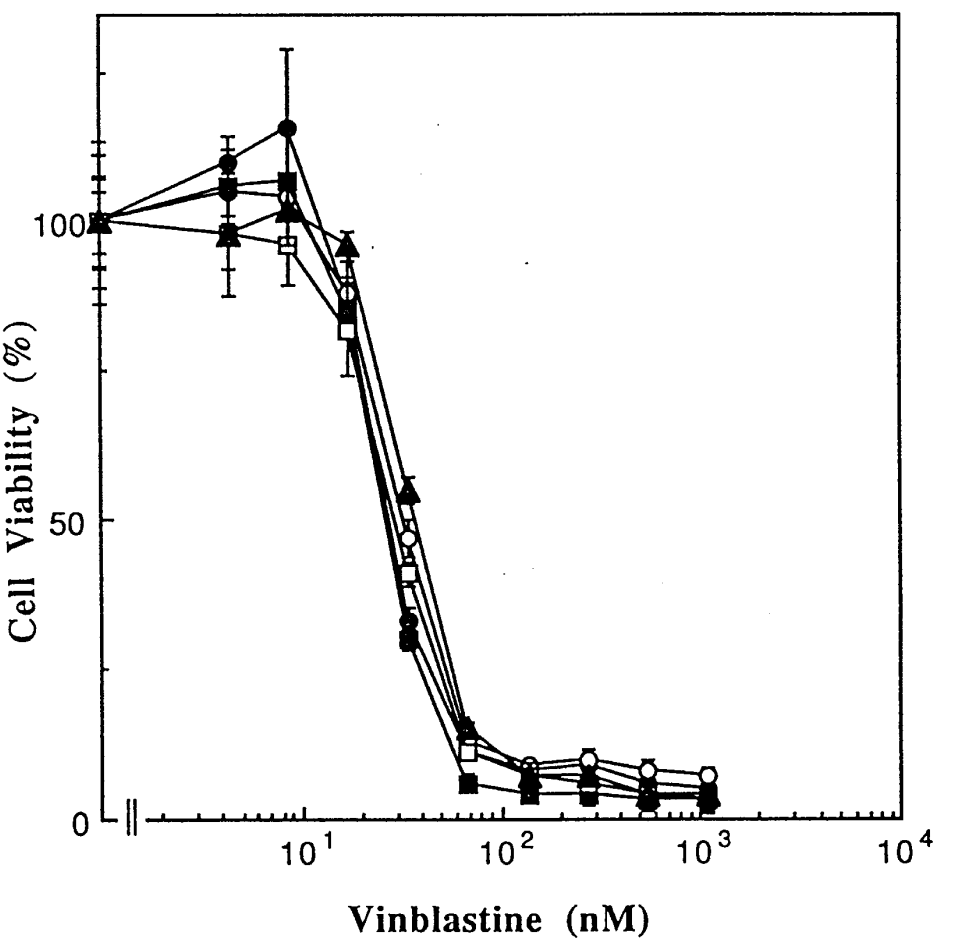

Figure 3.2. Resistance to vinblastine in the KG-1a cells and drug resistant sublines. Cells were exposed to serial dilutions of vinblastine for 4 days, after which cell viability was determined using the MTT assay as described in *Materials and Methods 3*. KG-1a (●), KG-1a/E15(o), KG-1a/E25(■), KG-1a/V8(□) and KG-1a/V16(▲) cells. *Points*, mean of triplicate determinations; *error bars*, standard deviations. Experiments were repeated at least three times and a representative result is shown.

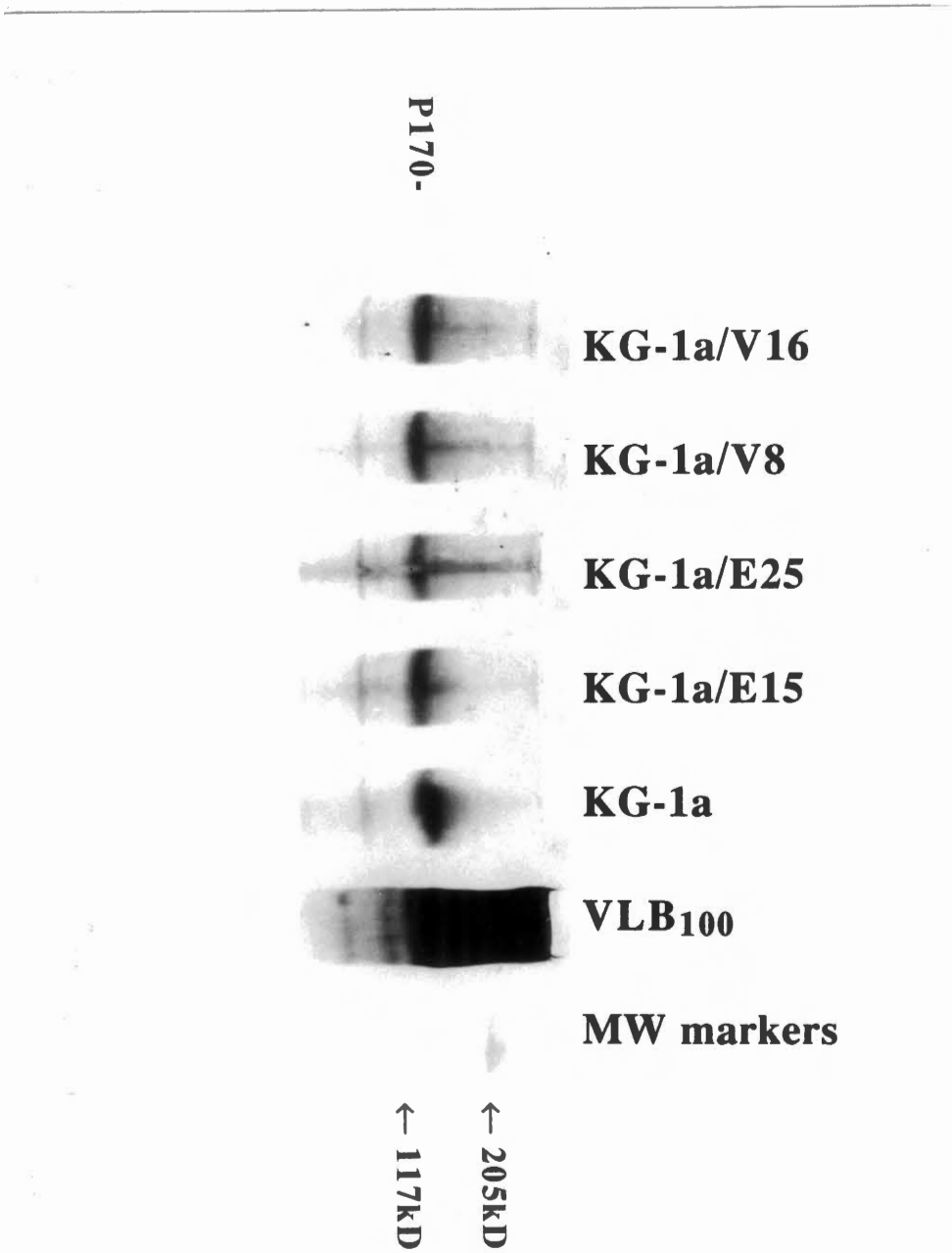

Figure 3.3. Western blot analysis of P-glycoprotein expression in KG-1a cells and drug resistant sublines. Plasma membrane fractions were prepared and analysed as described in *Materials and Methods 6.1*. Positions of molecular weight markers and P-glycoprotein (P170) are indicated.

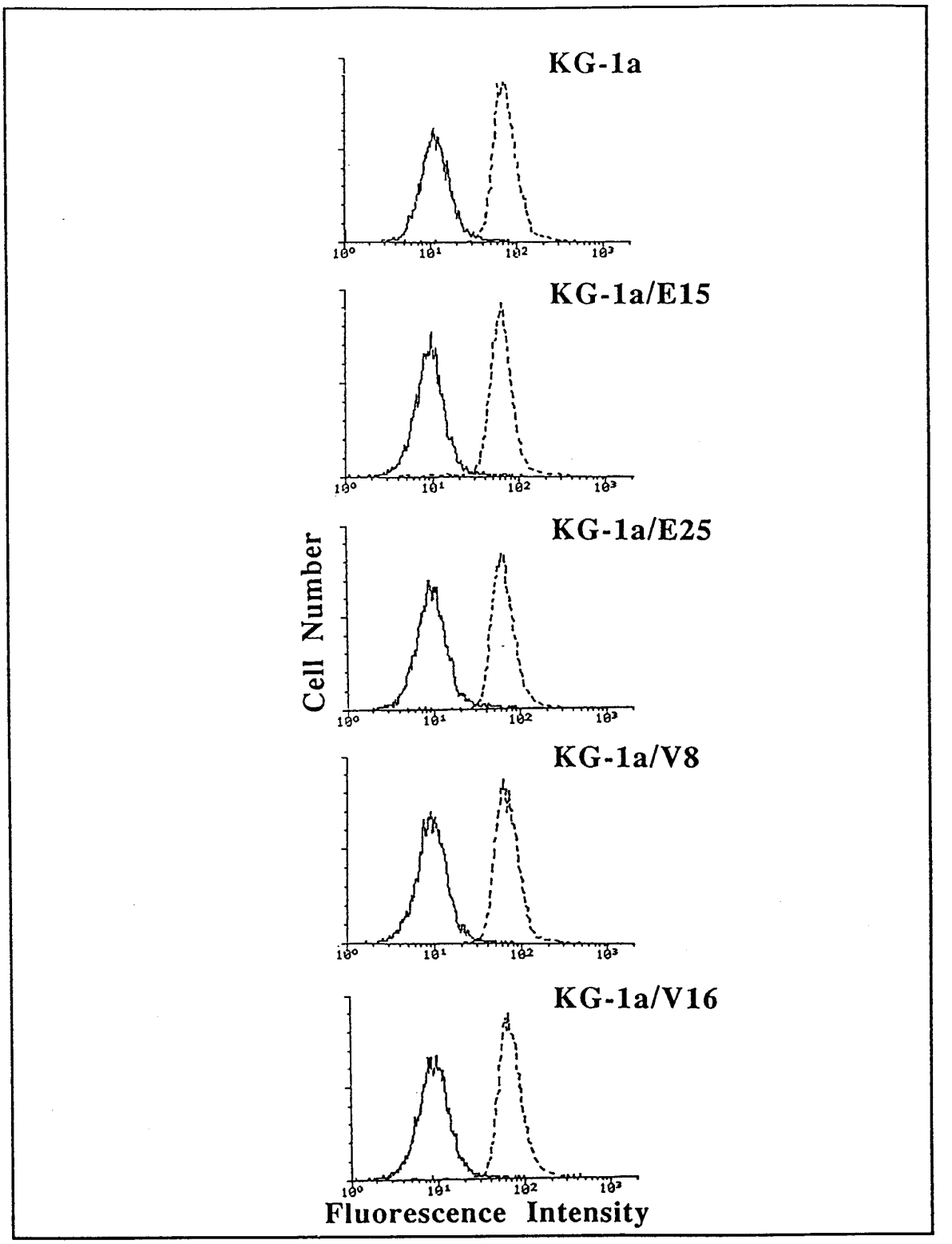

Figure 3.4. Rh123 accumulation in KG-1a cells and drug resistant sublines. Cells were incubated with Rh123 in the presence(---) or absence(—) of 10μM verapamil, after which fluorescence was measured by flow cytometry as described in *Materials and Methods 7*. Experiments were repeated twice and a representative result is shown.

accumulation was greatly increased by 10 μM verapamil in the parental KG-1a cells, indicating P-glycoprotein activity in these cells, which correlated with P-glycoprotein expression. Similar results were obtained for all of the drug treated sublines (Fig. 3.4).

3.2.3 Differentiation Markers

The parental KG-1a cells expressed the myeloid antigen CD13 (Fig. 3.5), very low levels of CD14 (Fig 3.5), the stem cell antigen CD34 (Fig. 3.6) and the megakaryocyte antigen CD61 (Fig. 3.6).

CD13 expression did not change in any of the drug treated sublines. CD14 expression was not affected by treatment with epirubicin, however treatment with vinblastine reduced CD14 expression, which became lower than the negative control (Fig. 3.5). CD34 expression did not change in the epirubicin treated sublines, while CD34 expression was slightly reduced in the vinblastine treated sublines (Fig. 3.6).

CD61 expression was less heterogeneous in the KG-1a/E15 and KG-1a/E25 sublines. However, binding of the negative control also increased, making it difficult to draw any conclusions about overall changes in CD61 expression. There was little change in CD61 expression in the KG-1a/V8 subline, while CD61 expression in the KG-1a/V16 subline was increased and more heterogeneous. The increases in CD61 expression and decreases in CD34 suggest megakaryocytic differentiation after treatment with vinblastine.

3.2.4 Cell Morphology of KG-1a sublines

The KG-1a cells grew in suspension, with some cells forming clumps when approaching confluency. This did not alter in the drug treated sublines. The drug treated KG-1a sublines did not show any increase in size.

3.3 The HEL Sublines

3.3.1 Development of Drug Resistance

HEL cells were treated with drug 7 times for 3 to 7 days over a period of 3 months, while the HEL/V8 subline was only exposed to vinblastine 6 times. The HEL/E10 and HEL/E15 sublines did not show increased resistance to epirubicin (Fig. 3.7) and were not cross-resistant to vinblastine (Fig. 3.8). The HEL/V1 and HEL/V8 sublines were not resistant to vinblastine (Fig. 3.8) and were not cross-resistant to epirubicin (Fig. 3.7).

3.3.2 P-glycoprotein Expression and Function

The parental HEL cells expressed P-glycoprotein (Fig. 3.9) and P-glycoprotein expression was not increased in the drug treated sublines. P-glycoprotein function was also assessed by Rh123 accumulation in the parental HEL cells and sublines (Fig. 3.10). When compared to the parental HEL cells, the drug treated sublines did not show any

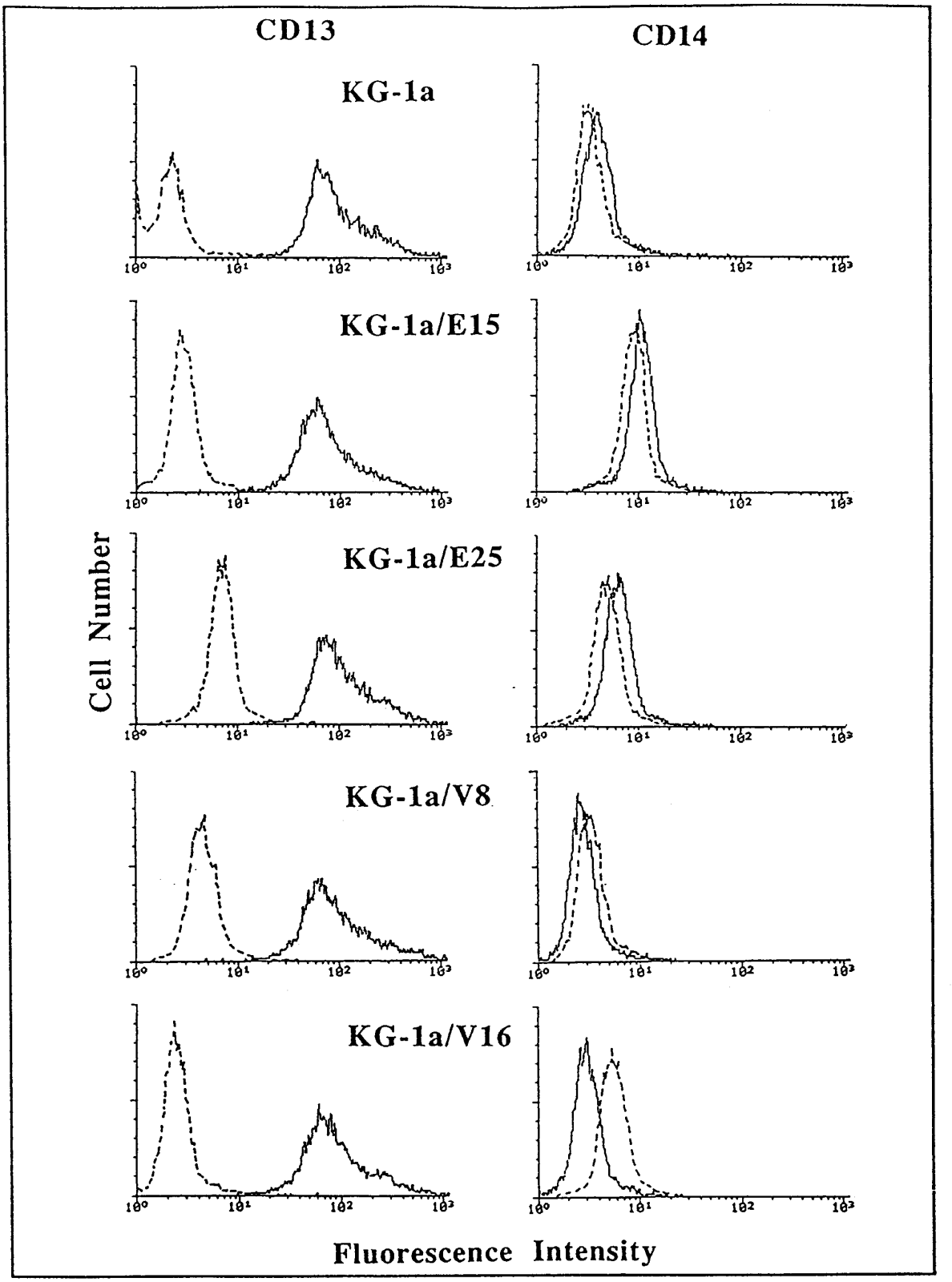

Figure 3.5. CD13 and CD14 expression of KG-1a cells and drug resistant sublines. Cells were analysed for expression of CD13 or CD14(—) by flow cytometry as described in *Materials and Methods 8*, and the fluorescence profile is compared to cells incubated with a negative control antibody(---). Experiments were repeated twice and a representative result is shown.

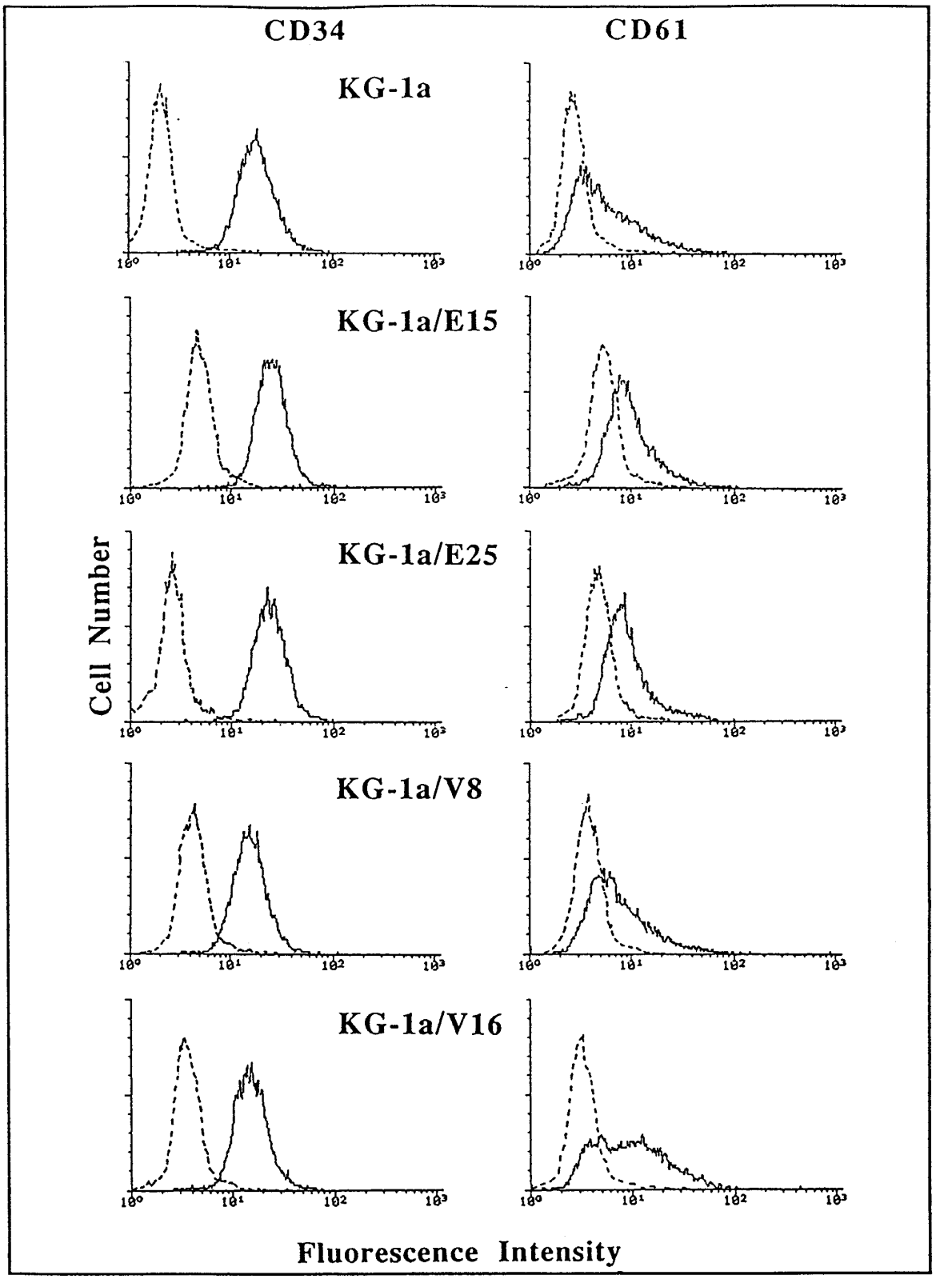

Figure 3.6. CD34 and CD61 expression of KG-1a cells and drug resistant sublines. Cells were analysed for expression of CD34 or CD61(—) by flow cytometry as described in *Materials and Method 8*, and the fluorescence profile is compared to cells incubated with a negative control antibody(---). Experiments were repeated twice, and a representative result is shown.

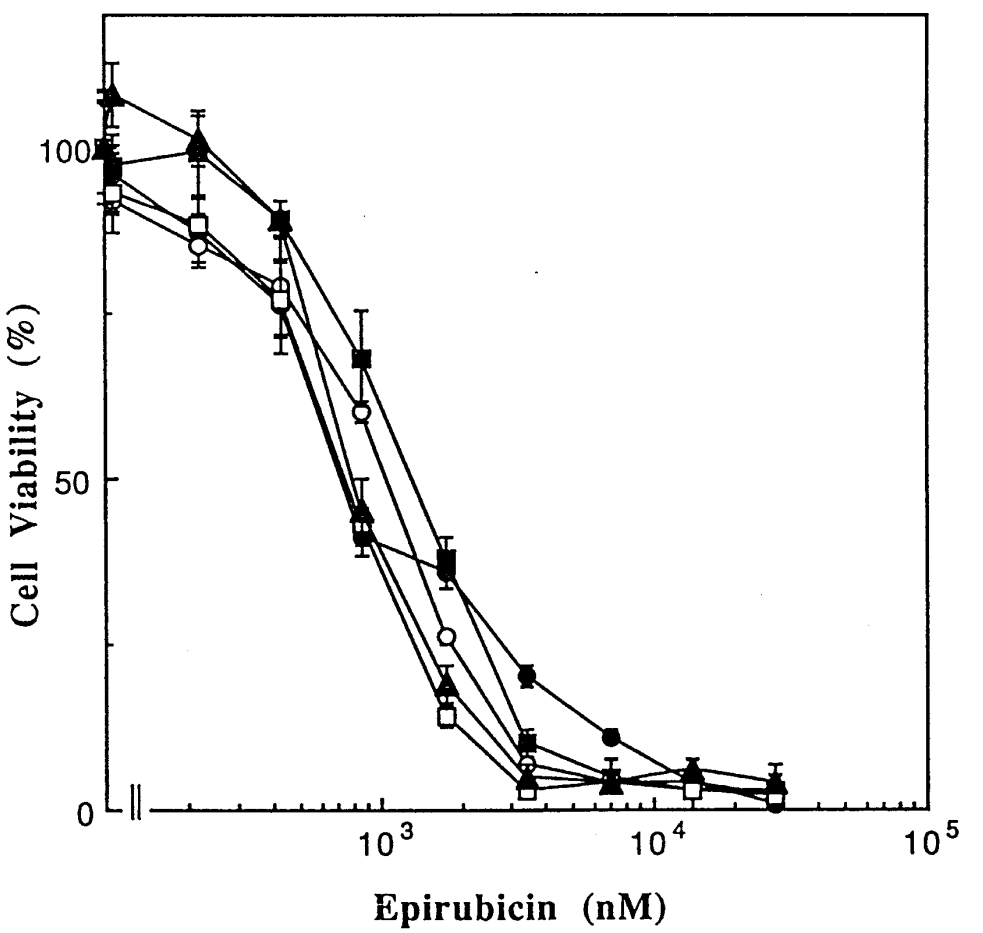

Figure 3.7. Resistance to epirubicin in the HEL cells and drug resistant sublines. Cells were exposed to serial dilutions of epirubicin for 4 days, after which cell viability was determined using the MTT assay as described in *Materials and Methods 3*. HEL(●), HEL/E10(o), HEL/E15(■), HEL/V1(□) and HEL/V8(▲) cells. *Points*, mean of triplicate determinations; *error bars*, standard deviations. The experiments were repeated at least three times and a representative result is shown.

