VERAPAMIL AND POLYUNSATURATED FATTY ACIDS AS RESISTANCE MODULATORS *IN VITRO*

by

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ABSTRACT

The development of resistance is thought to be a major cause of treatment failure in cancer patients. Multidrug resistance is a form in which tumour cells develop cross-resistance to a range of natural products. This is commonly associated with increased expression of P-glycoprotein, which appears to act as a membrane efflux pump. The aims of the present studies were firstly to further characterise the mechanisms by which verapamil can sensitise resistant tumour cells to cytotoxic drugs, and secondly to investigate polyunsaturated fatty acids as an alternative means of sensitising drug resistant cell lines.

Verapamil was shown to increase the sensitivity to anthracyclines of multidrug resistant human ovary and breast cancer cell lines that express high levels of P-glycoprotein, thus confirming previous observations. Two other modulators, quinidine and bepridil, also sensitised these two cell lines. In addition, four human non-small cell lung cancer cell lines were sensitised by verapamil but only one of these lines expressed P-glycoprotein. Neither quinidine nor bepridil altered the drug sensitivity of any of these four cell lines. The results clearly demonstrate that verapamil can modulate drug resistance in the absence of P-glycoprotein.

The drug resistant ovarian and breast cell lines showed reduced anthracycline accumulation compared with the drug sensitive parental cell lines. Verapamil increased drug accumulation in the drug resistant cell lines by two fold, yet sensitised the cells by at least 11 fold. Intracellular drug distribution was studied in the multidrug resistant cell lines by autoradiography. This method allowed quantification of drug associated with the nucleus and the cytoplasm. Most of the anthracycline daunorubicin was located in the nucleus in both drug sensitive and drug resistant cell lines. However, the ratio of nuclear to cytoplasmic grain counts was greater in the sensitive than in the resistant cell lines. Verapamil caused an increase in grain counts and a shift in the nuclear to cytoplasmic grain count ratio in the drug resistant cells to a level approaching that in the drug sensitive cell lines. It is concluded that verapamil sensitises cells not only by increasing the amount of drug accumulated but also by redistributing drug within the cell to the potential site of action of the anthracyclines, the nucleus. Furthermore, such effects on drug distribution may not be related to P-glycoprotein expression and this could account for the activity of verapamil in P-glycoprotein negative cell lines.

Studies of intracellular drug distribution led to the observation of a marked heterogeneity in drug accumulation and morphology in the parental and drug resistant ovarian cell lines. Clonal cell lines were isolated from the ovarian lines. For the drug sensitive cell line the heterogeneity in drug accumulation was shown to be caused by differences in cell size, such that intracellular drug concentrations were comparable. Two clonal cell lines derived from the resistant cell line maintained their multidrug resistant phenotype for at least 20 weeks, even in the absence of exposure to doxorubicin, the agent used to select and maintain the

resistant cell line. In contrast, four other clonal cell lines derived from the resistant cell line lost their resistant phenotype when cultured over a period of time in the absence of doxorubicin. This loss of resistance correlated with loss of expression and function of P-glycoprotein but both resistance and P-glycoprotein expression were restored by a single exposure to doxorubicin. Thus it appears that development of drug resistance *in vitro* by chronic exposure to doxorubicin can be associated with the selection of a number of different resistance phenotypes which can be maintained within a mixed population provided that the selection pressure is continued.

Growth of cells *in vitro* in the presence of fatty acids can alter the membrane composition and hence fluidity and permeability. Exposure of both doxorubicin and cisplatin resistant human ovarian cell lines to non-toxic concentrations of polyunsaturated fatty acids either before or during exposure to cytotoxic drugs did not modulate drug sensitivity. However, both gamma linolenic acid and eicosapentaenoic acid were cytotoxic alone even to drug resistant cell lines. Furthermore, isobologram analysis was used to demonstrate that this toxicity was additive with that of the cytotoxic drugs. Since the fatty acids have been given to patients without undue side effects these observations suggest that this additive toxicity to tumour cells could be exploited in the clinic.

CONTENTS

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CHAPTER 1

General Introduction

Chemotherapy has an important role in cancer treatment. With the introduction of more effective drugs and the optimisation of therapeutic strategies, clinical responses have steadily improved in patients with malignancies including Hodgkin's disease, testicular cancer and lymphocytic leukaemia (De Vita, 1982). Some encouraging results of treatment have also been achieved in patients with other common malignancies such as ovarian cancer, breast cancer and lung cancer. Unfortunately, the successes of chemotherapy are inevitably overshadowed by the eventual relapse of patients who initially responded. Implementation of the same regimen seldom results in a second remission due to the emergence of drug resistance. To make matters worse, in most cancers the development of resistance to a single drug or combination is often coupled with the emergence of cross resistance to a wide variety of other antineoplastic agents to which the tumour cells have not been exposed. This type of induced cross-resistance is termed 'acquired' as opposed to 'de novo' resistance which is used for tumours such as colon cancer which are resistant from the outset (Moscow and Cowan, 1988).

Anthracyclines

Daunorubicin was the first anthracycline to be isolated and recognised to have anti-tumour activity (Arcamone, 1985), particularly against leukaemias (Gale & Foon, 1987). Subsequently doxorubicin was isolated and shown to have a wider range of anti-tumour activities (Arcamone, 1985). It is now clear that doxorubicin has the widest spectrum of all chemotherapeutic agents and is highly effective in a number of tumour types (Weiss *et al*, 1986). Doxorubicin and daunorubicin are identical except that daunorubicin has an acetyl group at the C-9 position of the anthracycline ring and doxorubicin has a hydroxyacetyl group at this position (see Chapter 3 Fig. 1 for structures). This small difference results in enhanced lipophilicity of daunorubicin and more rapid metabolism and elimination (Gewirtz & Yanovich, 1987). Although widely used in chemotherapy the activity of anthracyclines is limited by cardiotoxicity. As a result a large number of analogues have been synthesised and are currently undergoing preclinical and clinical evaluation (Mross, 1991).

Although they have been in clinical use for many years the exact mechanism by which anthracyclines exert their cytotoxicity is as yet unknown. Anthracyclines exert multiple effects on cells. Those which have been considered the most important in killing tumour cells are: (*1*) intercalation into DNA; (*2*) inhibition of topoisomerase II; (*3*) interaction with the cell membrane and (*4*) free radical formation (Gianni *et al*, 1983; Powis, 1987).

It has generally been assumed that the binding of anthracyclines to DNA

occurs via intercalation of anthraquinone ring into the DNA helix. However, the possible existence of ternary complexes of doxorubicin, DNA and iron has also been discussed (Eliot *et al,* 1984). Topoisomerase II is an essential nuclear enzyme involved in DNA replication (Liu, 1989; Wang, 1985). Both the epipodophyllotoxins and the anthracyclines interfere with the activity of topoisomerase II (Beck & Danks, 1991). Although it is not known how drugs which inhibit this enzyme cause cell death it has been postulated that a stable complex is formed between the enzyme and DNA in the presence of the drug (Shen *et al*, 1989).

Direct cell membrane action is hypothesised as another cytotoxic effect of anthracyclines. This concept is supported by several observations which demonstrate that anthracyclines can exert their cytotoxic effect without entering cells (Tokes *et al*, 1982; Tritton & Yee, 1982). The interaction of anthracyclines with cell membranes can also result in changes in membrane lipid composition, membrane fluidity and permeability (Okano *et al*, 1984) as well as inhibition of plasma membrane enzymatic activities (Sun *et al*, 1984). It is also clear that free radical formation catalysed by intracellular enzymes, can be responsible for DNA strand breaks as well as cytoplasmic damage. The semiquinone free radical formed by reduction of the anthracycline quinone moiety can in turn react with molecular oxygen to yield superoxide anion and subsequently hydrogen peroxide and hydroxyl radical. These can then cause the membrane lipid peroxidation and DNA damage. Generation of free radicals is thought to be responsible for the cardiotoxicity of the anthracyclines (Davies & Doroshow, 1986; Doroshow &

Davies, 1986). Although cardiotoxicity is a major factor in determining the maximum tolerated dose of the anthracyclines, the observation that complete responses can be achieved suggests that the development of resistance is the key factor limiting their clinical efficacy (Kaye & Merry, 1985).

Multidrug drug resistance

Investigations on the cellular mechanisms of drug resistance have relied extensively on the use of model systems *in vitro*. Continuous cell lines selected for resistance to chemotherapeutic agents are derived by exposing cells chronically to a stepwise increasing concentration of a single drug. When the drug used is an anthracycline, *Vinca* alkaloid, podophyllotoxin or colchicine, resistance is induced not only to the selecting drug but also to the other members of this group of high molecular weight natural product amphipathic drugs (Biedler & Riehm, 1970; Dano, 1972; Kessel *et al*, 1968). In general, multidrug resistant cells are not cross resistant to cisplatin, bleomycin, alkylating agents or antimetabolites (Schurr *et al*, 1989; Supino *et al*, 1986; Twentyman *et al*, 1986a & b).