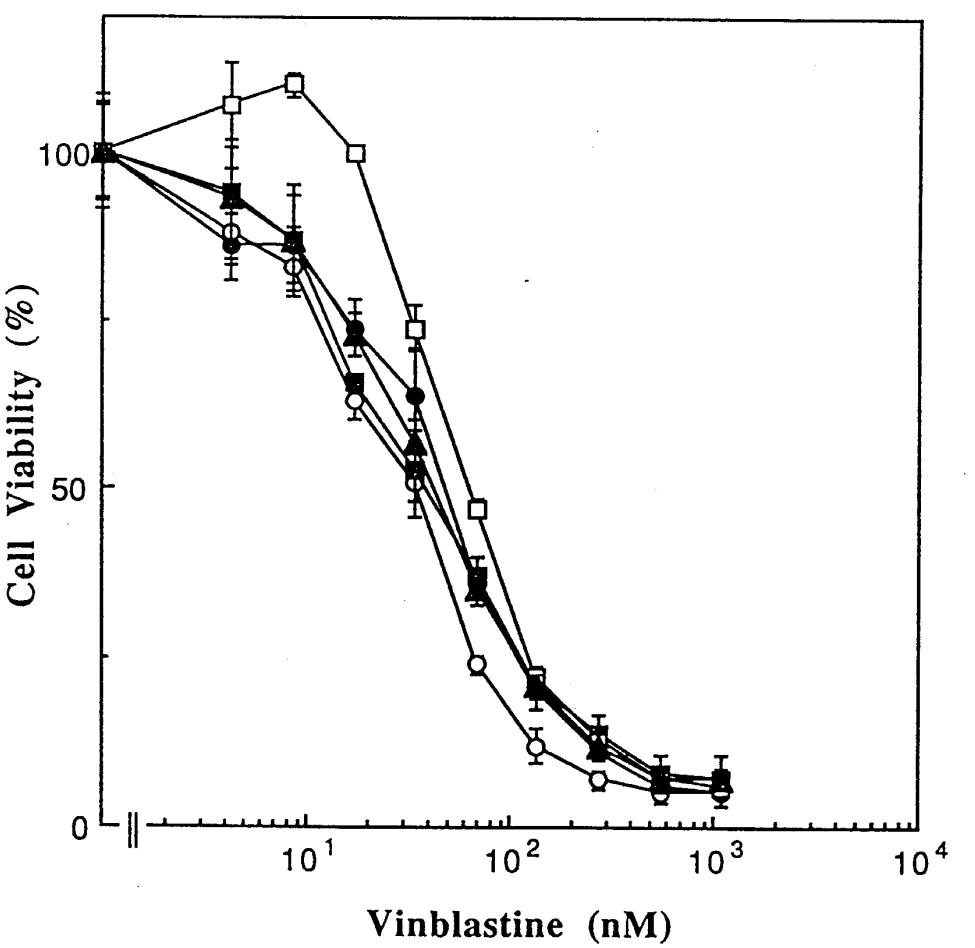

Figure 3.8. Resistance to vinblastine in the HEL cells and the drug resistant sublines. Cells were exposed to serial dilutions of vinblastine for 4 days, after which cell viability was determined using the MTT assay as described in *Materials and Methods 3*. HEL(●), HEL/E10(o), HEL/E15(■) HEL/V1(□) and HEL/V8(▲) cells. *Points*, mean of triplicate determinations; *error bars*, standard deviations. Experiments were repeated at least three times and a representative result is shown.

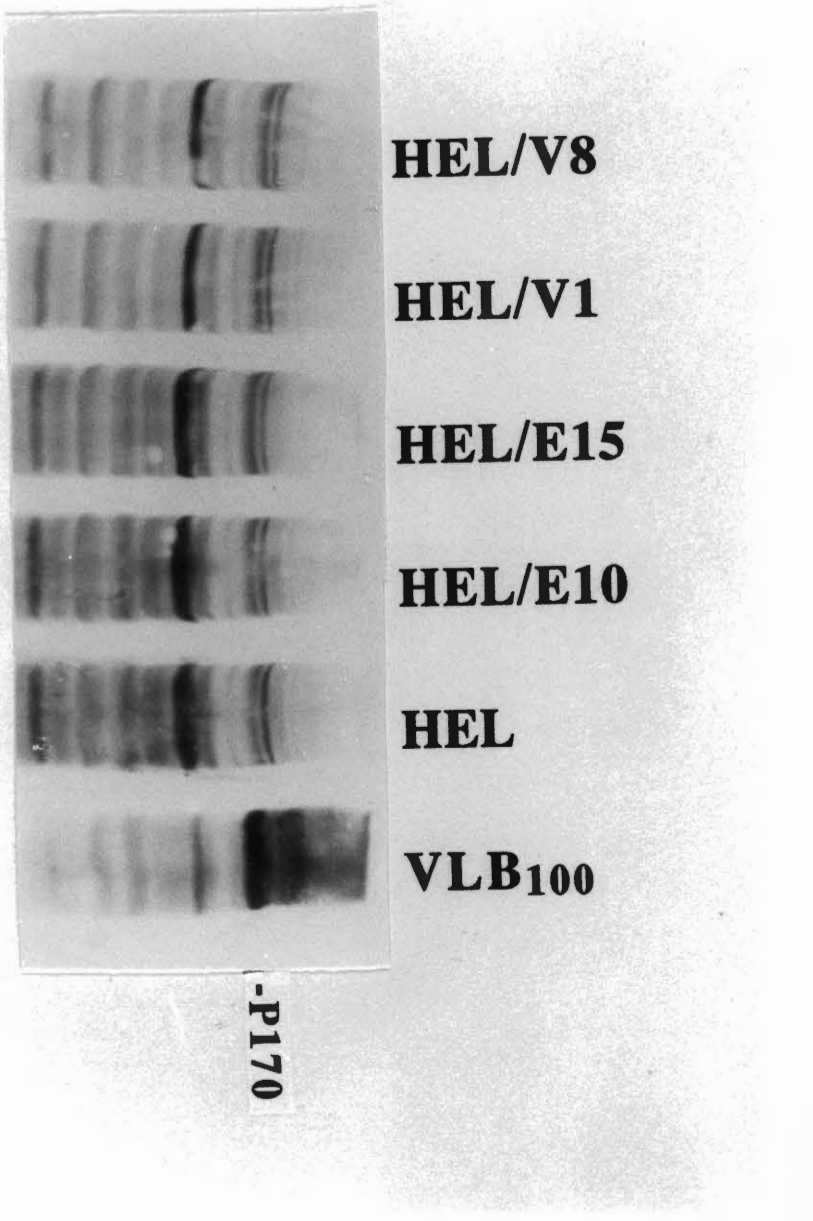

Figure 3.9. Western blot analysis of P-glycoprotein expression in HEL cells and drug resistant sublines. Plasma membrane fractions were prepared and analysed as described in *Materials and Method 6.1*. The position of P-glycoprotein (P170) is indicated.

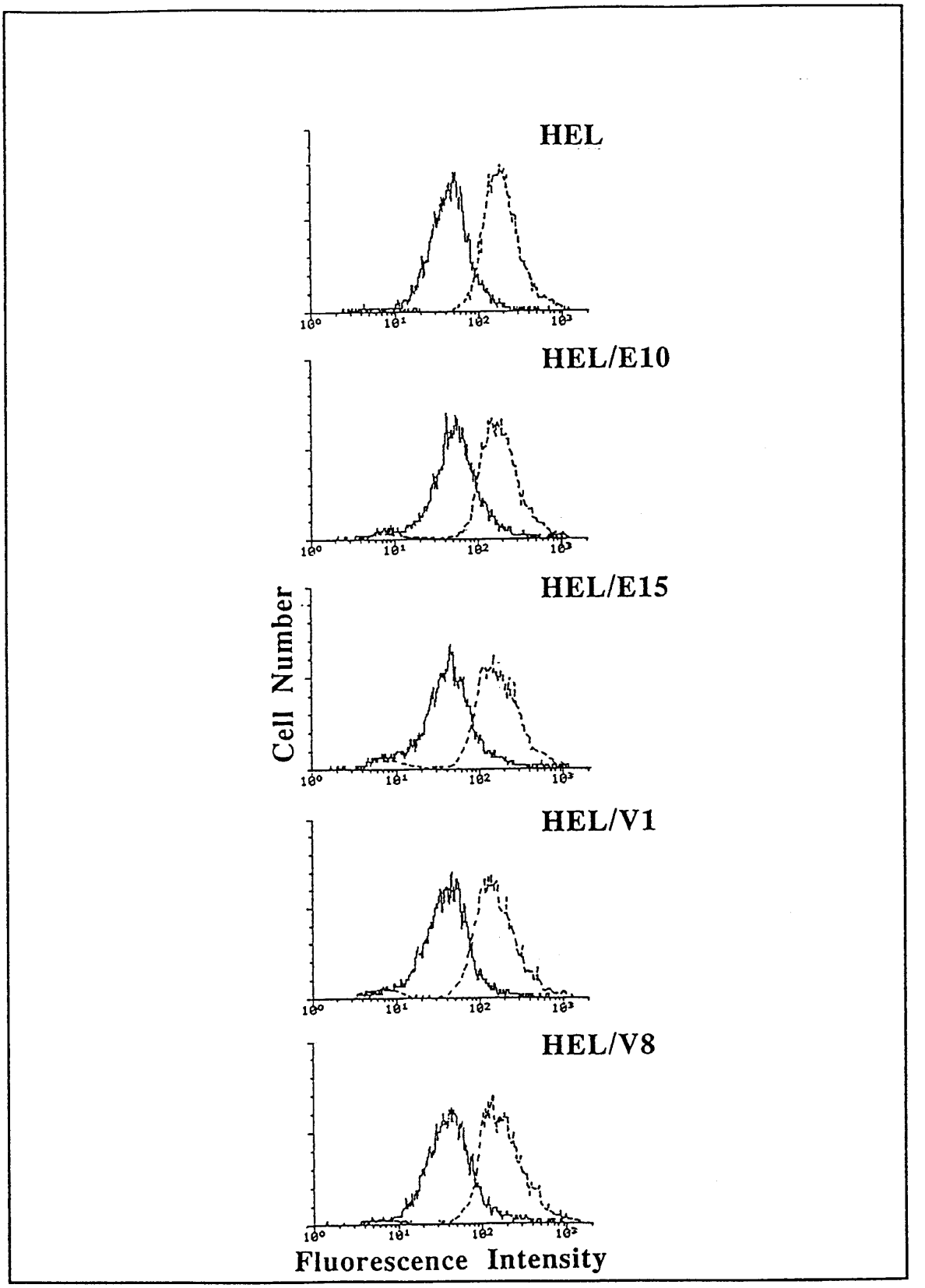

Figure 3.10. Rh123 accumulation in HEL cells and drug resistant sublines. Cells were incubated with Rh123 in the presence(---) or absence(—) of 10μM verapamil, after which fluorescence was measured by flow cytometry as described in *Materials and Methods 7*. Experiments were repeated twice and a representative result is shown.

reduction in Rh123 accumulation, indicating there was no change in P-glycoprotein activity in these cells. Rh123 accumulation in the parental HEL cells was greatly increased by 10 μM verapamil, indicating P-glycoprotein activity in these cells. Verapamil similarly increased Rh123 accumulation in all of the drug treated sublines (Fig. 3.10).

3.3.3 Differentiation Antigens

HEL cells expressed CD13, (Fig. 3.11), CD61 and glycophorin A (Fig. 3.12), and did not express CD14 (Fig. 3.11). CD13 expression did not change in the HEL/E10 subline, however their was a slight reduction in expression in the HEL/E15 subline (Fig. 3.12). Selection with vinblastine did not induce any changes in CD13 expression in either the HEL/V1 or HEL/V8 sublines. CD14 was not expressed by HEL cells, and expression did not change in any of the drug treated sublines.

CD61 expression was slightly increased in the both the epirubicin and the vinblastine treated HEL sublines (Fig. 3.12). The increases in CD61 expression correlated with the decreases in glycophorin A expression, which was slightly lower and more heterogeneous in all four of the drug treated sublines, indicating megakaryocytic differentiation after drug treatment (Fig. 3.12).

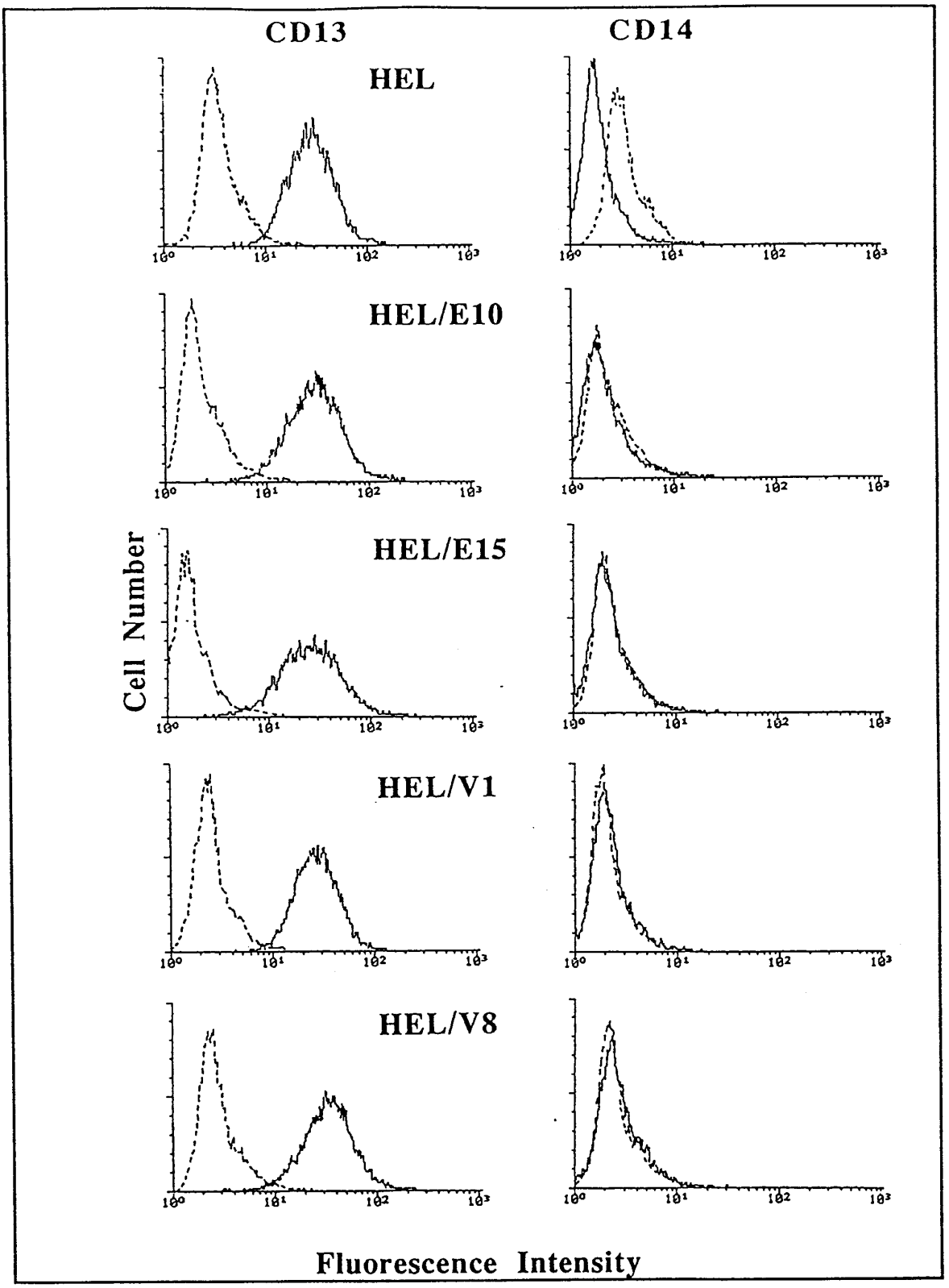

Figure 3.11. CD13 and CD14 expression of HEL cells and drug resistant sublines. Cells were analysed for expression of CD13 or CD14(—) by flow cytometry as described in *Materials and Methods 8*, and the fluorescence profile is compared to cells incubated with the negative control antibody(---). Experiments were repeated twice, and a representative result shown.

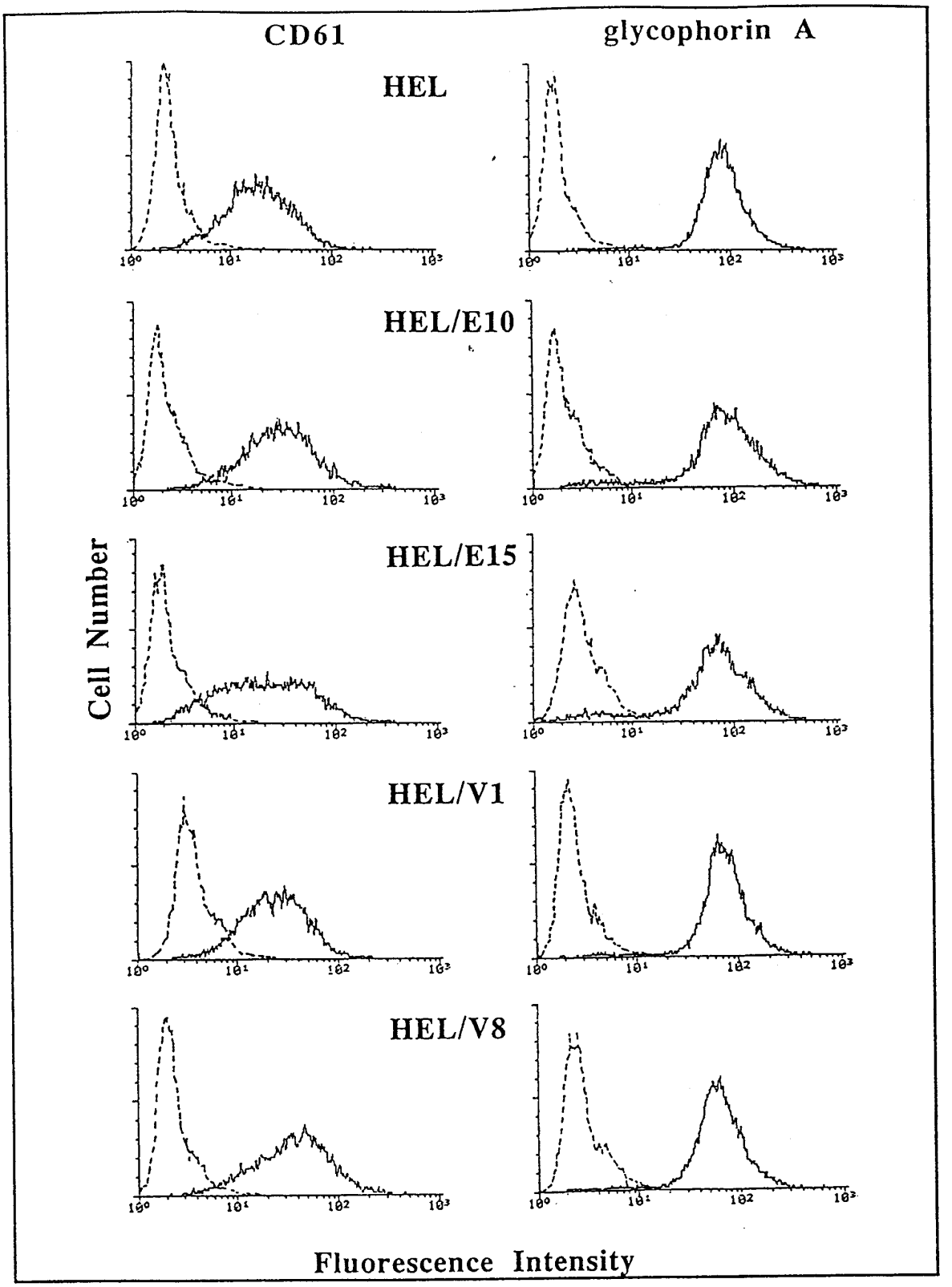

Figure 3.12. CD61 and glycophorin A expression of HEL cells and drug resistant sublines. Cells were analysed for expression of CD61 or glycophorin A(—) by flow cytometry as described in *Materials and Methods 8*, and the fluorescence profiles are compared to cells incubated with the negative control antibody(---). Experiments were repeated twice and a representative result is shown.

3.3.4 Cell morphology of HEL sublines

The HEL cells grew in suspension as single cells. The morphology of the drug treated sublines did not differ from the parental HEL cells, with no changes in cell size.

3.4 Discussion

Both the HEL and KG-1a cell lines expressed P-glycoprotein and treatment with chemotherapeutic drugs at low, clinically relevant levels did not induce further increases in drug resistance. Treatment with chemotherapeutic drugs did however induce some megakaryocytic differentiation in the vinblastine treated KG-1a sublines (Fig. 3.6), and the HEL sublines underwent megakaryocytic differentiation in response to both epirubicin and vinblastine (Fig. 3.12).

The parental U937 and K562 cell lines did not inherently express P-glycoprotein and were relatively sensitive to vinblastine and epirubicin. The results of all four cell lines are summarised in Table 3.1. When treated with chemotherapeutic drugs, these two cell lines rapidly became drug resistant and underwent changes in antigen expression. Therefore the expression of P-glycoprotein and the inherent drug sensitivity of leukaemic cell lines will determine their response to treatment with chemotherapeutic drugs.

Table 3.1: The association between MDR and the ability to undergo differentiation in response to drug treatment in human leukaemic cell lines.

Cell Line	Epirubicin IC_{50} (nM)	Vinblastine IC_{50} (nM)	P-gp Expression	Differentiation in response to drugs.
U937	110	3.0	NO	+++
K562	290	3.0	NO	++
HEL	650	30.0	YES	+
KG-1a	2500	25.0	YES	+

Selection with epirubicin and vinblastine in the KG-1a and HEL cell lines contrasts the effects observed in the drug resistant K562 and U937 sublines, both of which do not express P-glycoprotein. Both the K562 and U937 cells were induced to differentiate and to express P-glycoprotein and the MDR phenotype in response to treatment with either epirubicin or vinblastine. In contrast, the KG-1a cells which expressed P-glycoprotein and were the most drug resistant (Table 3.1), showed the least response to drug selection, with no change in drug resistance and little differentiation. The HEL cells expressed P-glycoprotein and were intermediate in their drug resistance. The HEL cells showed some

response to the drugs in terms of megakaryocytic differentiation. The presence of P-glycoprotein and the intrinsic level of drug resistance therefore influenced the ability to respond to drug treatment by undergoing differentiation or becoming drug resistant. The P-glycoprotein expressed in the KG-1a and HEL cells is functional, as demonstrated by the effect of verapamil on Rh123 accumulation (Fig. 3.4 and 3.10), therefore the ability of P-glycoprotein in the HEL and KG-1a cells to remove drug from the cells probably contributed to their reduced differentiation response. The drugs may not have reached the potential intracellular target or binding site through which they may stimulate differentiation.