Although not universally seen, the most common changes observed in MDR cell lines are (*1*) a reduction in steady-state cellular drug accumulation (Beck *et al*, 1983; Fojo *et al*, 1985; Riehm & Biedler, 1971; Tsuruo *et al*, 1982; Twentyman *et al*, 1986b); (*2*) an increase in drug extrusion rate (Dano, 1973; Fojo *et al*, 1985; Inaba *et al*, 1979), and (*3*) a hyperexpression of a specific membrane

glycoprotein with a molecular weight of 170,000 dalton known as P-glycoprotein (Juliano & Ling 1976; Kartner *et al*, 1983a & b; Ma *et al*, 1987). The latter is associated with both an amplification of the *mdr* gene family, including *mdr*1 which encodes P-glycoprotein (Bourhis *et al*, 1989; Keith *et al*, 1990; Riordan *et al*, 1985a; Van der Bliek *et al*, 1986, 1988) and an increased expression of the corresponding mRNA (Reeve *et al*, 1989; Scotto *et al*, 1986; Shen *et al*, 1986).

MDR cells expressing P-glycoprotein exhibit reduced intracellular drug accumulation and increased drug extrusion, and interference with P-glycoprotein increases drug accumulation (Broxterman *et al*, 1988; Hamada & Tsuruo, 1986; Rittmann-Grauer *et al*, 1990). This led to the suggestion that P-glycoprotein may actively exclude xenobiotics from resistant cells, resulting in lower intracellular drug accumulation and reduced toxicity (Juliano & Ling, 1976; Kartner *et al*, 1983a & b; Ling *et al*, 1983).

Direct evidence in support of this causal role for the P-glycoprotein in MDR has come from the isolation of complementary DNA for mouse (Gros *et al*, 1986b) and human (Chen *et al*, 1986) P-glycoprotein and from the subsequent transfection experiments. Transfection and expression of cloned complementary DNA encoding P-glycoprotein into drug sensitive NIH 3T3 cells is able to confer the MDR phenotype (Croop *et al*, 1987; Gros *et al*, 1986a; Ueda *et al*, 1987). Moreover, a single spontaneous mutation in *mdr*1 gene leading to substitution of glycine to valine at position of 185 of P-glycoprotein results in preferential resistance of an MDR variant to colchicine (Choi *et al*, 1988).

The deduction of the full length of P-glycoprotein primary sequence

predicts a protein 1280 amino acids long and consisting of two homologous halves connected by a 60 amino acid sequence (Chen *et al*, 1986; Gerlach *et al*, 1986; Gros *et al*, 1986b). Comparison of the deduced P-glycoprotein primary sequence with other available data has subsequently shown that the hydrophillic regions on each of two homologous halves share striking similarity with a class of bacterial transport proteins (Croop *et al*, 1987; Gros *et al*, 1986b; Ueda *et al*, 1987). Further analysis of the predicted structure of P-glycoprotein suggests that each half of the protein contains six potential transmembrane regions. This implies that the P-glycoprotein molecule can span the membrane 12 times to form a transmembrane configuration characteristic of a channel-like structure. Moreover, a highly conserved hydrophillic region, located on the predicted cytoplasmic domain, has the characteristics of an ATP-binding site (Chen *et al*, 1986; Gerlach *et al*, 1986; Gros *et al*, 1986b) and P-glycoprotein has been shown to have ATPase activity (Hamada & Tsuruo, 1988a & b). Thus the structure of the P-glycoprotein is consistent with a role for P-glycoprotein as an ATP-driven active drug efflux pump. In support of this hypothesis are the findings showing that ATP binds specifically to P-glycoprotein (Cornwell *et al*, 1987b) and the protein itself has ATPase activity (Hamada & Tsuruo, 1988a & b). Furthermore, the use of photoaffinity labelled *Vinca* alkaloids showed that drugs in the MDR category are able to bind directly to the P-glycoprotein.

P-glycoprotein has been predicted to have two binding sites for azidopine, a photoaffinity labelling agent (Bruggemann *et al*, 1989). Further evidence in support of the speculation that P-glycoprotein acts as a 'hydrophobic vacuum

cleaner', binding natural antineoplastic products and removing them from resistant cells, comes from the competition binding studies using photoaffinity labelling technique. In these experiments a range of MDR-related agents exhibited higher affinity to the membrane vesicles from P-glycoprotein hyperexpressing resistant cells than to those from wild type sensitive cells. The binding of these drugs to the membrane preparations was ATP-dependent (Cornwell *et al*, 1986a & b; Horio *et al*, 1988; Naito & Tsuruo, 1989).