Although the KG-1a sublines did not become more drug resistant in response to epirubicin or vinblastine, the KG-1a/V16 subline underwent megakaryocytic differentiation, with increased expression of CD61 and reduced CD34 expression. Similarly, the HEL sublines did not become more drug resistant in response to treatment with epirubicin or vinblastine, however they underwent megakaryocytic differentiation, with increased expression of CD61 and a reduction in glycophorin A expression. HEL cells have previously been reported to express megakaryoblastic features, and undergo further megakaryocytic differentiation in response to treatment with TPA (Ylänne *et al*, 1988).

The lack of response to the effects of cytotoxic drugs and expression of P-glycoprotein in the KG-1a and HEL cells corresponds to the clinical situation where P-glycoprotein expression correlates with poor response to treatment in leukaemias such as AML (Sato *et al*, 1990b).

The KG-1a cells also express the CD34 antigen, and a significant correlation between expression of CD34 and P-glycoprotein has been demonstrated in AML patients (Geller *et al*, 1990; Tiirikainen *et al*, 1992), with lower complete response rates in those pateints with cells expressing CD34 (Guinot *et al*, 1991; Thomas *et al*, 1992). Expression of CD34 indicates a primitive stem-cell-like phenotype, suggesting that the development of drug resistance may be associated with a less-differentiated phenotype. The lack of response to cytotoxic drugs in the treated KG-1a cells supports the clinical findings that P-glycoprotein and CD34 expression are of poor prognosis.

Expression of the MDR phenotype and P-glycoprotein prior to drug treatment therefore influences the response of human leukaemic cells to chemotherapeutic drugs. Cells which do not express P-glycoprotein may develop drug resistance and undergo differentiation, while expression of P-glycoprotein renders cells resistant to the effects of clinically relevant levels of chemotherapeutic drugs.

4 INDUCTION OF DIFFERENTIATION IN K562 AND K562/E15B CELLS

4.1 Introduction

Leukaemias are thought to arise from a blockage in the normal differentiation pathway of haematopoiesis. The maturation-arrested leukaemia cells are able to proliferate without differentiating, and a population of immature cancer cells arises. Differentiation therapy is a new approach to the treatment of leukaemia, whereby the differentiating agent is administered before chemotherapy in an attempt to overcome the block maturation of the leukaemic cells, also rendering the cells more sensitive to the effects of cytotoxic drugs. Retinoic acid (Castaigne *et al*, 1990; Bashford *et al*, 1991) and GM-CSF are currently undergoing clinical trial (Estey *et al*, 1992). It is important to know if these agents will cause changes in drug resistance, particularly in patients who may already be refractory to treatment.

Recent studies have saught to examine the effects of differentiating agents on P-glycoprotein expression, in order to determine any association between differentiation and the MDR phenotype. Inducers of differentiation which have been used to study the relationship between P-glycoprotein and differentiation *in vitro* include sodium butyrate, 12-O-tetradecanoylphorbol-13-acetate (TPA), and biological inducers of differentiation such as activin A.

Drug resistance and P-glycoprotein expression after treatment with TPA have been studied in a variety of leukaemia and solid tumour cell lines. Treatment of the P-glycoprotein expressing CEM/VLB$_{100}$ subline induced differentiation with increases in resistance to vinblastine (Beck *et al*, 1986b). Treatment of drug resistant KB-V1 cells with 200 nM TPA for up to 24 hours phosphorylated P-glycoprotein and also stimulated its drug transport activity, in a time- and dose-dependent manner (Chambers *et al*, 1990). Furthermore treatment of cell lines including the KG-1, KG-1a and K562 cells, and solid tumour cell lines such as the MCF-7 breast carcinoma with TPA increased the expression of P-glycoprotein (Chaudhary and Roninson, 1992).

Recently, PKC has been shown to directly phosphorylate P-glycoprotein on serine residues clustered in the linker region between the two halves of the molecule. This site is analogous to the regulatory domain on the CFTR protein, suggesting a putative regulatory domain on which PKC acts upon P-glycoprotein (Chambers *et al*, 1993). TPA is thought to act through PKC activity, and it has therefore been used in conjunction with PKC inhibitors to elucidate the role of PKC in drug resistance. This has been confirmed by transfection of P-glycoprotein expressing BC-19 cells with PKCα cDNA,

which conferred resistance to doxorubicin and vinblastine, with decreased drug accumulation and increased phosphorylation of P-glycoprotein (Yu *et al*, 1991).

Overexpression of PKC has also been observed in a variety of drug resistant cell lines. Drug resistant P388/ADR cells overexpress PKCα and PKCβ isoforms, compared to the drug sensitive P388 cells (Gollapudi *et al*, 1992), and Posada *et al* (1989) have shown that multidrug resistant human KB carcinoma cells have an 80% to 90% increase in basal PKC activity compared to the parental drug sensitive cells. Similarly, MDR MCF-7 cells have a 7-fold higher level of PKC activity compared to the drug-sensitive parental cells (Fine *et al*, 1988). These studies suggest that drug resistant cells have elevated levels of PKC, which may be responsible for phosphorylation of P-glycoprotein.

Staurosporine, an inhibitor of PKC, has also been used in conjunction with TPA to examine the role of PKC in drug resistance. Activation of *mdr1* gene expression by TPA has been shown to be inhibited by staurosporine (Chaudhary and Roninson, 1992). Treatment of P-glycoprotein expressing KB carcinoma cells with staurosporine also resulted in decreased resistance to doxorubicin (Posada *et al*, 1989), suggesting inhibition of PKC results in decreases in expression of the MDR phenotype. However, others have shown that staurosporine itself has been shown to reverse MDR, enhancing drug accumulation in MDR cells and reduce P-glycoprotein expression (Sato *et al*, 1990c; Sampson *et al* 1993; Wakusawa *et al*, 1993). Staurosporine and its derivatives are able to inhibit [^{3}H]-azidopine photoaffinty-labeling of P-glycoprotein, and reverse MDR by directly inhibiting the binding of drugs to P-glycoprotein, rather than inhibiting protein kinases (Sato *et al*, 1990c; Miyamoto *et al*, 1993). These studies suggest PKC is involved in the upregulation of drug resistance, and that TPA increases P-glycoprotein activity and function through PKC. Furthermore, staurosporine may act on P-glycoprotein directly, as well as through PKC.

Sodium butyrate has also been used to investigate the effect of differentiation on P-glycoprotein expression. Mickley *et al* (1989) reported large increases in P-glycoprotein expression in some colon carcinoma cell lines after treatment with sodium butyrate. This correlated with the degreee of differentiation, but without accompanying increases in resistance to cytotoxic drugs. Similarly, Bates *et al* (1992) showed that sodium butyrate induced P-glycoprotein expression in SW620 human colon carcinoma cell lines but with increases in drug accumulation rather than decreases, which was attributed to alterations in P-glycoprotein phosphorylation, resulting in a P-glycoprotein with altered drug affinities. Conversely, sodium butyrate treatment of a rat colon cancer cell line induced differentiation but did not induce *mdr*1 expression (Petit *et al*, 1993). Therefore it appears that sodium butyrate induces P-glycoprotein expression without P-glycoprotein function in some human colon cell lines, with varying degrees of differentiation.

The effects of sodium butyrate have also been studied in K562 cells. Shibata *et al* (1990) treated K562 and MDR K562 cells (K562/ADM and K562/VCR) with sodium butyrate, resulting in *mdr*1 expression. However, the P-glycoprotein induced in these cells was not functional, as found in the studies on colon carcinoma cells (Mickley *et al*, 1989), and sodium butyrate did not induce erythroid differentiation. Sodium butyrate treatment of the K562/VCR cells, demonstrated increased MRK16 binding, and therefore increased P-glycoprotein expression, which was accompanied by erythroid differentiation (Okabe-Kado *et al*, 1991). Sodium butyrate therefore appears to increase P-glycoprotein expression in drug resistant K562 cells.

Sodium butyrate has been used in the past in the treatment of leukaemia. However, very few studies have been undertaken, and it appears that the use of sodium butyrate was with little success, as it had no effect in the patients, and did not improve survival (Novodgrodsky *et al*, 1983; Miller *et al*, 1987).

Activin A, another inducer of erythroid differentiation in K562 cells, has been shown to enhance the sensitivity of vincristine resistant K562/VCR cells to MDR drugs, with decreased expression of P-glycoprotein (Okabe-Kado *et al*, 1991). Therefore erythroid differentiation may not correlate with either increases or decreases in P-glycoprotein expression.

The growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to increase erythroid differentiation in K562 cells (Tawhid *et al*, 1989). *In vivo*, GM-CSF stimulates the growth and function of neutrophils, monocytes and macrophages in human bone marrow. GM-CSF receptors are expressed on human neutrophils, eosinophils and monocytes (Moore, 1990).

Recombinant human GM-CSF (rhGM-CSF) has been shown to enhance the effects cytotoxic drugs in leukaemic cells and blast cells from leukaema patients (Cannistra *et al*, 1989; Bhalla *et al*, 1991; Hassan and Maurer, 1991; Waga *et al*, 1992). However little is known about its effects on drug resistant cells either *in vitro* or *in vivo*.

Any association between P-glycoprotein expression and differentiation is unclear. While P-glycoprotein is expressed on CD34+ human haematopoietic stem cells (Chaudhary and Roninson, 1991), which are undifferentiated, P-glycoprotein has also been detected in tumours which are well differentiated (Mickley *et al*, 1989). As K562 and U937 cells treated with the chemotherapeutic drugs vinblastine and epirubicin were induced to differentiate as well as develop various mechanisms of drug resistance, the effects of known differentiating agents on the drug resistance phenotypes of these two cell lines and drug resistant sublines were examined. TPA induces megakaryocytic differentiation in K562 cells (Yen *et al*, 1993), and monocytic differentiation in U937 cells (Hass *et al*, 1992) while sodium butyrate induces erythroid differentiation (Sutherland *et al*, 1986) in K562 cells.

The interaction of TPA and staurosporine on differentiation and drug resistance in K562, U937, K562/E15B and U937/E15 cells were therefore investigated in order to further understand the role of PKC in inducing drug resistance and differentiation. The effects of other inducers of differentiation, namely sodium butyrate and GM-CSF were also compared to TPA, in order to determine whether differentiation *per se* resulted in increases in drug resistance.

P-glycoprotein expression was determined using C219. When studying changes in P-glycoprotein expression in response to treatment with differentiation agents, it is important to also determine any changes in P-glycoprotein function, as some studies have found *mdr*1/P-glycoprotein expression can increase without accompanying increases in drug resistance (Shibata *et al*, 1990). P-glycoprotein function was therefore determined after induction of differentiation by cytotoxicity assays and also by changes in Rh123 accumulation in the presence of verapamil.

4.2 Induction with TPA

4.2.1 The Effect of TPA on Differentiation

Treatment of the K562 cells and the drug resistant K562/E15B subline with 1, 5 and 16 nM TPA for 18 h induced megakaryocytic differentiation which was defined by an increase in CD61 expression (Fig 4.1) and a decrease in glycophorin A expression (Fig. 4.2). Glycophorin A was not expressed in the K562 cells while low expression remained in the K562/E15B subline after treatment with 16 nM TPA. Four days treatment with TPA gave similar results, and therefore treatment for 18 h with 5 nM TPA was used in further experiments.

TPA has been shown to increase PKC activity (Slapak *et al*, 1993). In order to determine whether the effects of TPA were being mediated by PKC, cells were treated with the PKC inhibitor staurosporine alone and before addition of TPA. 30 nM staurosporine was chosen as this concentration has previously been shown to inhibit PKC activity in many other human cell lines (Chaudhary and Roninson, 1992).

Treatment with staurosporine alone induced some megakaryocytic differentiation, with increased expression of CD61 and decreased glycophorin A expression in both the K562 and K562/E15B cells, although the changes were less marked than after TPA treatment (Fig. 4.3). Pretreatment of the K562 and K562/E15B cells with staurosporine partially inhibited the increase in CD61 induced by TPA, while glycophorin A expression was further decreased (Fig. 4.3; staurosporine is abbreviated to STP in all figures). Therefore the responses to TPA and staurosporine were similar in the K562 and K562/E15B cells. To fully determine whether inhibition of PKC also inhibited megakaryocytic differentiation, it would be necessary to examine the effects of other inhibitors of PKC.

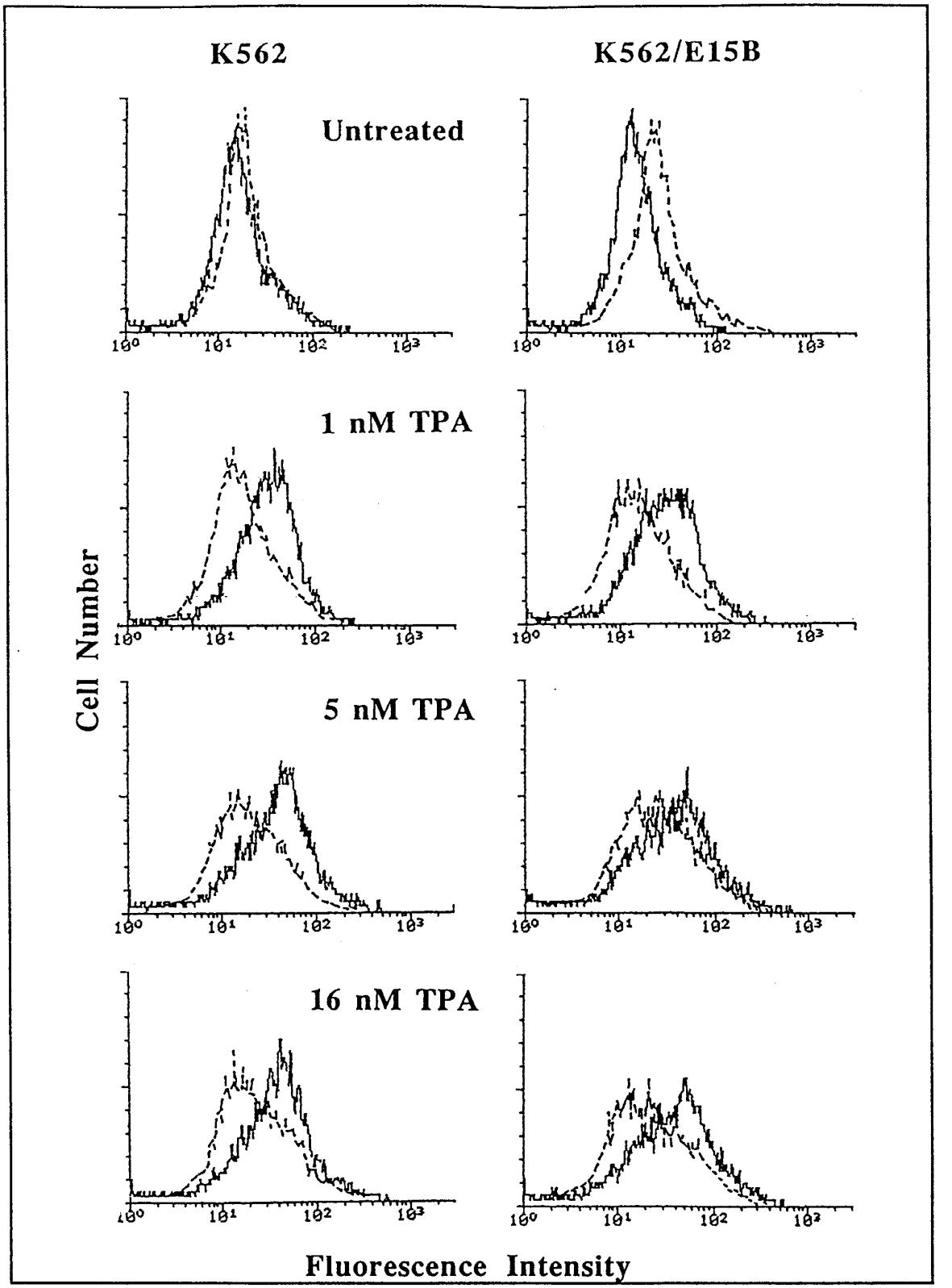

Figure 4.1. The effect of TPA treatment on CD61 expression in K562 and K562/E15B cells. Cells treated with TPA for 18 h after which they were analysed for expression of CD61(—) by flow cytometry as described in *Materials and Methods 8*, and the fluorescence profile is compared to cells incubated with the negative control antibody(---). Experiments were repeated twice and a representative result is shown.

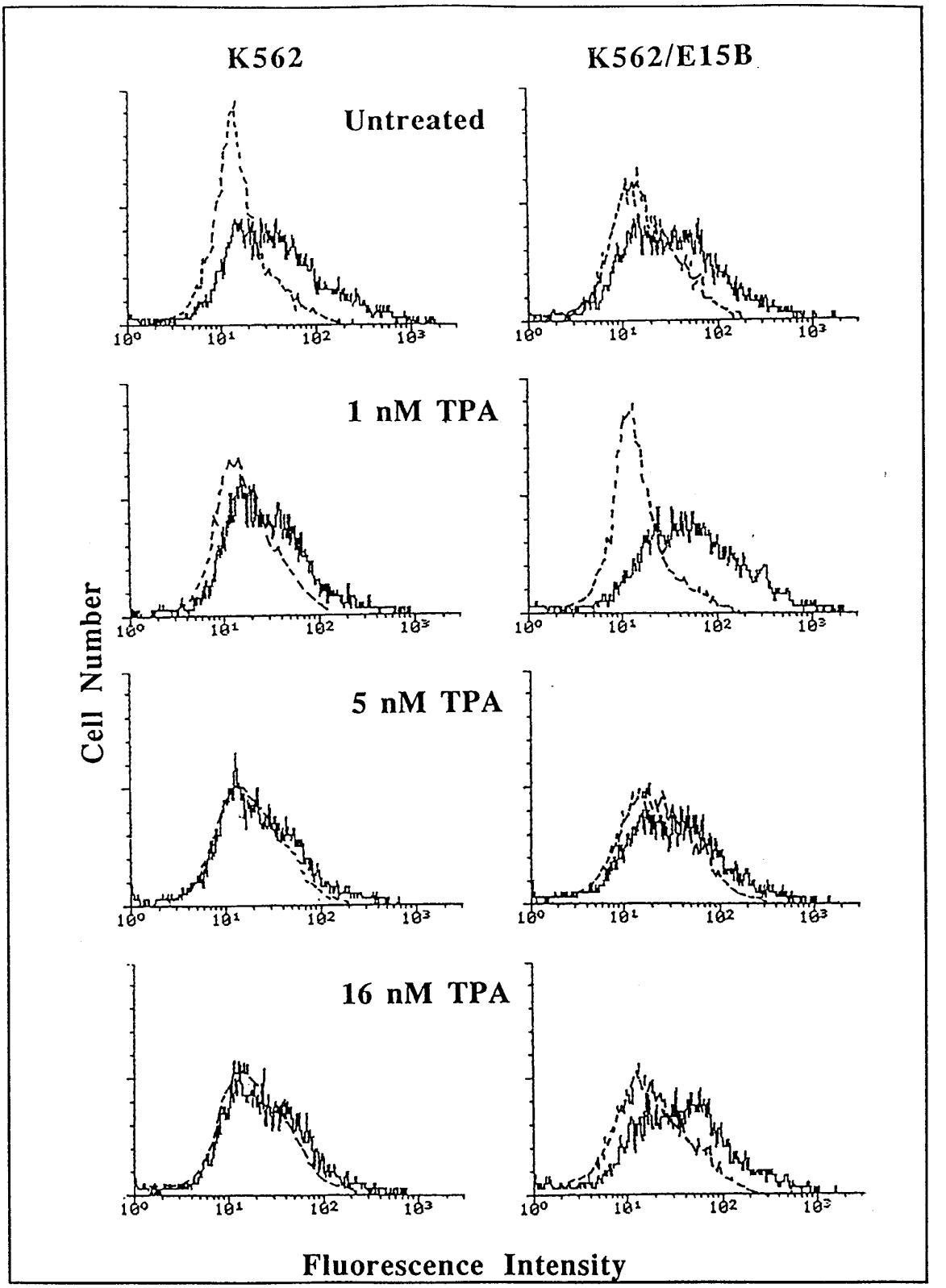

Figure 4.2. The effect of TPA treatment on glycophorin A expression in K562 and K562/E15B cells. Cells were treated with TPA for 18 h after which they were analysed for expression of glycophorin A(—) by flow cytometry as described in *Materials and Methods 8*, and the profile is compared to cells incubated with the negative control antibody(---). Experiments were repeated twice and a representative result is shown.

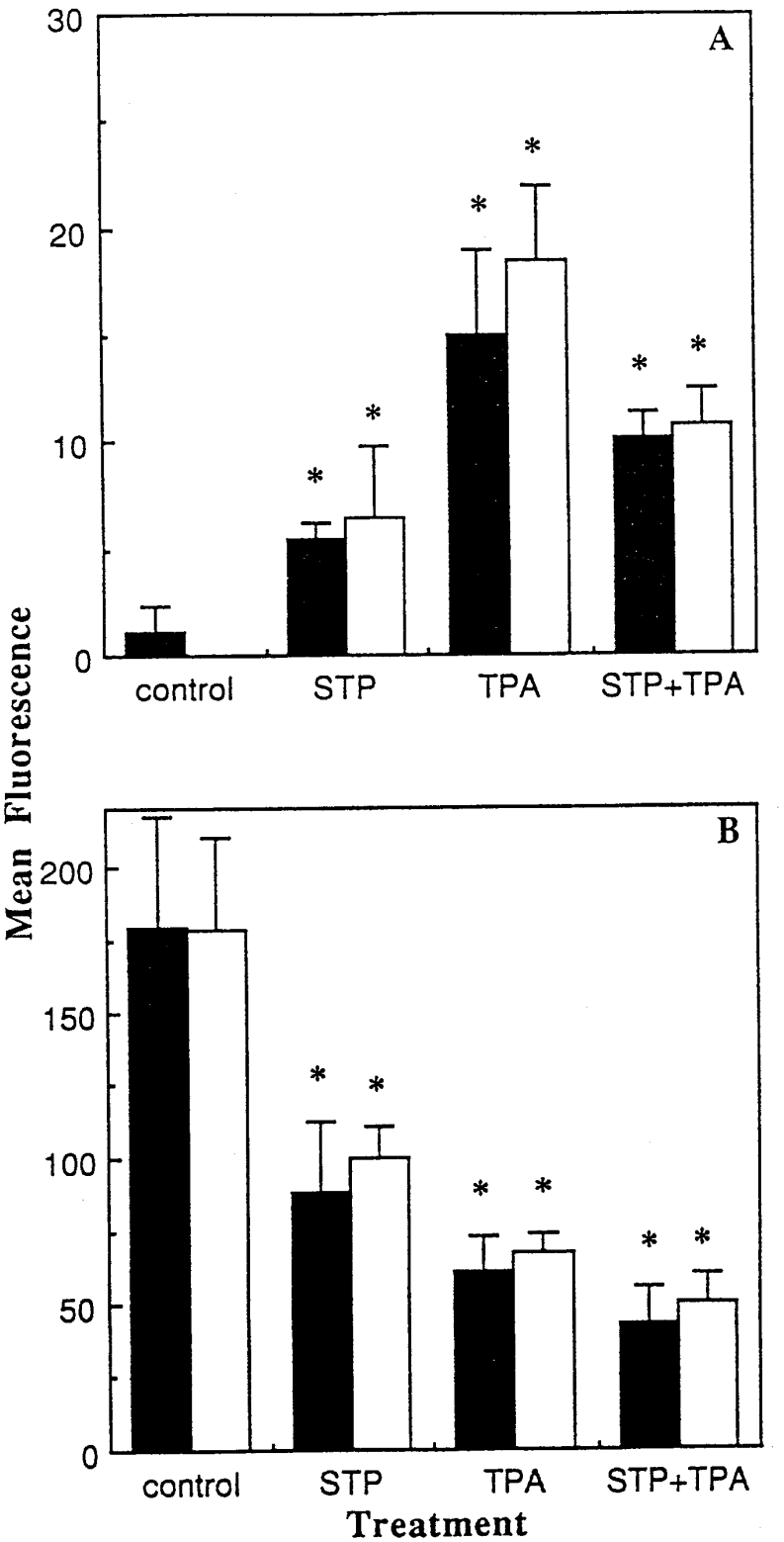

Figure 4.3. Summary of the effects of TPA and staurosporine on CD61 and glycophorin A expression in K562 and K562/E15B cells. A. CD61 expression and B. glycophorin A expression, K562 cells(□) and K562/E15B cells(■). Results are the mean of 3 experiments; *error bars*, standard error of the mean; * indicates significant difference ($p<0.05$) from the untreated cells using the student's t-test.

In order to determine whether changes in other drug resistance proteins might be associated with differentiation, expression of the drug-resistance and differentiation-associated protein CD36, detected by the monoclonal antibody MRK20, was investigated after treatment with TPA, in the presence and absence of staurosporine. The K562 cells did not express CD36, and expression was not changed by treatment with staurosporine or TPA alone, or pretreatment with staurosporine before the addition of TPA (Fig. 4.4). The K562/E15B cells expressed CD36. Expression was increased by treatment with staurosporine and decreased by treatment with TPA. Staurosporine partially inhibited the decrease in CD36 expression induced by TPA in the K562/E15B cells, with CD36 expression remaining similar to the untreated cells (Fig. 4.4).

4.2.2 The Effect of TPA on Drug Resistance

K562 cells and the K562/E15B subline were treated with TPA for 18 h, after which resistance to epirubicin and vinblastine were determined. The effect of TPA on both epirubicin and vinblastine resistance was variable, however the resistance in general tended to be increased. The results are summarised in Table 4.1 and representative results are shown in Fig. 4.5. TPA therefore increased drug resistance in both K562 cells and the K562/E15B subline.

Treatment of the K562 and K562/E15B cells with staurosporine and 5 nM TPA in combination was toxic to the cells, as growth in the control wells of the plates after 4 days was insufficient for the cytotoxicity assay to be valid.

Table 4.1. The effect of TPA on resistance to epirubicin and vinblastine in K562 and K562/E15B cells.

Treatment	Fold Increase[a]	
	Epirubicin	**Vinblastine**
K562 + 5 nM TPA	4.9 ± 2.7[b]	27.5 ± 23.8
K/E15B + 5 nM TPA	2.8 ± 1.6	3.1 ± 1.4

aFold increase was determined by dividing the IC_{50} of treated cells by the IC_{50} of the untreated cells.
bMean ± Standard deviation, n=3.

4.2.3 The Effect of TPA on P-glycoprotein Expression

P-glycoprotein expression was determined by Western blot analysis using the monoclonal antibody C219 after treatment of the K562 and K562/E15B cells with TPA in the presence and absence of staurosporine. The K562 cells appeared to have a faint band at 170kD, indicating low level P-glycoprotein expression (Fig. 4.6). However P-glycoprotein expression did not change in K562 cells with any of the treatments.

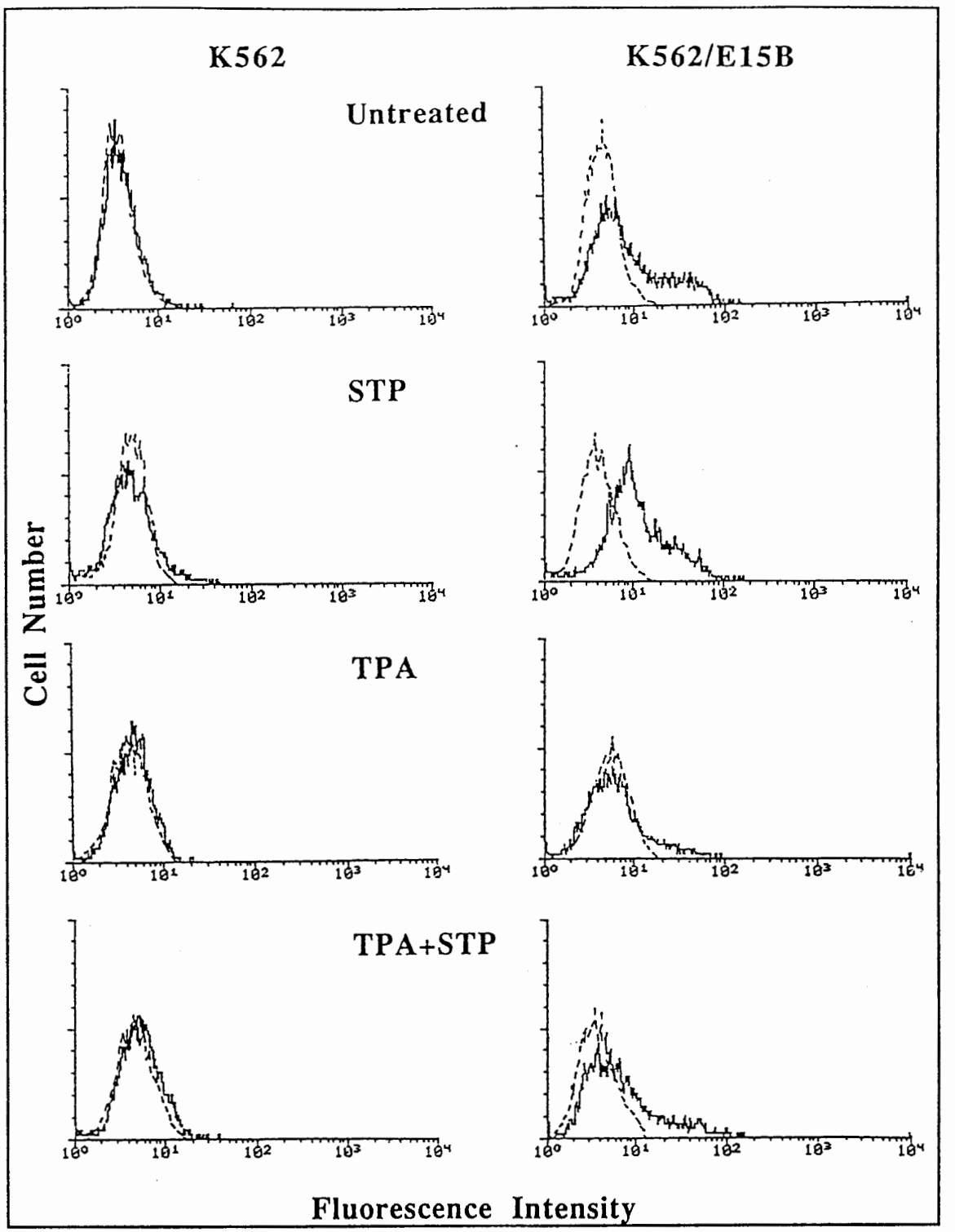

Figure 4.4. The effect of TPA and staurosporine on CD36 expression in K562 and K562/E15B cells. Cells were treated with TPA and/or staurosporine for 18 h after which they were analysed for expression of CD36(—) by flow cytometry as described in *Materials and Methods 8*, and the fluorescence profile is compared cells incubated with the negative control antibody(---). Experiments were repeated twice and a representative result is shown.

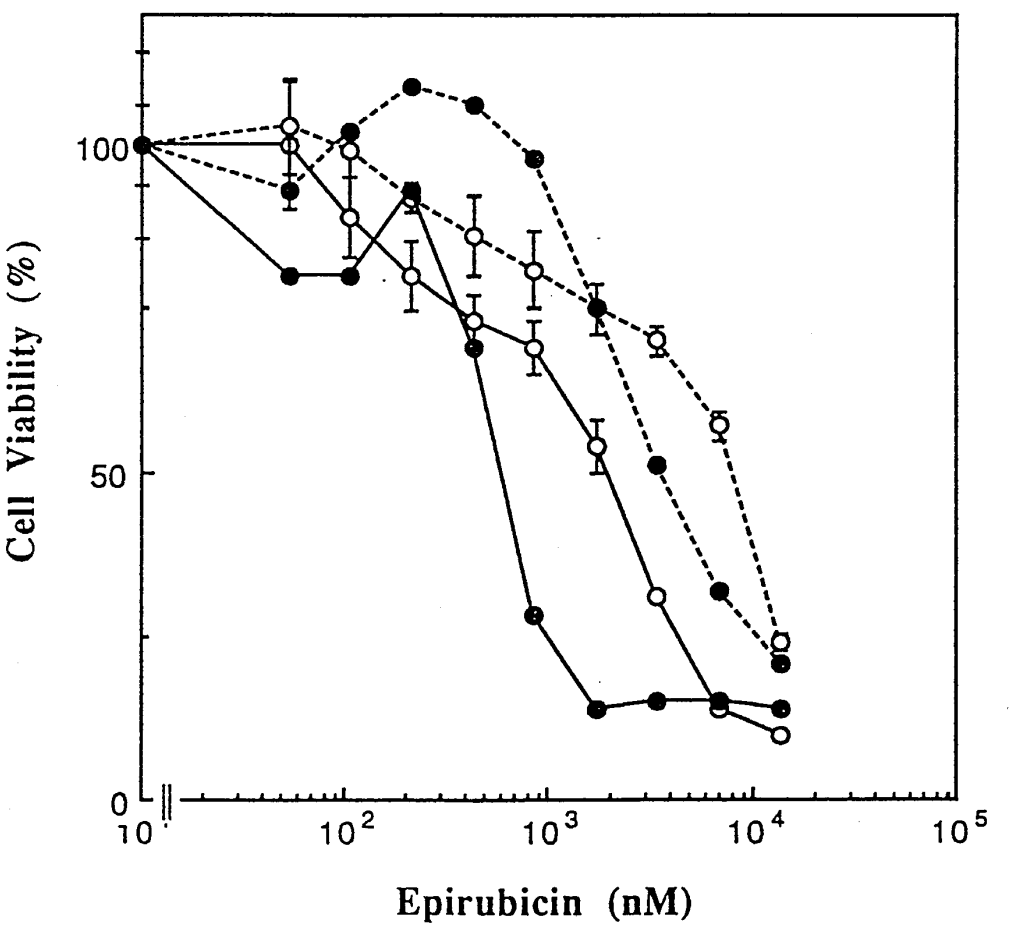

Figure 4.5. The effect of TPA on epirubicin resistance of K562 and K562/E15B cells. Cells were treated with 5 nM TPA(---) for 18 h and resistance is compared to untreated cells(—). K562(●) cells, K562/E15B cells (o). *Points*, mean of triplicate determinations; *error bars*, standard deviations. Experiments were repeated at least three times and a representative result is shown.

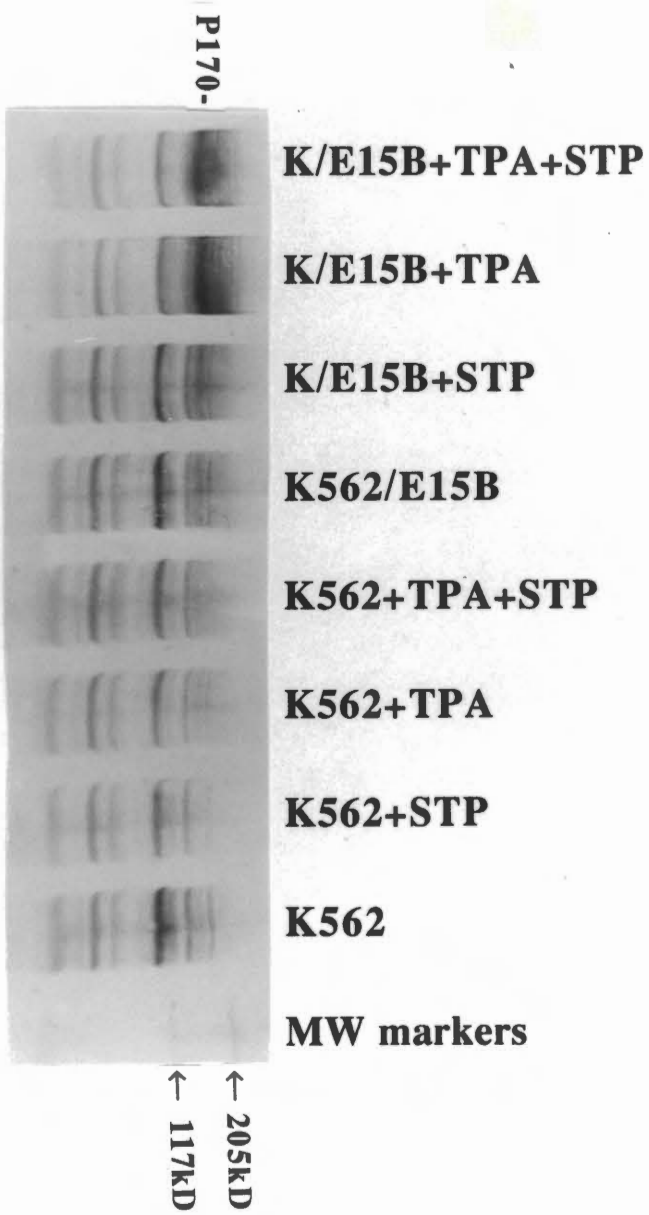

Figure 4.6. Western blot analysis of the effects of TPA and staurosporine on P-glycoprotein expression in K562 and K562/E15B cells. Cells were treated with TPA and/or staurosporine for 18 h, after which plasma membranes were prepared and analysed as described in *Materials and Methods 6.1*. The positions of molecular weight markers are indicated.

However, TPA induced a large increase in P-glycoprotein expression in the K562/E15B cells (Fig. 4.6). Pretreatment with staurosporine did not inhibit the increase in P-glycoprotein expression, and staurosporine alone also had no effect. Therefore TPA increased P-glycoprotein expression in the K562/E15B cells but not the K562 cells.

4.2.4 The Effects of TPA on Rh123 Accumulation

P-glycoprotein function was assessed by the ability of the cells to retain Rh123 after treatment with TPA and staurosporine, and the results are summarised in Figure 4.7. TPA significantly reduced Rh123 accumulation in the K562/E15B subline but did not significantly reduce Rh123 accumulation in the K562 cells. Staurosporine did not inhibit the reduction in Rh123 accumulation induced by TPA in the K562/E15B subline.

Verapamil had no effect on Rh123 accumulation in the K562 cells, nor did it increase Rh123 accumulation after treatment with TPA, staurosporine or TPA and staurosporine in combination (Fig. 4.8). However, verapamil increased Rh123 accumulation in the K562/E15B cells after treatment with staurosporine, TPA, and TPA and staurosporine in combination. These data indicate that TPA increases P-glycoprotein function in the K562/E15B subline, but not the K562 cells, and the ability of TPA to increase P-glycoprotein function is not inhibited by the PKC inhibitor staurosporine.

4.3 Induction with Sodium Butyrate

4.3.1 The Effect of Sodium Butyrate on Differentiation

Glycophorin A expression was determined after treatment of K562 and K562/E15B cells with 1.5 mM sodium butyrate. Glycophorin A expression and CD61 expression in the K562 and K562/E15B cells did not change after 18 h treatment (Fig. 4.9). However, after 4 days exposure to 1.5 mM sodium butyrate, glycophorin A expression in both cell lines was increased, with the emergence of 2 sub-populations, one of which did not express glycophorin A and one which was strongly positive (Fig. 4.10). These results indicate that sodium butyrate initially had little effect on glycophorin A expression, while longer exposure resulted in higher expression of glycophorin A and erythroid differentiation in a sub-population of K562 and K562/E15B cells.

Expression of CD61 was also induced by 4 days exposure to 1.5 mM sodium butyrate. Taken together with glycophorin A expression, this data suggests that sodium butyrate may induce both megakaryocytic and erythroid differentiation in K562 cells and the K562/E15B subline.

4.3.2 The Effect of Sodium Butyrate on Drug Resistance

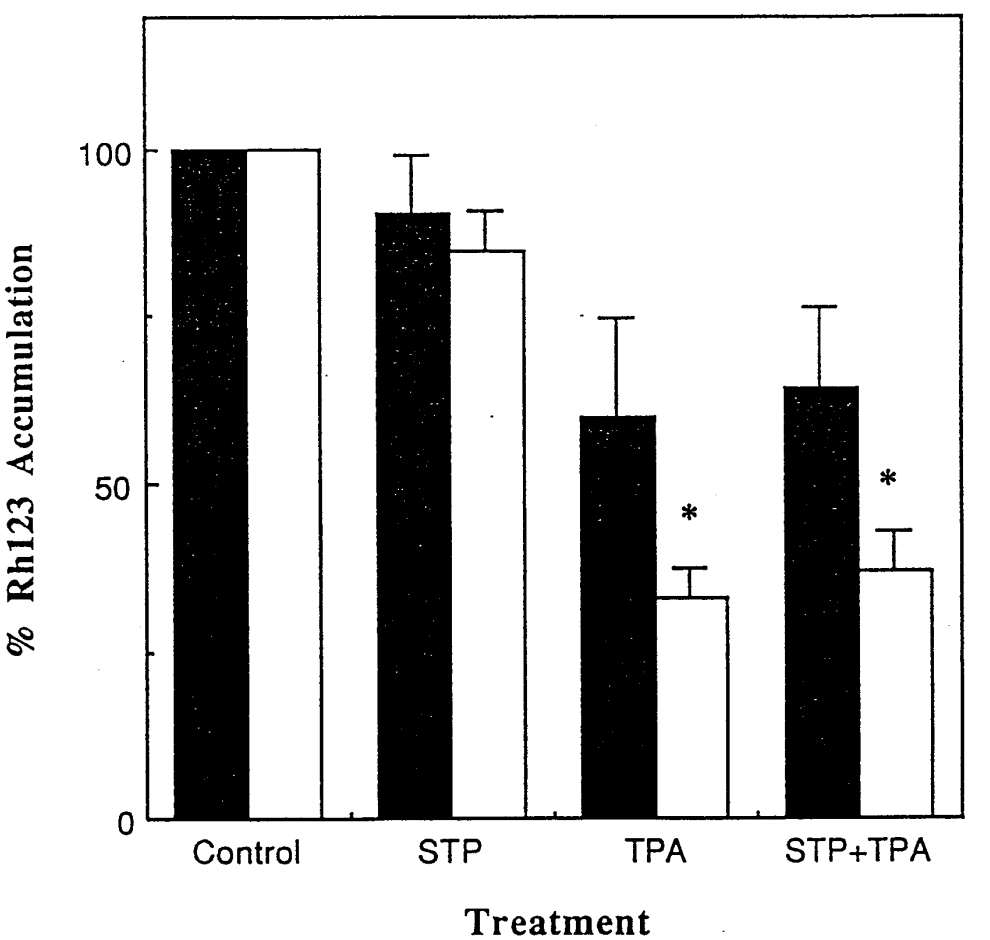

Figure 4.7. Summary of the effects of TPA and staurosporine on Rh123 accumulation in K562 cells and the K562/E15B subline. Cells were treated with TPA and/or staurosporine for 18 h, after which Rh123 accumulation was determined by flow cytometry as described in *Materials and Methods 7.* K562 cells(■) and K562/E15B cells(□). Results are the mean of two experiments and are calculated as a percentage of untreated cells; *error bars*, standard deviation; * indicates a significant difference ($p<0.01$) from the untreated cells using the student's t-test.

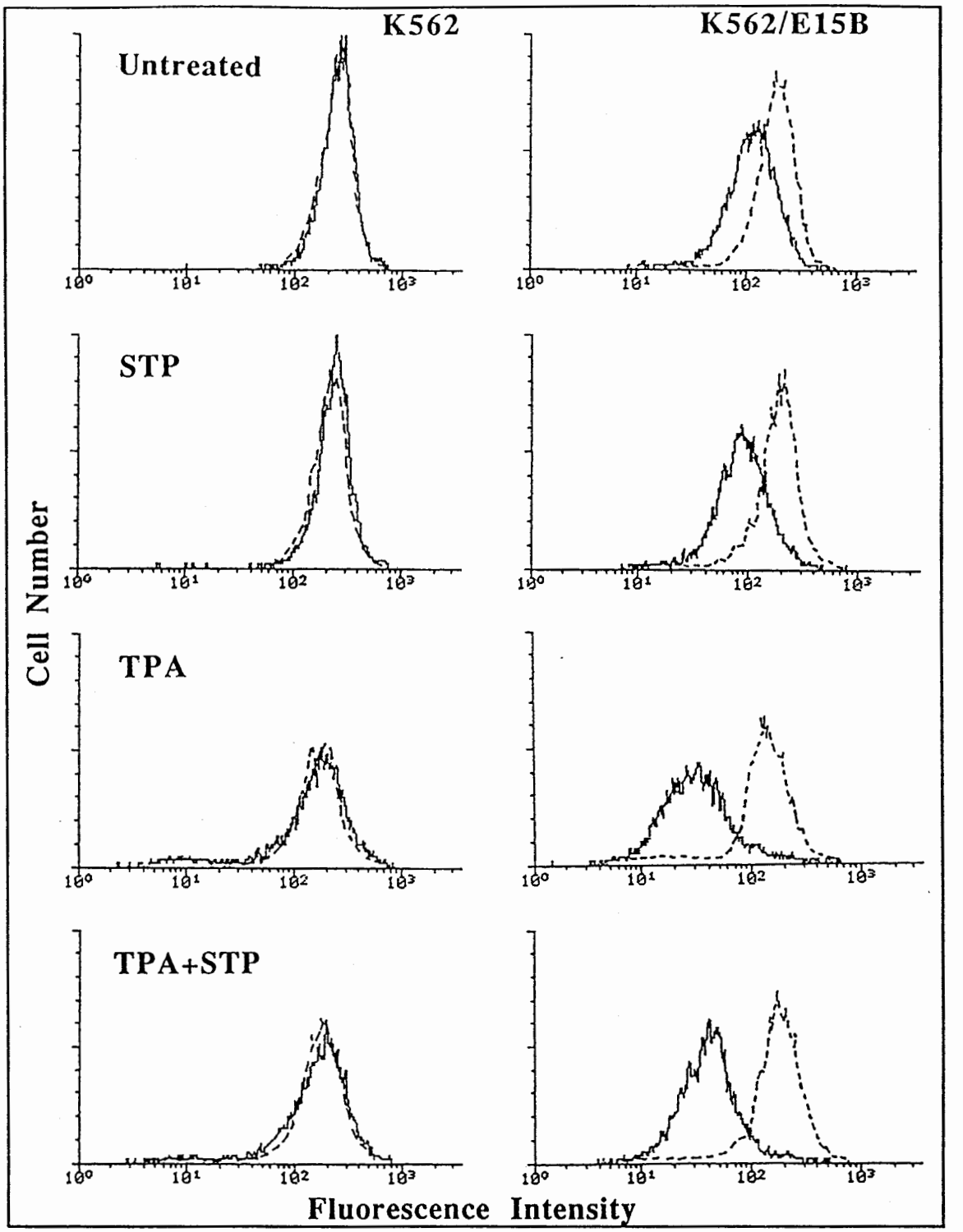

Figure 4.8. The effect of verapamil on Rh123 accumulation after treatment with TPA and staurosporine in K562 and K562/E15B cells. Cells were treated with TPA and/or staurosporine for 18 h, after which Rh123 accumulation in the presence(---) or absence(—) of 10 μM verapamil was determined by flow cytometry as described in *Materials and Methods 7*. The experiment was repeated twice and a representative result is shown.