Atypical multidrug resistance

Although the majority of multidrug resistant cell lines have been shown to overexpress P-glycoprotein it has become apparent that induction of drug resistance *in vitro* can give rise to cell lines with the characteristic cross-resistance phenotype of MDR but without overexpression of P-glycoprotein (Beck *et al*, 1987; Cass *et al*, 1989; Coley *et al*, 1991a; Mirski *et al*, 1987). Beck (1989) has used the term 'atypical MDR' to describe such cell lines. In many cases resistance is associated with decreased expression of topoisomerase II. On the whole such cell lines do not show reduced drug accumulation (Danks *et al*, 1987; Harker *et al*, 1989), but there are exceptions which may be associated with an alternative drug efflux mechanism (Mirski *et al*, 1987; Taylor *et al*, 1991; Twentyman *et al*, 1986b; Zijlstra *et al*, 1987a).

Expression of P-glycoprotein in human tissues and tumours

Identification of P-glycoprotein *in vitro* and the subsequent isolation of the gene and protein allowed the development of cDNA probes for *mdr*1 mRNA and monoclonal antibodies specific for the protein (Juliano & Ling, 1976; Hamada & Tsuruo, 1986; Kartner *et al*, 1985; Riordan *et al*, 1985b; Roninson *et al*, 1986; Van der Bliek *et al*, 1986). It is therefore possible to detect P-glycoprotein expression both at the level of mRNA by Northern analysis and at the level of the protein by both immunocytochemistry and Western analysis. One of the most important findings has been the detection of P-glycoprotein in a number of normal tissues in humans. These include adrenal gland, kidney, liver and large intestine. One of the advantages of immunohistochemistry is that it allows precise location of the marker expression. P-glycoprotein was shown to be expressed on the luminal surface of the kidney brush border, on the mucosal surface of large intestine and on the bile canalicular surface of hepatocytes. These locations suggest a role for P-glycoprotein in protecting against exogenous toxins and, possibly, in removal of such toxins from the body (Thiebault *et al*, 1987).

There have been a large number of reports of expression of P-glycoprotein in human tumours. Goldstein *et al* (1989) analysed more than 400 human tumours by mRNA slot blot. They showed expression in untreated tumours derived from colon, kidney, adrenal gland and liver. All of these tumour types are characteristically resistant at presentation and are derived from tissues with intrinsic expression of P-glycoprotein. Although this suggests an interesting link

it should be stressed that some of the mRNA analysed will originate from contaminating normal tissue in the tumour biopsy. Expression was also detected in carcinoid tumours, pancreatic tumours and cell lines established from non-small cell lung cancers. Ma *et al* (1987) showed expression of P-glycoprotein in acute non-lymphoblastic leukaemia and demonstrated a correlation between the proportion of blast cells that stained positive for P-glycoprotein and disease progression. Some tumours which do not appear initially to express P-glycoprotein have been found to express P-glycoprotein at relapse following chemotherapy, *e.g.*, ovary (Bourhis *et al*, 1989), acute myeloid leukaemias (Sato *et al*, 1990) and multiple myeloma (Dalton *et al*, 1989). A number of reports have noted expression of P-glycoprotein in untreated breast tumours (Keith *et al*, 1990; Schneider *et al*, 1989; Wishart *et al*, 1990). Wishart *et al* (1990) used immunocytochemistry and were able to demonstrated P-glycoprotein in the majority of tumours although expression was heterogeneous. More importantly they noted expression in the stromal cells as well as in the epithelial cells of the tumour. Furthermore, Weinstein *et al* (1991) demonstrated preferential expression of P-glycoprotein in areas of invasive growth of colon tumours. They suggest that P-glycoprotein expression may correlate with the degree of malignancy of a tumour but it is also possible that the P-glycoprotein has a role in invasion and metastasis.

There have been a few attempts to correlate P-glycoprotein expression with response to treatment (Pirker *et al*, 1991; Salmon *et al*, 1989). The most convincing of these studies was that of Chan *et al* (1990) who developed the

methodology to detect P-glycoprotein by immunohistochemistry in formalin fixed paraffin embedded tissue. This allowed a retrospective study of childhood sarcoma over a nine year period. They were able to demonstrate a clear correlation between P-glycoprotein expression and response to treatment and survival.

Resistance modulators

A number of agents are able to sensitise MDR cell lines to cytotoxic drugs. These resistance modulators belong to various groups of drugs which by themselves are non-cytotoxic. The most potent show some structural similarity in that they are typically hydrophobic molecules featuring two planar aromatic rings and a tertiary basic nitrogen atom with a positive charge at physiological pH (Rothenberg & Ling, 1989). The calcium channel blocker verapamil was the first such agent identified (Tsuruo *et al*, 1981). Verapamil was shown to sensitise vincristine resistant P388 leukaemia cells to both vincristine and vinblastine and sensitisation was accompanied by increased drug accumulation.