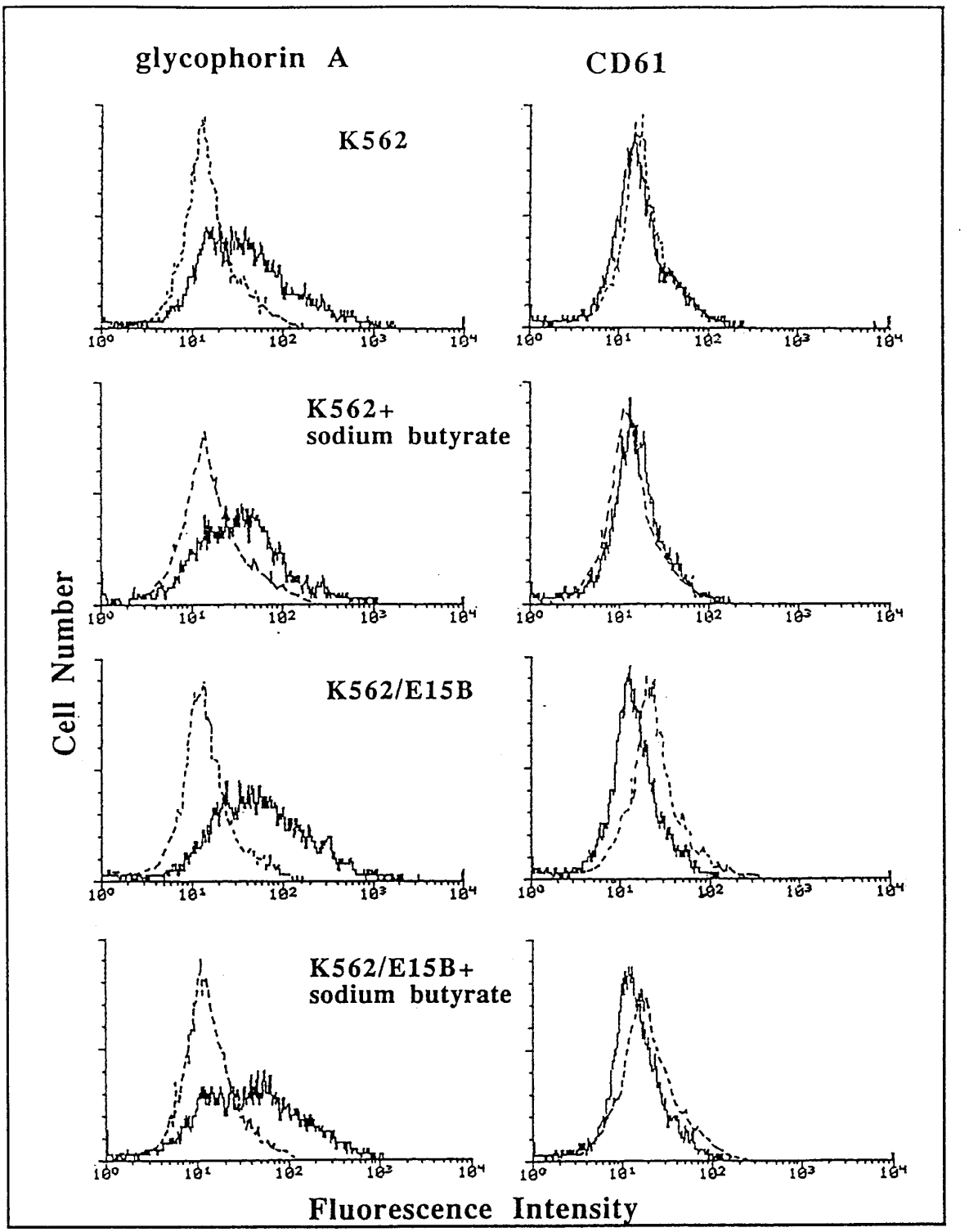

Figure 4.9. The effect of 18 h treatment with sodium butyrate on glycophorin A and CD61 expression in K562 and K562/E15B cells. Cells were treated with 1.5 mM sodium butyrate for 18 h, after which they were analysed for expression of glycophorin A and CD61(—) by flow cytometry as described in *Materials and Methods 8*. Fluorescence profiles are compared to cells incubated with the negative control antibody(---). The experiment was repeated at least three times and a representative result is shown.

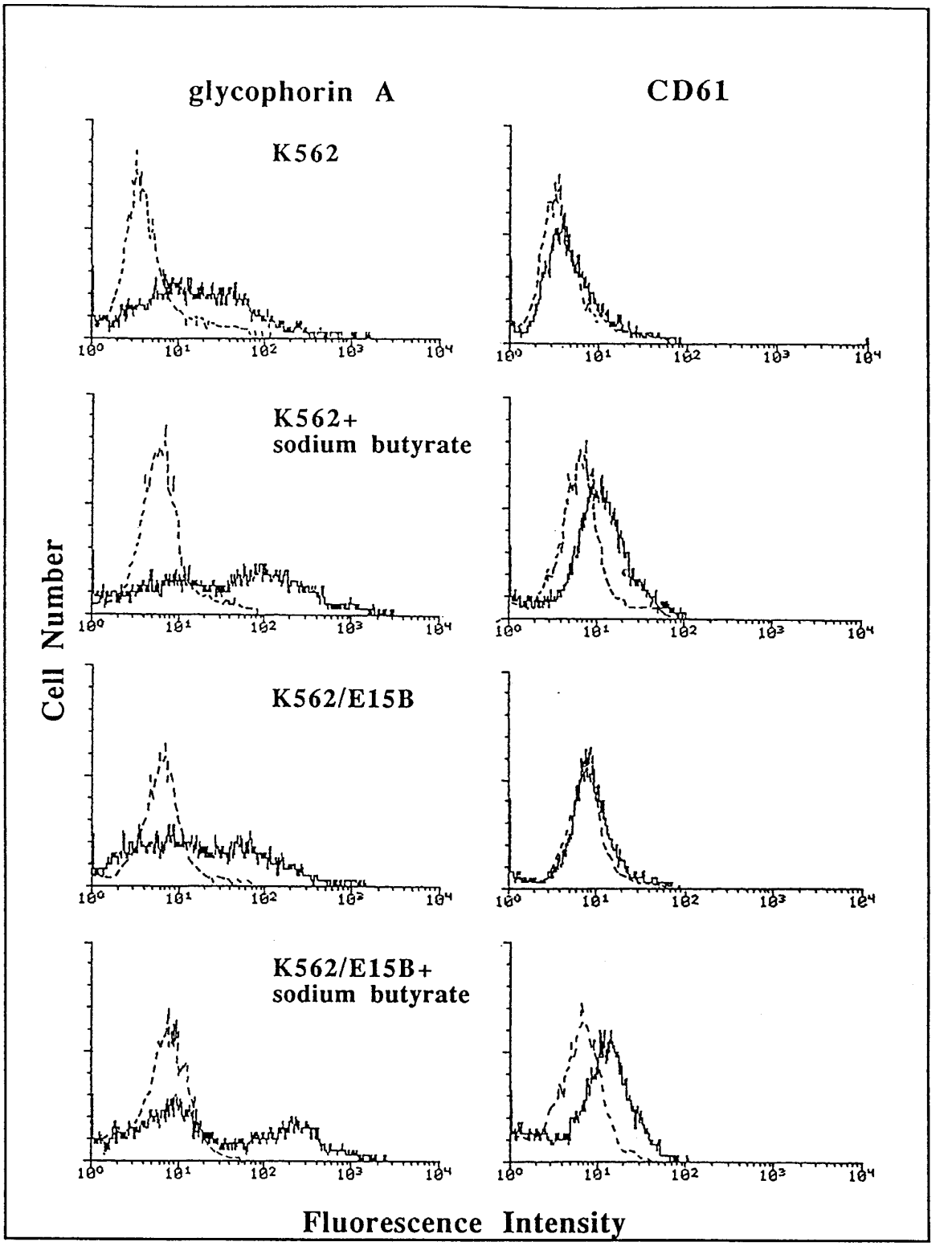

Figure 4.10. The effect of 4 d treatment with sodium butyrate on glycophorin A and CD61 expression in K562 and K562/E15B cells. Cells were treated with 1.5 mM sodium butyrate for 18 h after which expression of CD61 and glycophorin A(—) were determined by flow cytometry as described in *Materials and Methods 8*. Fluorescence profiles are compared to cells incubated with the negative control antibody(---). The experiment was repeated at least three times and a representative result is shown.

15 mM sodium butyrate was also used to investigate changes in drug resistance and P-glycoprotein expression as other studies have shown that higher doses increase P-glycoprotein expression (Shibata *et al*, 1990). Treatment of the K562 cells with 1.5 mM sodium butyrate had no effect on resistance to epirubicin or colchicine. Colchicine was tested as sodium butyrate had been shown to change colchicine resistance in a study by Bates *et al* (1992). Treatment of the K562/E15B subline with 1.5 mM sodium butyrate produced a small but statistically significant increase in resistance to colchicine, with no effect on epirubicin resistance. 15 mM sodium butyrate decreased resistance to epirubicin in the K562 cells, with no effect on colchicine resistance, while both epirubicin and colchicine resistance were both decreased after treatment of the K562/E15B cells with 15 mM sodium butyrate. These results are summarised in Table 4.2. Therefore 1.5 mM and 15 mM sodium butyrate had differing effects on drug resistance in the K562 and K562/E15B cells.

Table 4.2. The effects of sodium butyrate on drug resistance in K562 and K562/E15B cells.

Treatment	Epirubicin IC_{50} (μM)	Colchicine IC_{50} (μM)
K562	0.600 ± 0.141	0.007 ± 0.001
K562+1.5mM NaB	0.415 ± 0.191 (1.4)[a]	0.007 ± 0.001 (0.0)
K562+15mM NaB	0.225 ± 0.106 **(2.7)**[a]	0.005 ± 0.007 (1.6)[a]
K562/E15B	1.200 ± 0.000	0.022 ± 0.000
K/E15B+1.5mM NaB	1.850 ± 0.778 [1.5][b]	0.030 ± 0.000 [1.4]*
K/E15B+15mM NaB	0.550 ± 0.070 **(2.2)**[a]*	0.011 ± 0.006 **(2.1)**[a]

a Number in parentheses indicates fold-decrease in resistance.
b Number in square parentheses indicates fold-increase in resistance.
*Indicates $p<0.05$ using the student's t-test, n=2.
Bold type indicates more than a 2-fold difference between sodium butyrate treated cells and untreated cells.

4.3.3 The Effects of Sodium Butyrate on P-glycoprotein Expression

K562 cells and K562/E15B subline were treated for 18 h with 1.5 and 15 mM sodium butyrate, after which P-glycoprotein expression was detected by Western blotting using C219 (Fig. 4.11). Treatment of K562 cells with 1.5 and 15 mM sodium butyrate had no effect on P-glycoprotein expression. Treatment of the K562/E15B subline with 1.5 and 15 mM sodium butyrate increased P-glycoprotein expression (Fig. 4.11).

4.3.4 The Effect of Sodium butyrate on Rh123 Accumulation

The effect of sodium butyrate on P-glycoprotein function in the K562 and K562/E15B cells was investigated by Rh123 accumulation. Neither 1.5 or 15 mM sodium butyrate

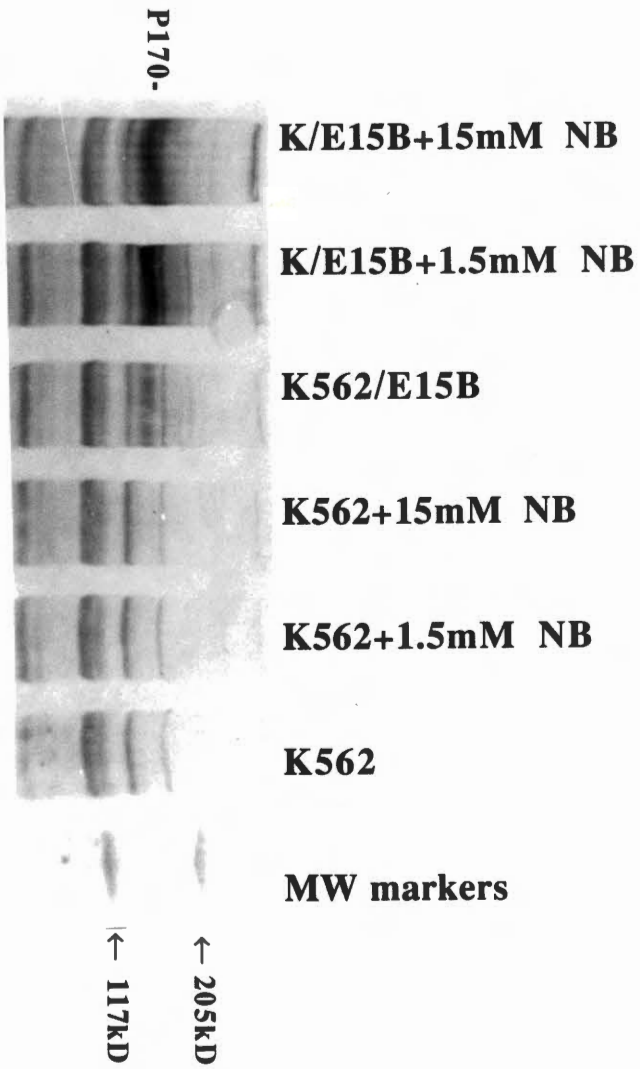

Figure 4.11. Western blot analysis of the effect of sodium butyrate on P-glycoprotein expression in K562 and K562/E15B cells. Cells were treated for 18 h with 1.5 or 15 mM sodium butyrate, after which plasma membranes were prepared and analysed as described in *Materials and Methods 6.1*. Positions of the molecular weight markers and P-glycoprotein (P-170) are indicated.

had any effect on Rh123 accumulation in the K562 cells (Fig. 4.12). Rh123 accumulation was decreased by 1.5 mM but not 15 mM sodium butyrate in the K562/E15B cells, indicating increased P-glycoprotein function after treatment with 1.5 mM sodium butyrate. The increase in P-glycoprotein function is in agreement with the increase in P-glycoprotein detected by C219. However, the increase in P-glycoprotein expression induced by 15 mM sodium butyrate was not paralleled by a decrease in Rh123 accumulation.

4.4 Induction with GM-CSF

4.4.1 The Effect of GM-CSF on Differentiation

GM-CSF had little effect on glycophorin A or CD13 expression in either the K562 cells or the K562/E15B subline after both 18 and 42 h exposure.

4.4.2 The Effect of GM-CSF on Drug Resistance

The K562 cells were initially exposed to 20, 100 or 1000 ng/ml of GM-CSF for 18 and 42 h in order to determine a suitable dose with which to treat the cells. 18 h treatment with GM-CSF had little effect on vinblastine cytotoxicity in the K562 cells at any of the three concentrations (Fig. 4.13). After 42 h treatment, 20 ng/ml GM-CSF sensitised the K562 cells to vinblastine, while 100 and 1000 ng/ml GM-CSF had no effect, and therefore 20 ng/ml GM-CSF was used. However, further experiments showed that treatment with 20 ng/ml GM-CSF for 1-2 days produced variable responses in both the K562 cells and the K562/E15B subline (Fig. 4.14). In general, GM-CSF either slightly sensitised the K562 cells or had no effect, while there was a trend for the K562/E15B subline to become 2 to 10-fold more drug resistant after GM-CSF treatment. GM-CSF therefore increased drug resistance in the K562/E15B subline, but not in the parental K562 cells.

4.5 DISCUSSION

4.5.1 Induction with TPA

Both K562 cells and their MDR subline underwent megakaryocytic differentiation in response to TPA, as determined by increased expression of the platelet glycoprotein CD61 (Fig. 4.1), and decreased expression of the erythroid antigen glycophorin A (Fig. 4.2). Pre-treatment of K562 and K562/E15B cells with staurosporine partially inhibited the induction of megakaryocytic differentiation induced by TPA. Therefore, megakaryocytic differentiation induced by TPA is likely to be mediated by PKC. Similar results have been observed in the THP-1 monocytic cell line, in which macrophage-like

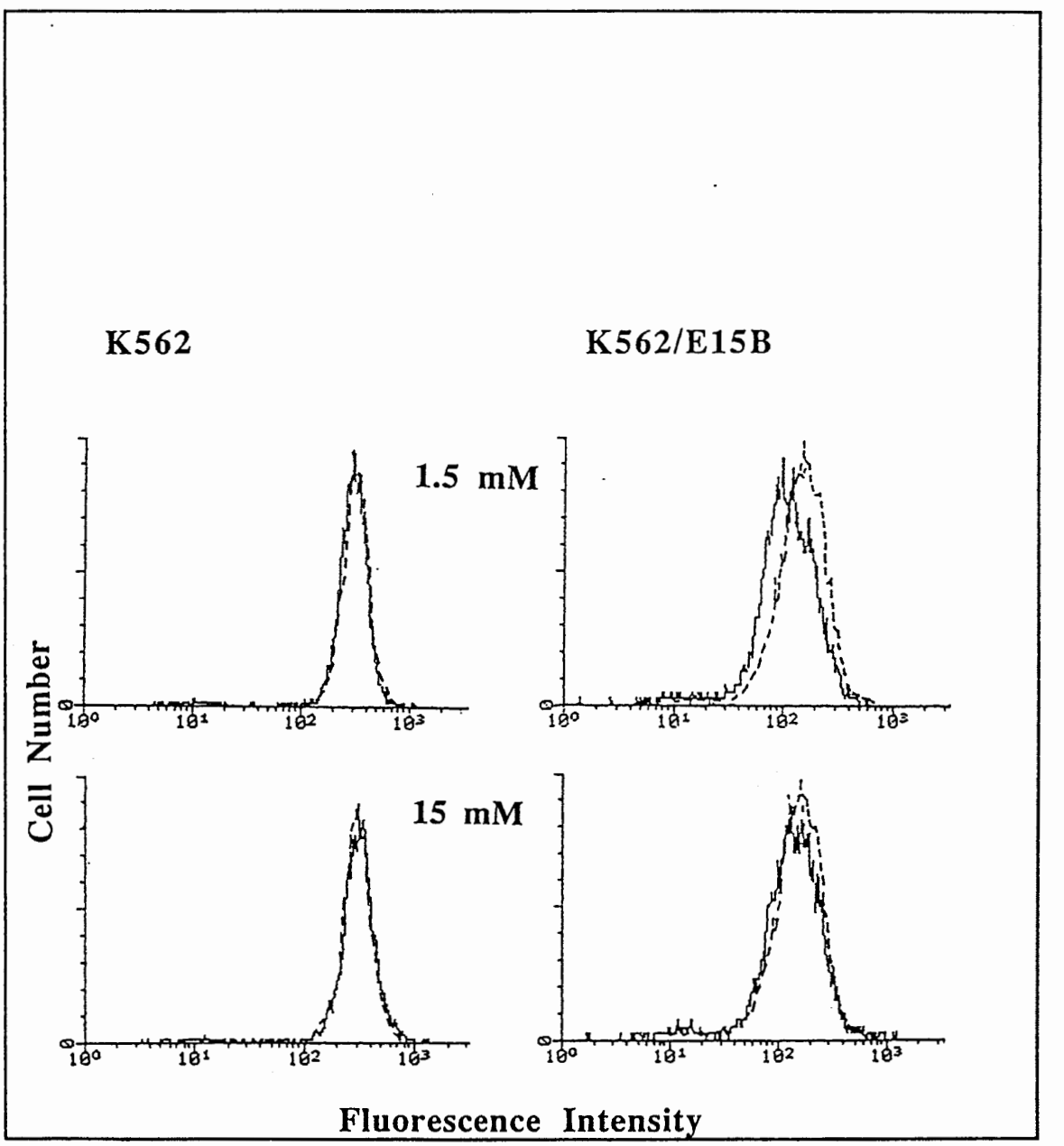

Figure 4.12. The effect of sodium butyrate treatment on Rh123 accumulation in K562 and K562/E15B cells. Cells were treated with 1.5 or 15 mM sodium butyrate(—) for 18 h, after which Rh123 accumulation was determined by flow cytometry as described in *Materials and Methods 7.* Fluorescence profiles of treated cells are compared to untreated cells(---). The experiment was repeated twice and a representative result is shown.

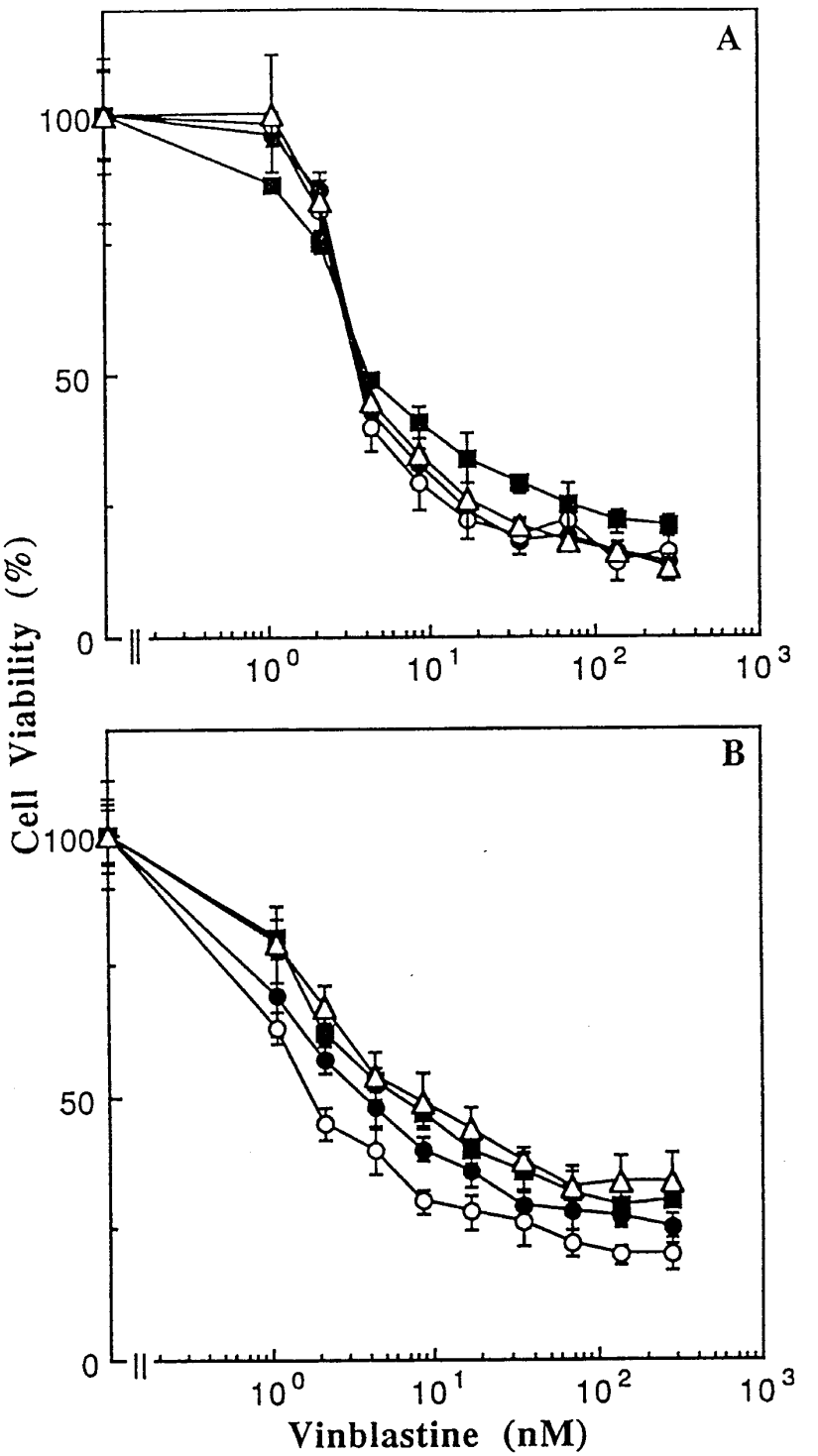

Figure 4.13. Determination of the optimum dose of GM-CSF to induce changes in drug resistance in K562 cells. K562 cells(●) were treated for A. 18 h or B. 42 h with GM-CSF at 20 ng/ml(o), 100 ng/ml(■) and 1 μg/ml(Δ), after which cells were incubated with serial dilutions of vinblastine for 4 days, and cell viability was determined using the MTT assay, as described in *Materials and Methods 3. Points*, mean of triplicate determinations; *error bars*, standard deviations. The experiment was repeated at least three times and a representative result is shown.

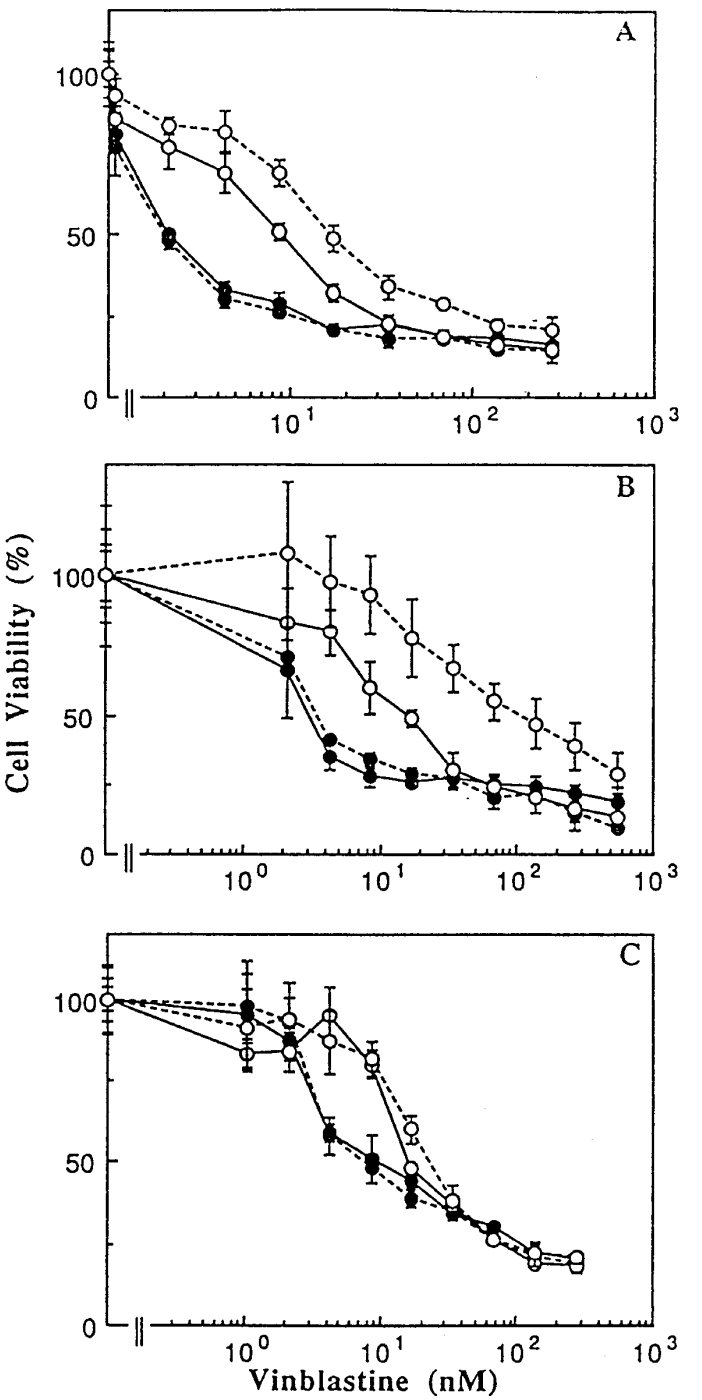

Figure 4.14. The effect of 42 h treatment with GM-CSF on vinblastine resistance in K562 and K562/E15B cells. K562(●) and K562/E15B(o) cells were treated with 20 ng/ml GM-CSF(---) for 42 h, after which cells were incubated with serial dilutions of vinblastine and cell viability was determined using the MTT assay and compared to untreated cells(—), as described in *Materials and Methods 3*. A., B. and C. show the results of three separate experiments. *Points*, mean of triplicate determinations; *error bars*, standard deviations.

differentiation in response to TPA was inhibited by pretreatment with staurosporine (Barendsen *et al*, 1990).

Staurosporine alone also induced CD61 expression in both the K562 and K562/E15B cells. Staurosporine is an inhibitor of PKC while TPA is an activator of PKC, yet they both elicited a similar cellular response in the K562 cells and the K562/E15B subline. However it is unlikely that staurosporine itself activated PKC, as if this was the case, staurosporine and TPA would have an additive effect, with more CD61 expression than after treatment TPA alone. Staurosporine has been shown to mimic TPA and induce cellular maturation of cultured mouse keratinocytes (Dlugosz and Yuspa, 1991). Furthermore, sphinganine, another PKC inhibitor, enhanced doxorubicin- and daunorubicin-induced differentiation in HL-60 cells, further suggesting that inhibition of PKC activity enhances differentiation (Yung *et al*, 1992). Therefore, taken with the results of other studies, the ability of staurosporine to induce megakaryocytic differentiation in K562 cells and the K562/E15B subline indicate that both inhibition and activation of PKC can induce differentiation.

The effect of TPA on CD36 expression was examined using the monoclonal antibody MRK20 (Ishii *et al*, 1992). MRK20 recognizes an 85kD protein expressed by the adriamycin resistant K562/ADM cell line, which has amino acid and nucleotide sequence homologous to CD36, a cell surface adhesion molecule found on endothelium, platelets and monocytes (Sugimoto *et al*, 1993). CD36 was not expressed by the parental K562 cells and expression of CD36 in the K562 cells did not change after treatment with either staurosporine, TPA or staurosporine and TPA in combination.

The K562/E15B subline expressed CD36, indicating that their differentiation status differed from K562 cells. CD36 has also been detected in doxorubicin resistant K562 cells, and it is a marker of drug resistance as well as differentiation (Ohtsu *et al*, 1989). TPA treatment reduced CD36 expression, and staurosporine partially inhibited the reduction in CD36 induced by TPA, confirming that inhibition of PKC inhibits differentiation in K562/E15B cells. As TPA induced megakaryocytic differentiation, a decrease in antigens not associated with megakaryocytic differentiation, such as CD36, would be expected. As with CD61, staurosporine alone also increased CD36 expression and inhibited the effect of TPA on CD36 expression by staurosporine, further suggesting a role for PKC in differentiation in the K562 and K562/E15B cells

Decreased expression of CD36 has been observed in HEL cells treated with TPA (Ishii *et al*, 1992). It has been suggested that the CD36 is characteristic of monocytic cells, as it is co-expressed with CD14. Although the K562/E15B cells do not express CD14, CD36 is still useful as a marker of drug resistance and differentiation in the K562 and K562/E15B cells.

TPA treatment of K562 cells caused an increase in drug resistance (Fig. 4.5) and decreased Rh123 accumulation (Fig. 4.7). However there were no changes in P-glycoprotein expression (Fig. 4.6) and verapamil had no effect on Rh123 accumulation (Fig. 4.8). This suggests that increased drug resistance is due to a mechanism other than P-glycoprotein. However, in drug resistant K562/E15B subline, TPA treatment caused an increase in P-glycoprotein expression (Fig. 4.6), increased drug resistance (Fig. 4.5), and a verapamil sensitive decrease in Rh123 accumulation (Fig. 4.8). This is consistent with TPA inducing an increase in P-glycoprotein mediated MDR. Staurosporine had no effect on TPA induced changes in either the K562 cells or the drug resistant K562/E15B subline, suggesting changes were not mediated by PKC.

Chaudhary and Roninson (1992) showed that TPA increased P-glycoprotein expression in cells already expressing P-glycoprotein, and this increase was mediated by PKC. They also demonstrated induction of P-glycoprotein mRNA with TPA treament in K562 cells , and the increase was mediated by PKC, whereas in this study TPA did not induce P-glycoprotein expression in K562 cells, and PKC did not appear to be involved in the upregulation of P-glycoprotein expression in the K562/E15B subline. However they used 16 nM TPA, and the higher dose may have resulted in more cell damage and induction of P-glycoprotein through PKC, whereas in this study 5 nM TPA was used. Chaudhary and Roninson (1992) also a cDNA-PCR assay for *mdr*1 mRNA, rhodamine 123 accumulation and staining with the UIC-2 monoclonal antibody which reacts with an external epitope on P-glycoprotein (Mechetner *et al*, 1992), and not C219, and this may also account for the differences.

The effects of staurosporine itself on P-glycoprotein expression are still unclear. Staurosporine and its derivatives have been shown to reduce P-glycoprotein expression and reverse multidrug resistance (Sampson *et al*, 1993; Wakusawa *et al*, 1993). However staurosporine had no effect on P-glycoprotein expression in the K562 or K562/E15B cells.

4.5.2 Induction With Sodium Butyrate

The apparent absence of erythroid differentiation (no increase in glycophorin A expression) after exposure of the K562 cells and the K562/E15B subline to sodium butyrate for 18 h was not initially expected, as sodium butyrate is commonly recognised as an inducer of erythroid differentiation in K562 cells (Sutherland *et al*, 1986). Others have reported that K562 cells have heterogenoeus commitment to erythroid differentiation (Vainchenkner *et al*, 1981), and that sodium butyrate does not increase expression of glycophorin A expression in K562 cells (Villeval *et al*, 1983). However, Glycophorin A expression did increase after 4 days exposure to sodium butyrate, indicating that longer exposure to sodium butyrate was necessary to induce expression of glycophorin A, and

differentiation occured only in a sub-population cells. In HL-60 cells, uptake of retinoic acid is cell cycle-dependent, and all cells must reach the required stage of the cell cycle for retinoic acid uptake to occur. Interaction with the intracellular target is also cell cycle specific (Yen and Albright, 1984). Therefore 2 cycles are needed before differentiation occurs and sodium butyrate may also act in this manner.

Sodium butyrate upregulated P-glycoprotein expression in the K562/E15B subline but not in the K562 cells after 18 h. Sodium butyrate therefore only induced changes in P-glycoprotein expression in cells which were already drug resistant and expressing P-glycoprotein, which was also observed after treatment with TPA.

While 1.5 mM sodium butyrate had little effect on drug resistance, 15 mM butyrate increased drug sensitivity in both the K562 and K562/E15B cells despite increased P-glycoprotein expression. Similarly, Bates *et al* (1992), reported that 2 mM sodium butyrate induced P-glycoprotein expression in SW620 human colon carcinoma cells with increased colchicine resistance, but decreased resistance to vinblastine, actinomycin-D and doxorubicin. Bates *et al* (1992) proposed that although P-glycoprotein expression was increased by sodium butyrate, phosphorylation of P-glycoprotein was in fact decreased, which changes in the specificity of P-glycoprotein transport. Similarly, treatment of the K562/E15B subline with sodium butyrate may have increased expression of a form of P-glycoprotein with a decreased affinity for drug transport. The decrease in drug resistance in the K562 cells, which do not express P-glycoprotein, is suggestive of mechanism other than P-glycoprotein.

Sodium butyrate had little effect on the parental K562 cells, with no induction of P-glycoprotein, no change in Rh123 accumulation and only a slight sensitisation to drug, which was not significant. In the drug resistant K562/E15B subline, results were dependent on the dose of sodium butyrate. At 1.5 mM, the K562/E15B subline showed increased P-glycoprotein expression, decreased Rh123 accumulation and a slight increase in resistance to colchicine but not to epirubicin. This suggests induction of functional P-glycoprotein. However, after treatment with 15 mM sodium butyrate, while P-glycoprotein expression increased, the cells were sensitive to epirubicin and colchicine, with no change in Rh123 accumulation, suggesting a non-functional P-glycoprotein. Alternatively, as C219 also cross reacts with the *mdr3* gene product (Schinkel *et al*, 1991), it is possible that the P-glycoprotein detected by C219 after treatment of the K562/E15B subline with 15 mM sodium butyrate could be the mdr3 gene product.

4.5.3 Induction with GM-CSF

Treatment with GM-CSF had little effect on vinblastine cytotoxicity in the K562 cells, whereas the K562/E15B cells tended to become more drug resistant (up to 10-fold) after pretreatment with GM-CSF. The K562/E15B cells have lower glycophorin A expression

and higher CD13 expression than the K562 cells, suggesting a more myeloid phenotype, which could account for the increase in their responsiveness to GM-CSF.

Earlier *in vitro* studies have demonstrated that AML blasts became more sensitive to daunorubicin after treatment with GM-CSF (Santini *et al*, 1990). Other studies using similar levels of GM-CSF to those used here (10-40 ng/ml) have shown that GM-CSF significantly enhanced daunorubicin cytotoxicity in blasts from untreated AML patients (Waga *et al*, 1992). These studies suggest that GM-CSF may enhance the effectiveness of chemotherapeutic drugs.

However, a more recent *in vivo* study has shown that treatment of AML patients with high-dose cytosine arabinoside, daunorubicin and GM-CSF decreased remission rates relative to therapy with Ara-C and daunorubicin alone (Estey *et al*, 1992), indicating that growth factor treatment may not be beneficial. GM-CSF has also been found to inhibit the induction of cell death by apoptosis in the myeloid cell line 7-M12 (Lotem *et al*, 1991), suggesting that GM-CSF may enhance cell survival. Inhibiton of cell death could explain the increased cell survival of the K562/E15B cells after GM-CSF, and this evidence together with the findings of Estey *et al*, (1992) suggests that growth factors may not always be beneficial in the treatment of leukaemic cells. The increase in drug resistance seen in the K562/E15B cells is therefore clinically relevant, and highlights the need to study drug resistance before and after administration of growth factors in leukaemia patients. The results with GM-CSF in the K562/E15B subline also varied from experiment to experiment (Fig. 4.14). Responsiveness of fresh AML cells to different growth factors has also been found to be variable, both among patients and among different colony stimulating factors (Cannistra *et al*, 1989; Lista *et al*, 1990; Waga *et al*, 1992).

GM-CSF did not induce differentiation in K562 and K562/E15B cells, although others have shown that K562 cells undergo erythroid differentiation when grown in soft-agar in the presence of GM-CSF (Tawhid *et al*, 1989).

Together with the differences between the K562 cells and the K562/E15B subline in reponse to TPA and sodium butyrate in terms of P-glycoprotein expression, the response of the K562/E15B subline to GM-CSF further demonstrates that induction of differentiation as a form of therapy may not necessarily be advantageous in treatment of leukaemias in which P-glycoprotein is expressed.

5 INDUCTION OF DIFFERENTIATION IN U937 AND U937/E15 CELLS

5.1 Introduction

The effects of TPA and GM-CSF on drug resistance and P-glycoprotein were also examined in the U937 cells and the drug resistant U937/E15 subline as a comparison to the K562 cells and the K562/E15B subline. As sodium butyrate did little to the K562 cells, it was not used to treat the U937 cells. All experiments with TPA were performed using 5 nM TPA and 30 nM staurosporine. TPA is known to induce monocytic differentiation in U937 cells (Hass e*t al*, 1992), as does GM-CSF (Zuckerman *et al*, 1988).

5.2 Induction with TPA

5.2.1 The Effect of TPA on Differentiation

Treatment of the U937 and U937/E15 cells with TPA induced monocytic differentiation, as the cells became adherent with increased CD13 expression (Fig. 5.1) and a slight increase in CD14 expression increased (Fig 5.2). The increase in CD13 was not effected by staurosporine, while staurosporine appeared to inhibit the increase in CD14 expression induced by TPA. However, staurosporine increased expression of CD13 in the U937 cells, with an accompanying decrease in CD14 expression, suggesting granulocytic differentiation and loss of the monocytic phenotype.

The U937/E15 cells have lost expression of CD13 (Fig. 5.1), and express low levels of CD14 (Fig. 5.2). Neither staurosporine or TPA induced expression of CD13 in the U937/E15 cells (Fig. 5.1). Staurosporine, TPA and staurosporine in combination with TPA all decreased CD14 expression in the U937/E15 subline (Fig. 5.2).

CD61 and glycophorin A expression in the U937 cells did not change after any of the treatments (Fig. 5.3 and Fig. 5.4). However, in the U937/E15 cells TPA reduced glycophorin A expression (Fig 5.4), but did not increase expression of CD61 (Fig. 5.3). Staurosporine induced expression of the megakaryocytic antigen CD61, while TPA and staurosporine together increased CD61 expression, with a further decrease in glycophorin A expression.

The U937 cells expressed CD36, and the U937/E15 subline expressed higher levels than the parental U937 cells (Fig. 5.5). The U937 cells also bound much higher levels of the control antibody 1D4.5 than the U937/E15 cells. CD36 expression increased in the U937 cells after TPA treatment, while TPA treatment produced a decrease in expression of CD36 in the U937/E15 cells. Staurosporine did not inhibit the changes in CD36 induced by TPA in the U937 cells and the U937/E15 subline. CD36 expression became

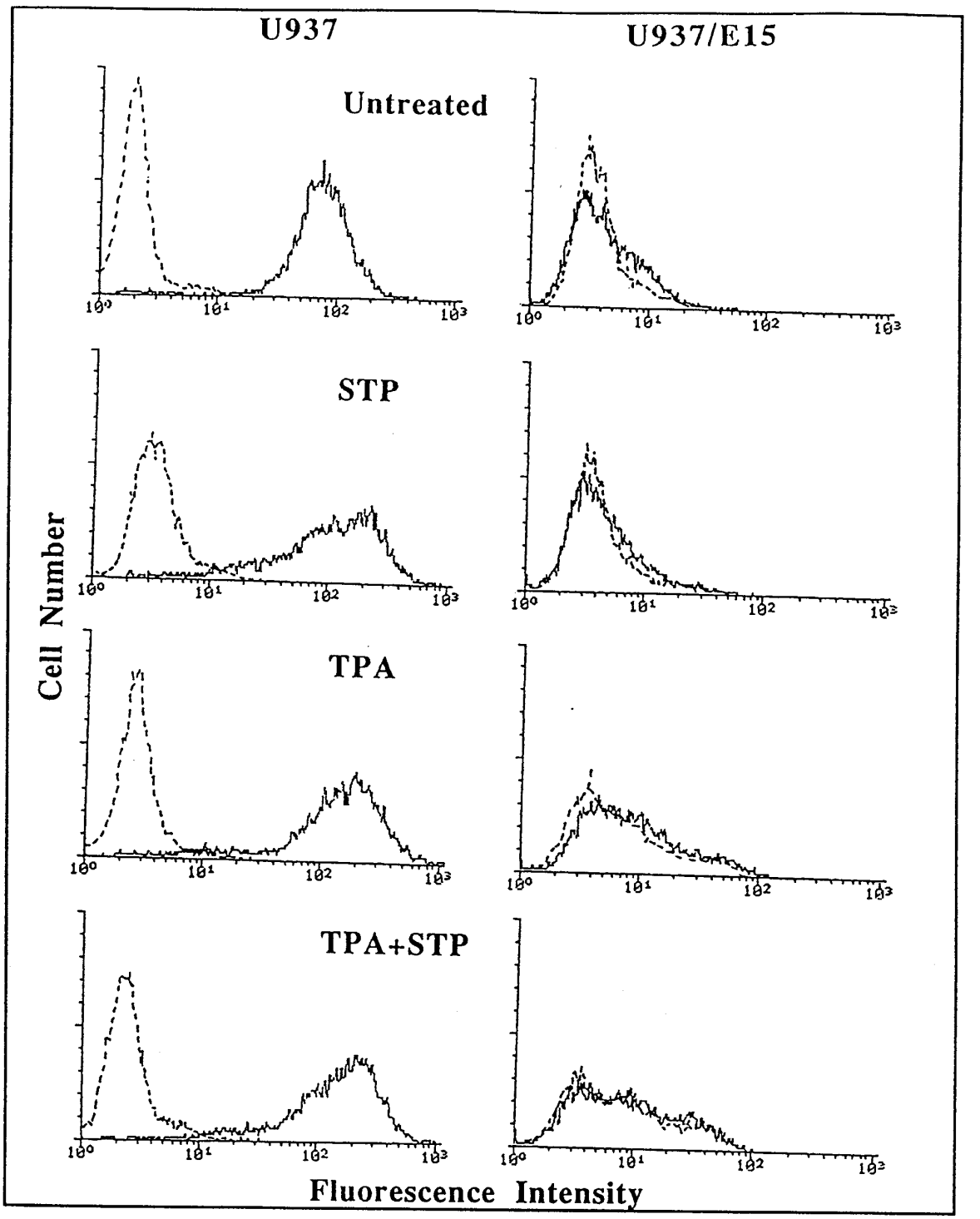

Figure 5.1. The effect of TPA and staurosporine on CD13 expression in U937 and U937/E15 cells. Cells were treated with TPA and/or staurosporine for 18 h, after which they were analysed for CD13 expression(—) by flow cytometry as described in *Materials and Methods 8*, and the fluorescence profile is compared to cells incubated with the negative control antibody(---). The experiment was repeated at least three times and a representative result is shown.

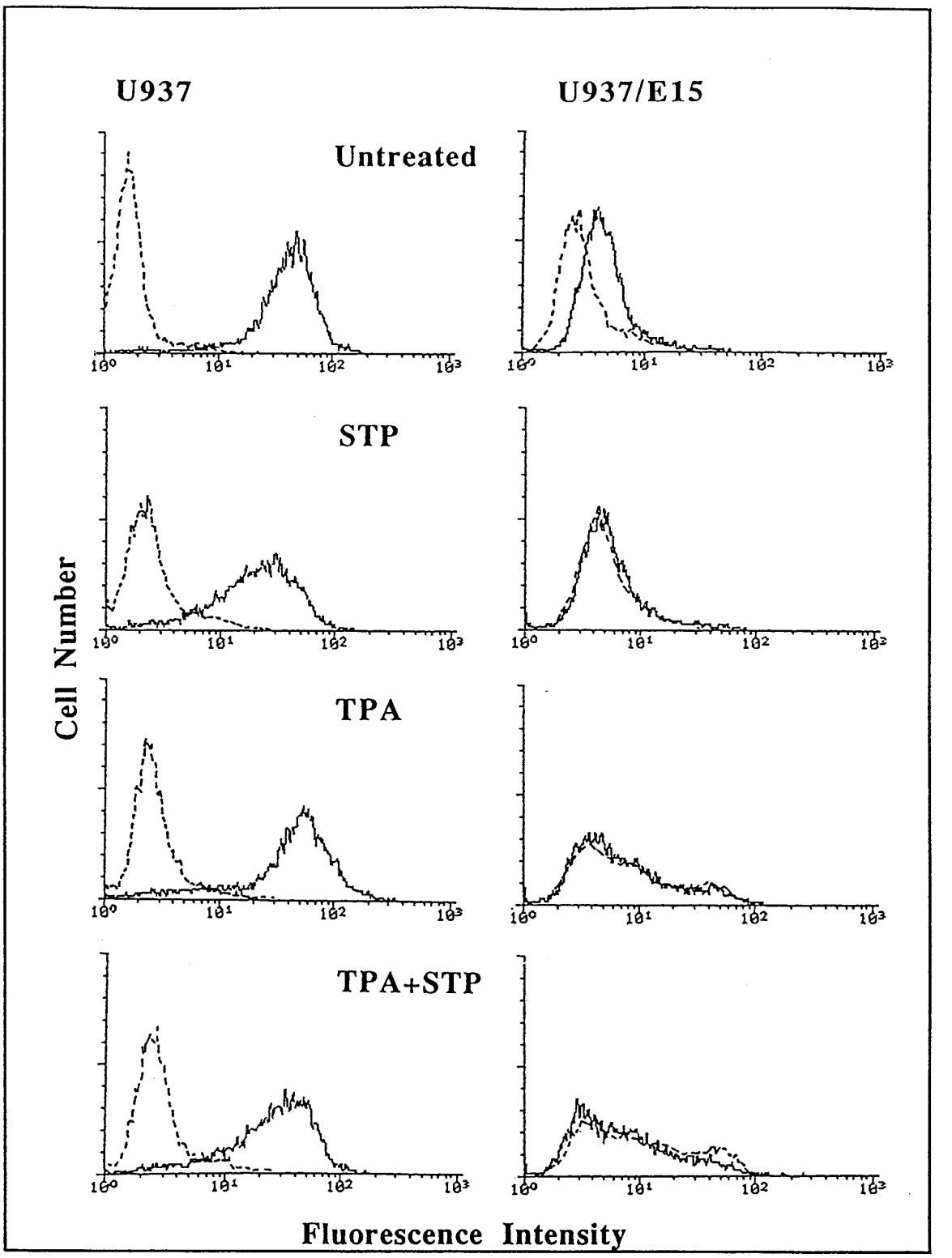

Figure 5.2. The effect of TPA and staurosporine on CD14 expression in U937 and U937/E15 cells. Cells were treated with TPA and/or staurosporine for 18 h, after which they were analysed for CD14 expression(—) by flow cytometry as described in *Materials and Methods 8*, and the fluorescence profile is compared to cells incubated with the negative control antibody(---). The experiment was repeated at least three times and a representative result is shown.

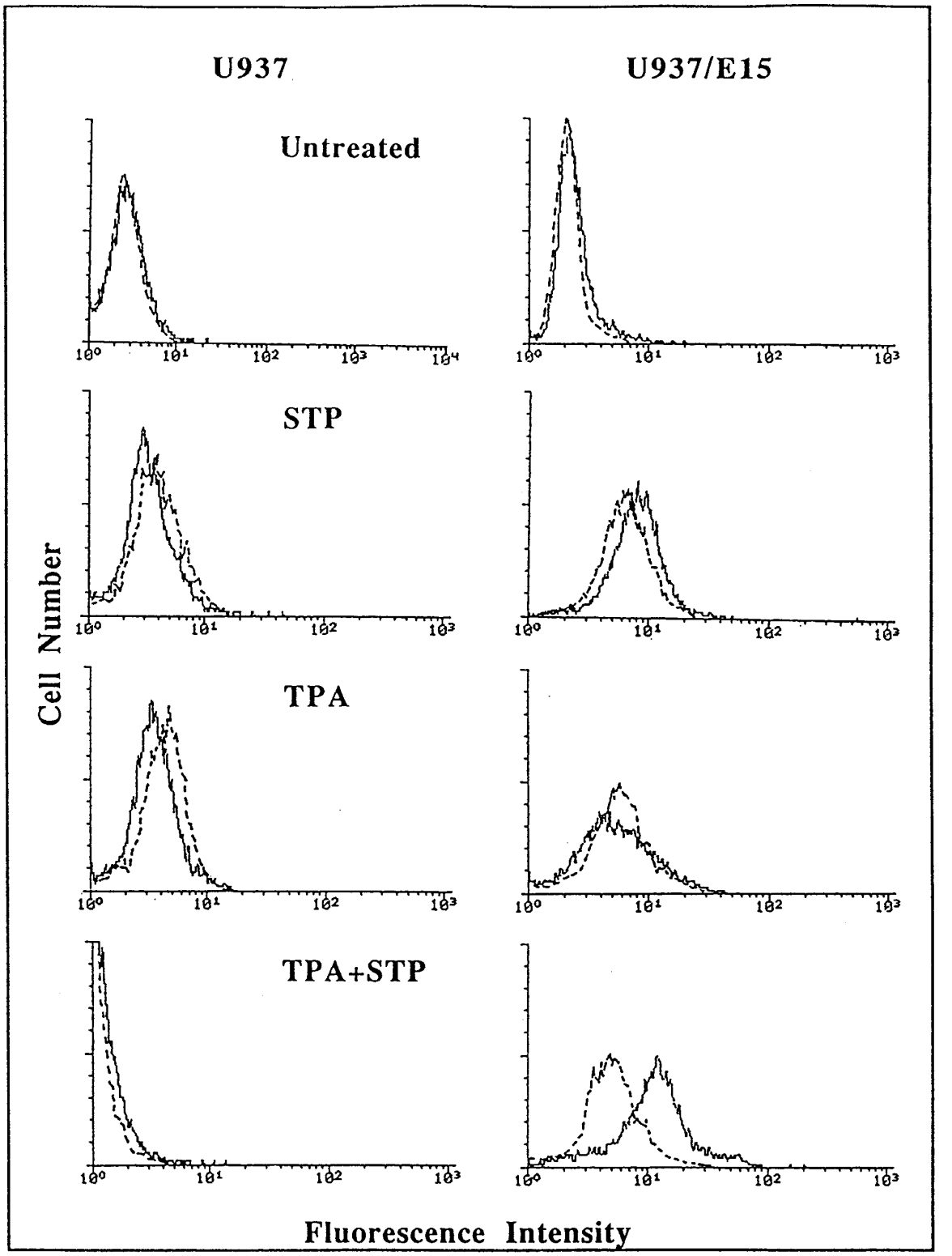

Figure 5.3. The effect of TPA and staurosporine on CD61 expression in U937 and U937/E15 cells. Cells were treated with TPA and/or staurosporine for 18 h, after which they were analysed for CD61 expression(—) by flow cytometry as described in *Materials and Methods 8*, and the fluorescence profile is compared to cells incubated with the negative control antibody(---). The experiment was repeated twice and a representative result is shown.

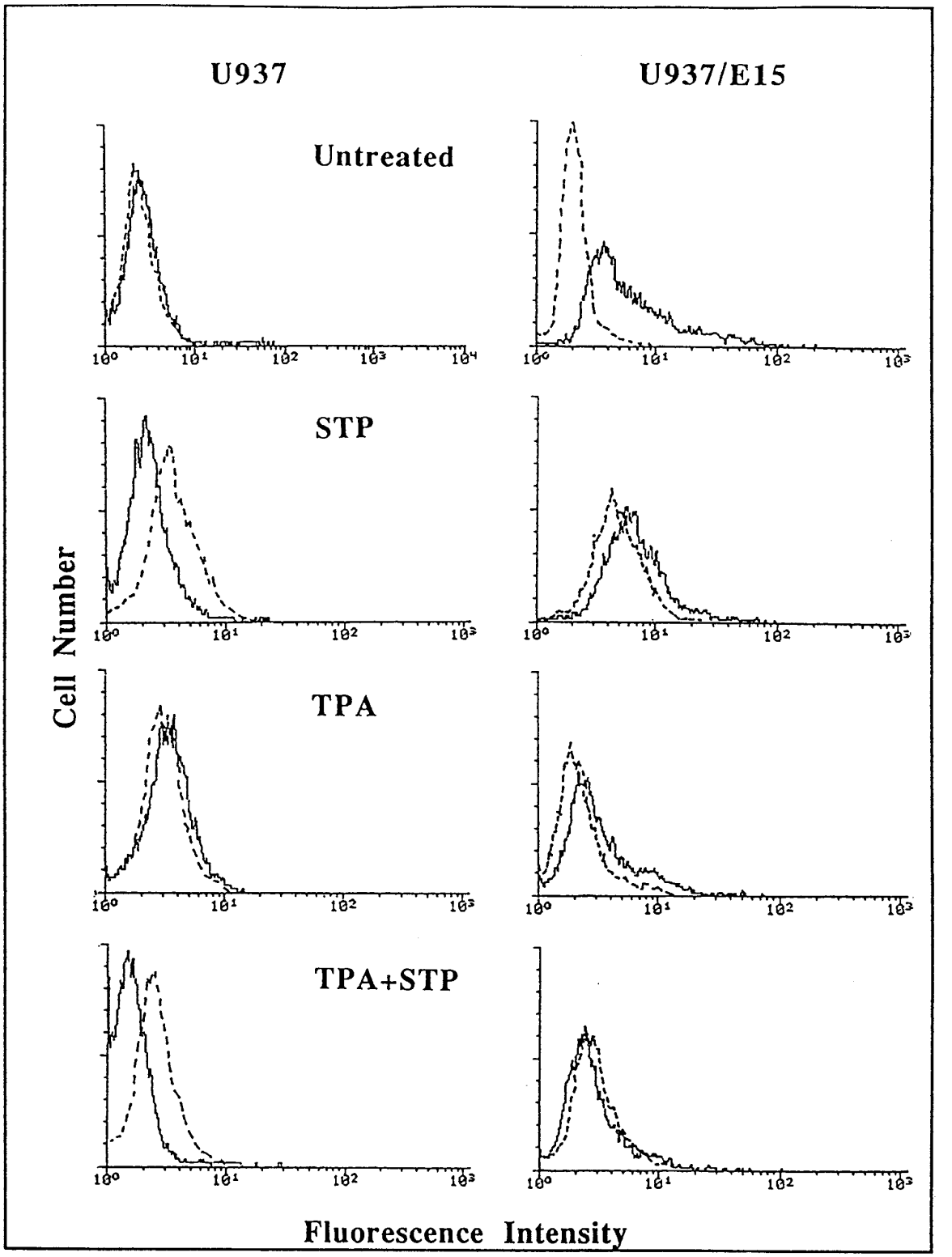

Figure 5.4. The effect of TPA and staurosporine on glycophorin A expression in U937 and U937/E15 cells. Cells were treated with TPA and/or staurosporine for 18 h, after they were analysed for expression of glycophorin A(—) by flow cytometry as described in *Materials and Methods 8*, and the fluorescence profile is compared to cells incubated with the negative control antibody(---). The experiment was repeated twice and a representative result is shown.

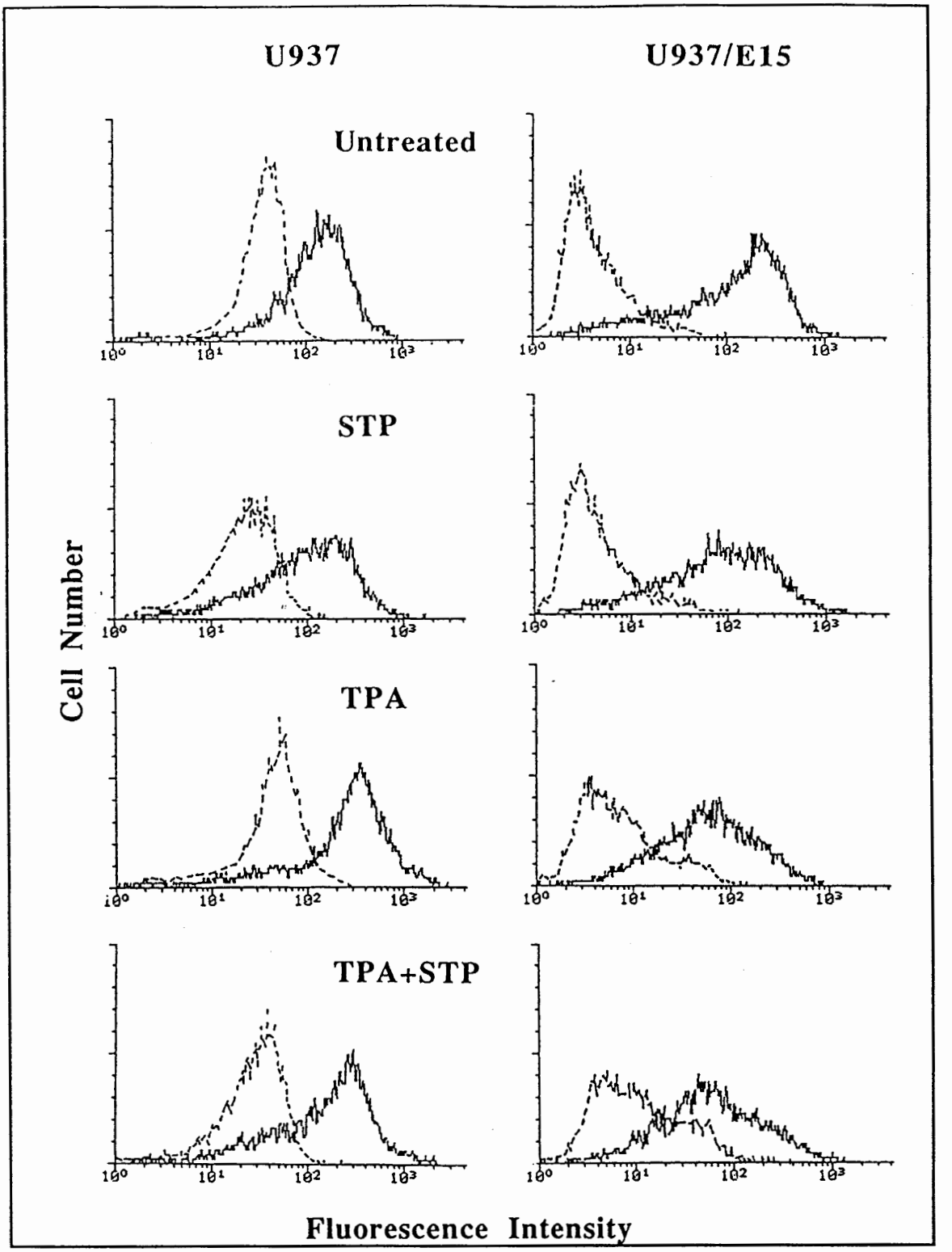

Figure 5.5. The effect of TPA and staurosporine on CD36 expression in U937 and U937/E15 cells. Cells were treated with TPA and/or staurosporine for 18 h, after which they were analysed for expression of CD36(—) by flow cytometry as described in *Materials and Methods 8*, and the fluorescence profile is compared to cells incubated with the negative control antibody(---). The experiment was repeated at least three times and a representative result is shown.

more heterogeneous in both the U937 and U937/E15 cells after treatment with staurosporine.

5.2.2 The Effect of TPA on Drug Resistance

The effects of TPA on drug resistance were not examined after TPA treatment as insufficient cells survived for the duration of the cytotoxicity assay, making the assay invalid.

5.2.3 The Effect of TPA on P-glycoprotein Expression

The effect of 18 h treatment with TPA and staurosporine on P-glycoprotein expression was examined in U937 cells and the drug resistant U937/E15 subline. U937 cells did not express P-glycoprotein while the U937/E15 cells expressed low levels of P-glycoprotein (Fig. 5.6). Treatment of the parental U937 cells with either TPA, staurosporine alone or before the addition of TPA did not induce expression of P-glycoprotein as detected by Western blot analysis (Fig. 5.6). However, TPA induced a large increase in P-glycoprotein expression in the U937/E15 subline, and this increase was not inhibited by staurosporine (Fig. 5.6). Staurosporine alone had no effect on P-glycoprotein expression in the U937/E15 cells. TPA therefore increased P-glycoprotein expression in the U937/E15 subline, but not the parental U937 cells.

5.2.4 The Effect of TPA on Rh123 Accumulation

The U937 and U937/E15 cells were treated with TPA in the presence or absence of staurosporine for 18 h, and the results are summarised in Fig. 5.7. Staurosporine induced a decrease in Rh123 accumulation in the U937 cells, but had no effect on Rh123 retention in the U937/E15 cells (Fig. 5.7). TPA induced a decrease in Rh123 retention in both the U937 and U937/E15 cells, and this was not inhibited by staurosporine. In the U937 cells staurosporine enhanced the effect of TPA, resulting in a further decrease in Rh123 retention.

Verapamil failed to restore Rh123 accumulation in the U937 cells after each treatment (Fig. 5.8). Rh123 retention was partially restored by verapamil in the TPA treated U937/E15 cells, but not the level of the untreated cells (Fig. 5.8).

5.3 Induction with GM-CSF

5.3.1 The Effect of GM-CSF on Differentiaton

Treatment of the U937 cells with GM-CSF for 18 and 42 h had little effect on differentiation, with no changes in expression of CD13 and a small decrease in CD14 after 42 h treatment (data not shown).

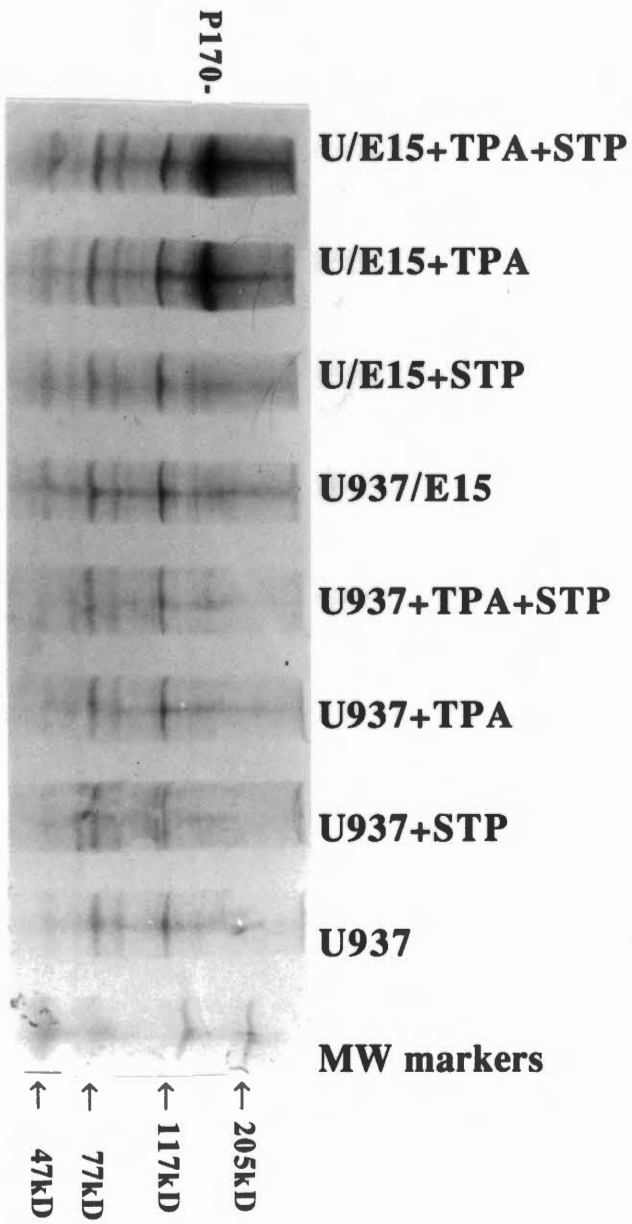

Figure 5.6. Western blot analysis of the effects of TPA and staurosporine on P-glycoprotein expression in U937 and U937/E15 cells. Cells were treated for 18 h with TPA and/or staurosporine, after which plasma membranes were prepared and analysed as described in *Materials and Methods 6.1*. Positions of molecular weight markers and P-glycoprotein (P-170) are indicated.

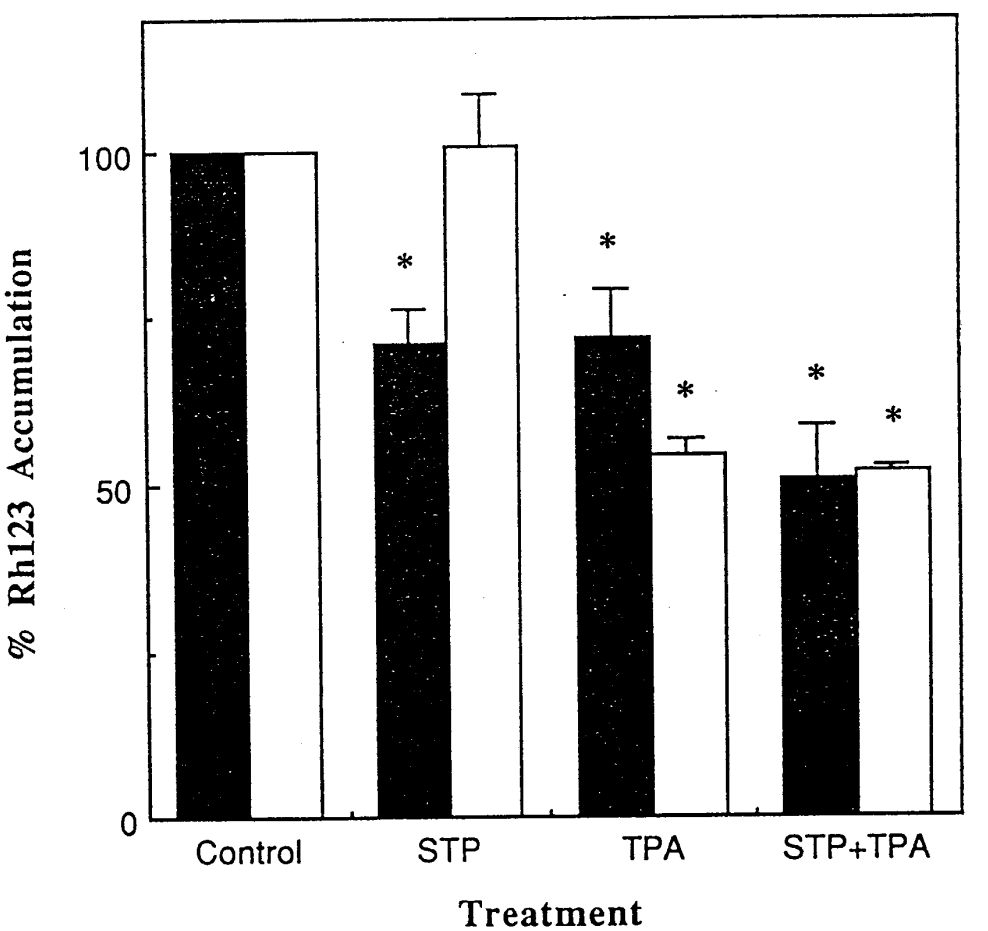

Figure 5.7. Summary of the effects of TPA and staurosporine on Rh123 accumulation in U937 and U937/E15 cells. Cells were treated with TPA and/or staurosporine for 18h, after which Rh123 accumulation was determined by flow cytometry as described in *Materials and Methods 7.* U937 cells(■) and U937/E15 cells(□). Results are the mean of three experiments and are calculated as a percentage of untreated cells; *error bars*, standard deviation; * indicates a significant difference ($p<0.01$) from untreated cells using the student's t-test.

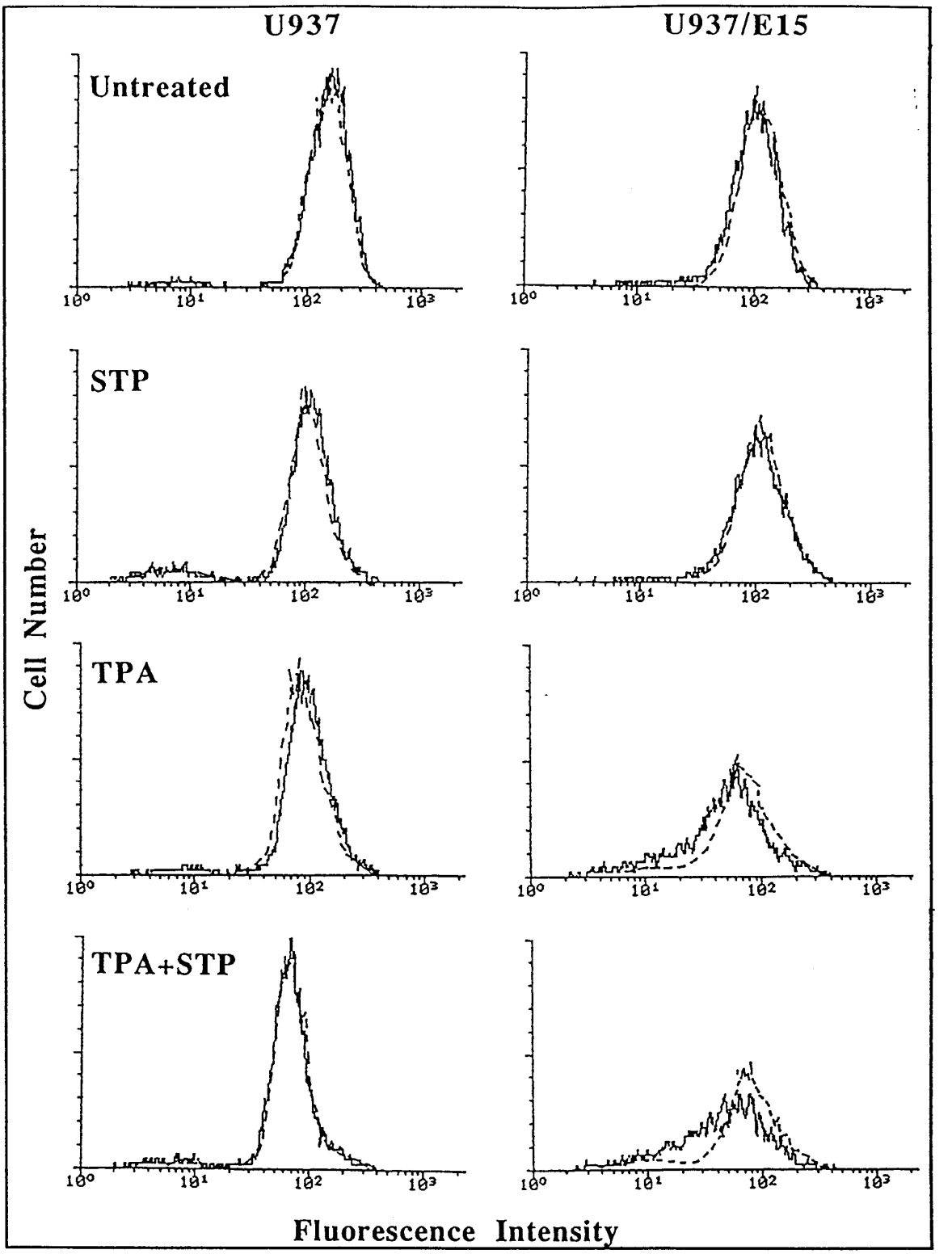

Figure 5.8. The effect of verapamil on Rh123 accumulation in U937 and U937/E15 cells after treatment with TPA or staurosporine. Cells were treated with TPA and/or staurosporine for 18 h, after which Rh123 accumulation in the presence(---) or absence of 10 μM verapamil(—) was determined by flow cytometry as described in *Materials and Methods 7*. The experiment was repeated twice and a representative result is shown.

Treatment of U937/E15 cells with GM-CSF for both 18 and 42 h induced increases in both CD13 and CD14 expression (Fig. 5.9). Glycophorin A expression was not changed by GM-CSF (data not shown). These results indicate that development of drug resistance and subsequent dedifferentiation in the U937/E15 cells altered their ability to differentiate in response to GM-CSF as compared to the parental U937 cells.

5.3.2 The Effect of GM-CSF on Drug Resistance

U937 cells were initially treated with 20, 100 and 1000 ng/ml GM-CSF for 42 h in order to determine which concentration would have the greatest effect (Fig. 5.10). 100 ng/ml was chosen as it produced a slightly greater decrease in vinblastine cytotoxicity than 20 ng/ml. Treatment of U937 and U937/E15 cells with 100 ng/ml GM-CSF had no effect on vinblastine resistance in either cell line (Table 5.1).

Table 5.1 The Effects of GM-CSF on vinblastine cytotoxicity in U937 and U937/E15 cells.

	100ng/ml	Vinblastine IC_{50} (nM)	
Cell Line	GM-CSF	18 hours	42 hours
U937	-	1.05±0.250[a]	1.55±0.541
	+	1.05±0.250	1.20±0.416
U937/E15	-	6.50±1.000	5.33±0.577
	+	8.50±4.500	6.17±1.167

aMean ± standard deviation, n=3.

5.4 Discussion

5.4.1 Induction with TPA

TPA induced monocytic differentiation in U937 cells, as they became adherent with slightly increased expression of CD14, which is characteristic of monocyte/macrophage-like differentiation. Staurosporine appeared to inhibit the ability of TPA to induce monocytic differentiation in the U937 cells.

TPA also induced increased expression of CD13 in the U937 cells, and pre-treatment with staurosporine did not inhibit the increase in expression of CD13 induced by TPA. This has also been demonstrated in another monocytic cell line, THP-1, in which TPA induced differentiation which was also inhibited by staurosporine (Barendsen *et al*, 1990). The involvement of PKC in monocytic differentiation in the U937 cells and megakaryocytic differentiation in the K562 and K562/E15B cells suggests that these aspects of haematopoietic differentiation may be controlled by PKC.

CD36, a cell surface adhesion antigen (Sugimoto *et al*, 1993) is expressed by U937 cells and increased after TPA treatment. TPA therefore induced a macrophage-like

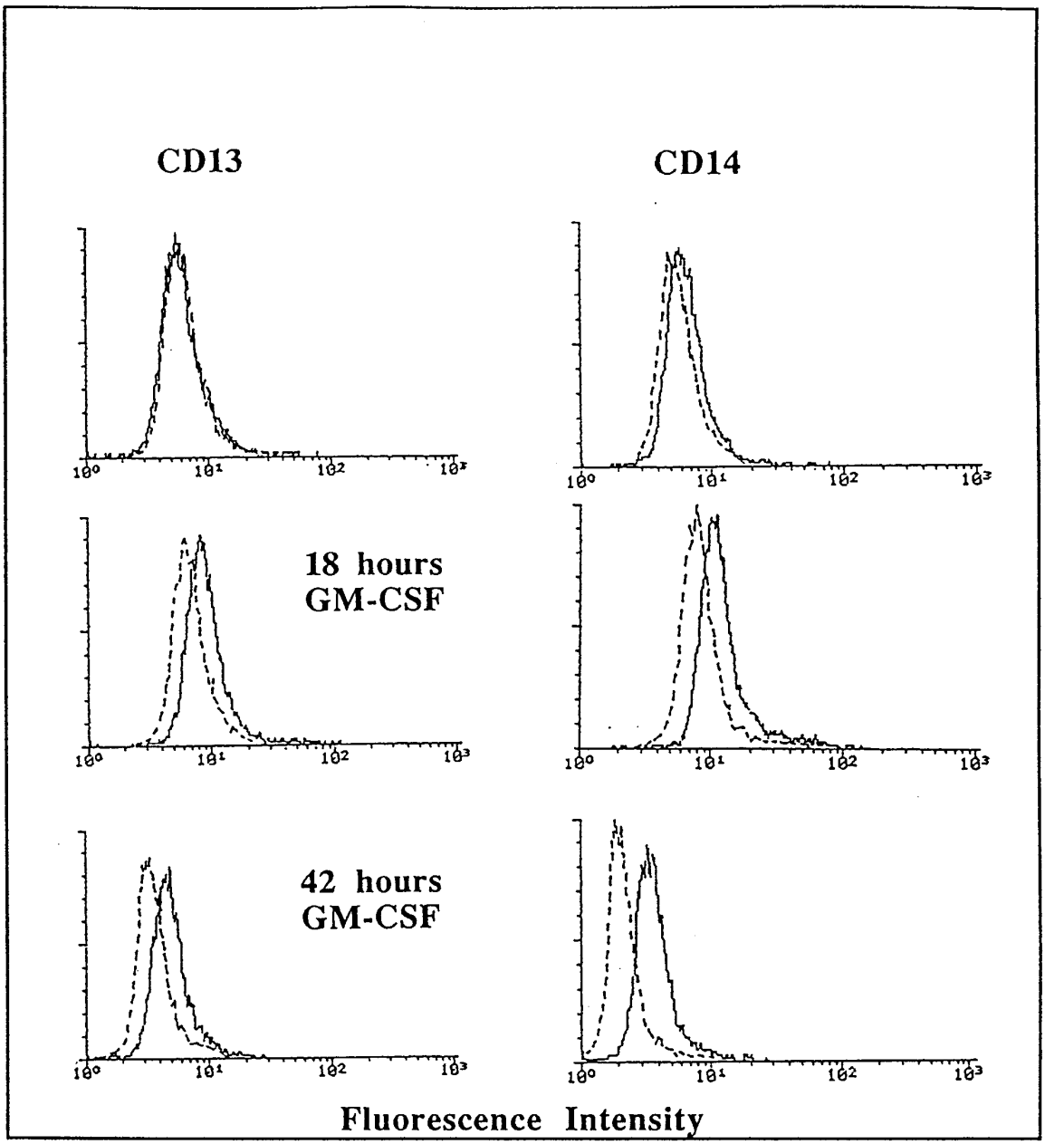

Figure 5.9. The effect of GM-CSF treatment on CD13 and CD14 expression in U937/E15 cells. Cells were treated with 100 ng/ml GM-CSF for 18 and 42h, after which cells were anlysed for expression of CD13(—) by flow cytometry as described in *Materials and Methods 8*, and the fluorescence profile is compared to cells incubated with the negative control antibody(---). The experiment was repeated twice and a representative result is shown.

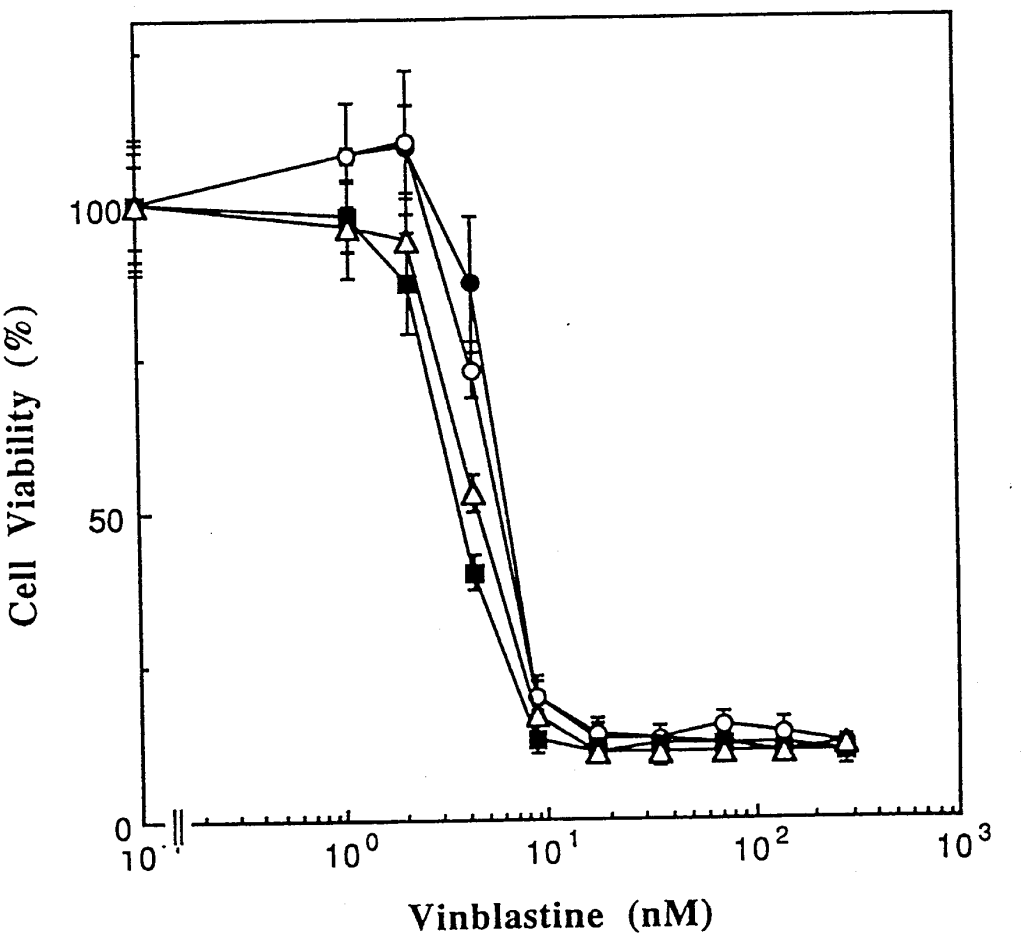

Figure 5.10. Determination of the optimum dose of GM-CSF to induce changes in drug resistance in U937 cells. U937 cells(●) were treated for 42 h with 20 ng/ml (O), 100 ng/ml(■) or 1 μg/ml(Δ) GM-CSF, after which cells were incubated with serial dilutions of vinblastine for 3 days, and cell viability was determined using the MTT assay as described in *Materials and Methods 3*. *Points*, mean of triplicate determinations; *error bars*, standard deviations. The experiment was repeated at least three times and a representative result is shown.

phenotype in U937 cells, with slightly increased expression of CD14 and CD36. The increase in CD36 expression however was not inhibited by staurosporine, suggesting PKC was not mediating the increase.

In contrast the U937/E15 subline which expresses glycophorin A, but with little CD13 or CD14 expression, did not become adherent after exposure to TPA. CD13 did not change in the U937/E15 cells after TPA treatment, while CD14 was further decreased.

This suggests that the U937/E15 cells have differentiated along the erythroid lineage to such an extent that they no longer respond to TPA. Similar results have been obtained using the MDR HL60/vinc cell line, which failed to differentiate in response to TPA. Insensitivity to TPA was attributed to vincristine resistance/P-glycoprotein expression, which resulted in defects in PKC-mediated signaling (Slapak *et al*, 1993). The inability of the HL-60/vinc cell line to undergo differentiation has been attributed to the inability to form a TPA inducible AP-1 complex (Ma *et al*, 1992), a transcription factor which binds to a sequence in the enhancer regions of TPA responsive genes (Lee *et al*, 1987), and would normally lead to differentiation. Similarly, the U937/E15 subline does not differentiate to macrophage-like cells, which could be due to an inability to form a TPA-inducible AP-1 complex.

However, TPA treatment of the U937/E15 subline decreased glycophorin A expression with no effect on CD61. Staurosporine induced an increase in CD61 expression, with a corresponding loss of glycophorin A expression, and staurosporine together with TPA further decreased glycophorin A expression and increased CD61 expression. The response of the U937/E15 subline resembled megakaryocytic differentiation reported by Yen *et al* (1993), whereby staurosporine induced megakaryocytic differentiation in K562 cells, which was enhanced when the cells were also treated with TPA. Yen *et al* (1993) proposed that differentiation due to both TPA and staurosporine suggested PKC activation was not necessarily an essential step leading to megakaryocytic differentiation. The response to staurosporine and TPA in combination with staurosporine, and lack of response to TPA treatment in the U937/E15 cells suggest an uncoupling of PKC and differentiation.

Expression of CD36 has been associated with drug resistance (Sugawara *et al*, 1988), and this is confirmed by the increased CD36 expression in the drug resistant U937/E15 subline. TPA reduced CD36 expression in the U937/E15 subline. Staurosporine did not inhibit the decrease, again suggesting that PKC is not involved in the regulation of CD36. Ishii *et al* (1992) have suggested that CD36 could be expressed on monocytic cells in association with CD14, and its expression may be regulated at an earlier stage of diferentiation than CD14. Expression of CD36 and low expression of CD14 by the U937/E15 cells, which are dedifferentiated, supports this theory.

However the changes in CD36 and glycophorin A expression demonstrate that the U937/E15 cells are still capable of responding to TPA, although it is far removed from the response of parental U937 cells. It would be of interest to examine the effects of other inducers of differentiation, such as sodium butyrate, on these cells, and examine their ability to differentiate along the various haematopoietic lineages, comparing changes in drug resistance.

The ability of the PKC inhibitor staurosporine to inhibit differentiation induced by TPA in both the K562 and U937 cells supports the suggestion that TPA induces differentiation through PKC. It cannot be disregarded that TPA may have other biochemical effects that result in differentiation as well as changes in drug resistance in leukaemic cells.

While TPA induced monocytic differentiation in the U937 cells it had little effect on their drug resistance phenotype. In the U937/E15 subline, TPA did not induce any changes in expression of differentiation markers. However, TPA treatment induced increased P-glycoprotein expression and decreased Rh123 accumulation which was verapamil sensitive, indicating that the increased P-glycoprotein was functional.

As staurosporine did not inhibit the ability of TPA to increase P-glycoprotein expression in the U937/E15 and K562/E15B sublines, it is unlikely that PKC is mediating the increases in P-glycoprotein. There are many different agents which have shown to induce P-glycoprotein expression and act upon the *mdr*1 promoter, suggesting that the induction of P-glycoprotein may be a general stress-response mechanism. The *mdr1* gene promoter has a heat shock consensus element (Chin *et al*, 1990a), and P-glycoprotein/*mdr*1 expression can be induced as a response to a variety of stresses including cytotoxic drugs themselves (Chin *et al*, 1990b; Kato *et al*, 1992), as well as heat shock and the toxic metals sodium arsenite and cadmium (Chin *et al*, 1990a). Overexpression of *mdr* is also induced by carcinogens and associated with resistance to cytotoxic drugs in rat liver preneoplastic nodules (Fairchild *et al*, 1987). If P-glycoprotein expression is a general response mechanism to enviromental stress, it is therefore likely that exposure of a cell to toxic agents such as TPA induces P-glycoprotein expression as a protective mechanism, and it is likely that this has occured in these cells, through a mechanism other than PKC.

TPA therefore induced differentiation in the U937 cells, but not the MDR U937/E15 subline. PKC appeared to be involved in differentiation in U937 cells, while PKC and differentiation appeared to be uncoupled in the U937/E15 subline. Increases in P-glycoprotein expression and function after TPA treatment were detected only in the U937/E15 subline, and PKC did not appear to mediate the increase in expression. Similarly, TPA only induced an increase in P-glycoprotein expression and function in the K562/E15B subline, and not the parental K562 cells.

5.4.2 Induction with GM-CSF

Exposure to GM-CSF did not affect drug resistance or differentiation in the U937 cells. However, the U937/E15 cells, which are dedifferentiated, were induced to differentiate in response to GM-CSF after both 18 and 42 h exposure, with increases in CD13 and CD14. GM-CSF induces differentiation along both of these lineages in normal haematopoietic precursor cells (Moore, 1990). Studies using GM-CSF to induce differentiation have shown that U937 cells are only slightly susceptible to GM-CSF, demonstrating increased expression of a macrophage cell surface marker after exposure (Zuckerman *et al*, 1988), while a more recent study has shown that U937 cells express the high affinity cell surface receptor for GM-CSF (Kloke *et al*, 1992). The U937 cells did not differentiate in response to GM-CSF, whereas the U937/E15 subline did, suggesting they may have increased expression of cell surface receptors. The difference in response to GM-CSF further exemplifies the differences between the U937 cells and the U937/E15 subline. The response of the U937/E15 subline to GM-CSF treatment confirms that the cells could still respond to differentiating agents, as CD13 and CD14 expression increased after GM-CSF treatment.

Drug resistance in the U937/E15 subline did not change in response to GM-CSF, which differed from the K562/E15B cells, which were more drug resistant after 42 h exposure to GM-CSF. When extrapolated to the clinical situation this suggests that GM-CSF may increase drug resistance in some types of leukaemia, and not others.

Differentiation induced by TPA in the K562, K562/E15B and U937 cells appeared to be mediated by PKC, while PKC was not involved in the U937/E15 subline. The increases in P-glycoprotein observed in the drug resistant sublines however were not mediated by PKC. Furthermore, the response of the K562/E15B subline to all inducers of differentiation, and the response of the U937/E15 subline to TPA all suggest induction of differentiation in cells which are already multidrug resistant further increases MDR. Translated into the clinical situation, this suggests that differentiation may not necessarily be a beneficial adjunct to chemotherapy.

CONCLUSIONS

Treatment of the K562 and the U937 cells with low, clinically relevant levels of epirubicin and vinblastine resulted in expression of the MDR phenotype. Drug resistance in the K562 sublines was most likely due to induction of P-glycoprotein. Treatment with 10 ng/ml epirubicin and 1 ng/ml vinblastine resulted in detectable P-glycoprotein expression with C219, while lower drug concentrations (5 ng/ml epirubicin and 0.5 ng/ml vinblastine) did not. The drug resistant K562 sublines were cross-resistant to other natural product drugs, and drug resistance was reversed by verapamil in cytotoxicity assays. The drug resistant K562 sublines also showed reduced Rh123 accumulation, although verapamil only increased Rh123 accumulation in the K562/E15 subline, which leads to controversy as to which method of assessing P-glycoprotein function is correct.

Drug treatment also caused differentiation in the K562 sublines. There was no obvious relationship between expression of P-glycoprotein and antigenic markers of differentiation in the epirubicin treated sublines. The K562/E15B subline was atypical, as glycophorin A expression was lower, and CD13 expression was higher than the K562 cells. The vinblastine treated sublines showed abberant antigen expression, with increases in both glycophorin A and CD13, which correlated with increases in P-glycoprotein expression.

The development of drug resistance in the K562/E15B subline did not alter their ability to undergo differentiation in response to TPA, and both the K562 and K562/E15B cells underwent megakaryocytic differentiation, with expression of CD61 and decreased glycophorin A expression. The response to sodium butyrate was also the same in the K562 cells and the K562/E15B subline, with erythroid differentiation and increased glycophorin A expression. GM-CSF did not induce differentiation in the K562 cells or the K562/E15B subline. Therefore, the development of drug resistance in the K562/E15B subline did not affect their ability to undergo further differentiation.

The U937 cells are a well-differentiated cell line, and did not express P-glycoprotein. Drug resistance to both MDR and non-MDR drugs was rapidly induced in the U937 sublines, with expression of multiple mechanisms of drug resistance, including the MDR phenotype and P-glycoprotein. As such, the drug resistant U937 sublines provide a model with which to examine the emergence and co-expression of drug resistance as observed in the clinical situation, whereby cells become resistant to both MDR and non-MDR drugs. The parental U937 cells did not express P-glycoprotein, and it is likely that drug resistance in these cells was induced and not selected, as in the K562 cells.

Resistance to the MDR drugs was reversed by verapamil in the U937 sublines, while verapamil only increased Rh123 accumulation in the U937/V8 subline, again highlighting a disparity between these two methods of assessing P-glycoprotein function. The drug resistant sublines were sensitised to doxorubicin, chlorambucil and cisplatinum by BSO, suggesting glutathione-related mechanisms also contributed to resistance in the U937

sublines. The increased DNA content in the drug resistant U937 sublines may also account for their cross-resistance to drugs such as VP-16.

Treatment with both epirubicin and vinblastine resulted in expression of glycophorin A, a loss of CD13 expression and reduction in CD14 expression in the U937 sublines, suggesting these myeloid cells have become less differentiated.

The U937 cells responded to treatment with TPA by undergoing monocytic differentiation, with increases in CD14 expression, while the U937/E15 subline did not respond to TPA treatment. Conversely, the U937 cells did not respond to treatment with GM-CSF, while the U937/E15 subline differentiated, with increases in CD13 and CD14 expression. Therefore, unlike the K562/E15B subline, the response of the U937/E15 subline to inducers of differentiation differed from the parental U937 cells.

In contrast to the K562 and U937 cells, treatment of the KG-1a and HEL cells with epirubicin and vinblastine did not induce further drug resistance due to their inherent drug resistance and expression of P-glycoprotein. Epirubicin had no effect on differentiation in the HEL cells, while vinblastine, despite the presence of functional P-glycoprotein, induced megakaryocytic differentiation, with increased CD61 expression and reduced CD34 expression. Both epirubicin and vinblastine induced megakaryocytic differentiation in the HEL sublines, with increased expression of CD61 and decreased glycophorin A expression. Therefore vinblastine induced differentiation in all four cell lines, regardless of their inherent P-glycoprotein expression. The response to epirubicin was less consistent in the four cell lines. The U937 and HEL cells were the most well differentiated cell lines and underwent differentiation in response to epirubicin, whereas lspond well to treatment and are well-differentiated, such as the U937 cells, may still be of poor prognosis and relapse with multiple mechanisms of drug resistance.

There were some exceptions to the general trends seen in the drug resistant sublines. Although the K562/E10B subline expressed low levels of P-glycoprotein, detected only by immunocytochemistry and flow cytometry, it is unlikely that the P-glycoprotein was functional, due to lack of cross-resistance to drugs other than the anthracyclines, and no change in Rh123 accumulation compared to the K562 cells. Another anomaly was the inability of verapamil to increase Rh123 accumulation in the epirubicin treated U937 sublines, despite the increased cytotoxicity in the presence of verapamil. This leads to the question as to which methods are the best to detect P-glycoprotein, particularly in clinical samples with low expression of P-glycoprotein. From these results, both functional assays and detection of P-glycoprotein expression together would appear to be necessary, as one method alone could give unreliable results.

Treatment of cells with differentiating agents gave an insight into the relationship between differentiation, multidrug resistance and P-glycoprotein expression. TPA treatment of the K562 and U937 cells induced a drug resistant phenotype, but this was

not accompanied by P-glycoprotein expression. TPA did however upregulate P-glycoprotein expression in the drug resistant K562/E15B and U937/E15 sublines, suggesting P-glycoprotein was functional. This was accompanied by verapamil sensitive decreases in Rh123 accumulation, and increased drug resistance in the K562/E15B sublines. The increases in P-glycoprotein did not appear to be mediated by PKC. Sodium butyrate induced increased expression of P-glycoprotein in the K562/E15B subline, suggesting the induced P-glycoprotein was not functional. GM-CSF increased drug resistance only in the K562/E15B subline, with no effect on other cells. P-glycoprotein and drug resistance therefore only increased in cells already expressing P-glycoprotein. In the clinical situation, this suggests that treatment of leukaemia with differentiation therapy may not be beneficial in patients already refractory to chemotherapy due to P-glycoprotein expression.

Future Directions

The U937 cells provide a unique model with which to study the emergence of drug resistance after treatment with low drug concentrations. As resistance was rapidly induced in the U937 sublines, the processes leading to to the expression of multiple mechanisms of drug resistance could be readily studied at the molecular level. This study also highlights the problems with detecting multidrug resistance in vivo. There is still much to be understood in the standard detection methods for P-glycoprotein, including the proposed functional assay using Rh123 accumulation.

The K562 and U937 cells also provide useful models to study the regulation of antigens associated with differentiation in response to drug treatment, as well as the role of PKC in differentiation. As TPA did not appear to induce P-glycoprotein through PKC in the K562/E15B and U937/E15 sublines, this suggests that P-glycoprotein was induced by another mechanism, and investigation of other modes of TPA-induction in these cell lines could provide useful information regarding mechanisms through which P-glycoprotein expression is regulated. It is possible that treatment with TPA enhances P-glycoprotein expression through the heat shock response element located in the *mdr*1 promoter (Kioka *et al*, 1992), and further studies examining the response of the heat shock element to TPA treatment may demonstrate another mechanism by which P-glycoprotein expression is induced or upregulated.

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