Following this discovery, various types of human (Cass *et al*, 1989; Fojo *et al*, 1985; Rogan *et al*, 1984; Tsuruo *et al*, 1983a & b; Twentyman *et al*, 1986a; Willingham *et al*, 1986) and animal (Chatterjee *et al*, 1990; Kessel & Wilberding, 1985; Ramu *et al*, 1983b; Supino *et al*, 1988; Twentyman *et al*, 1990) MDR cell lines have been shown to be sensitised to a number of MDR drugs in the

presence of verapamil. In addition, numerous other agents have been identified as active in drug resistance modulation in experimental models, including bepridil (Schuurhuis *et al*, 1987), quinidine (Tsuruo *et al*, 1984), perhexiline maleate (Ramu *et al*, 1984a), cyclosporins (Slater *et al*, 1986; Twentyman, 1988), reserpine (Beck *et al*, 1988), tamoxifen (Ramu *et al*, 1984b; Chatterjee & Harris, 1990) and other related compounds (Cole *et al*, 1989; Dusre *et al*, 1989; Jamali *et al*, 1989; Kessel, 1986). In most cases sensitisation is accompanied by increased drug accumulation and it is thought that resistance modulators act via inhibition of the drug efflux mechanism associated with P-glycoprotein (Rogan *et al*, 1984; Tsuruo *et al*, 1983a). A number of photoaffinity analogs of both cytotoxic drugs associated with the MDR phenotype and resistance modulators have been shown to bind to P-glycoprotein (Beck *et al*, 1988; Busche *et al*, 1989; Cornwell *et al*, 1986a & b; Foxwell *et al*, 1989; Qian & Beck, 1990; Safa *et al*, 1987 & 1989; Safa, 1988; Yang *et al*, 1988). The resistance modulators can displace the cytotoxic drugs from P-glycoprotein but not a photoaffinity analog of ATP suggesting that modulators interfere with drug binding to the pump and not the energy supply to the pump (Cornwell *et al*, 1987a & b).

Resistance modulation in the clinic

Following the identification of P-glycoprotein in several human tumour types there have been a number of clinical trials of resistance modulators. An

initial trial of verapamil in addition to chemotherapy for ovarian cancer failed to show an improved response (Ozols *et al*, 1987). However this trial identified cardiotoxicity as the major dose limiting factor in the use of verapamil. As a result, the plasma levels achieved in patients are between 3 and 12 times lower than the most effective concentration *in vitro* (Cairo *et al*, 1989; Dalton *et al*, 1989; Kerr *et al*, 1986). Although Dalton *et al* (1989) showed promising results in patients with multiple myeloma treated with verapamil in addition to chemotherapy the study was far too small to draw any real conclusions about the effectiveness of modulators in the clinic. Similarly, Sonneveld & Nooter (1990) reported the activity of cyclosporin-A as a modulator in the clinic based on observations on a single patient.

Thus there is little evidence as yet that resistance modulators can be effective in patients. Verapamil is clearly not the most effective agent due to its cardiological activity and there is a need to identify more suitable agents for clinical use. This can be done either by trial and error with modulators identified *in vitro* or by obtaining a clearer understanding of the mechanism of action of modulators in order to design better agents.

Aims of the thesis

The main aims of these studies can be summarised as follows:

(*1*) to characterise verapamil as a resistance modulator

(*2*) to determine whether verapamil affects intracellular drug distribution

(*3*) to find alternative means of overcoming drug resistance

Lay-out of the thesis

Each chapter begins with a self-contained Introduction and therefore full details of the rational behind the various individual stages of the work will not be discussed here.

A number of observations have indicated that verapamil can sensitise cells to cytotoxic drugs by mechanisms other than inhibition of P-glycoprotein. Cole *et al* (1989) reported that verapamil could sensitise a doxorubicin resistant cell line that does not express P-glycoprotein. Furthermore, several studies have indicated a discrepancy between the magnitude of the effects of verapamil on drug sensitivity and its effects on drug accumulation (Ford *et al*, 1991; Kessel & Wilberding, 1984; Rogan *et al*, 1984). Because of these findings, the experimental work of the thesis begins with a comparison of three different resistance modulators using a panel of cell lines that includes both P-glycoprotein positive and negative cell lines (Chapter 2). The results clearly establish verapamil as a modulator with a broad spectrum of activity that extends to cell lines that do not express P-glycoprotein.

In Chapter 3 evidence is presented that verapamil can alter the distribution of drug within multidrug resistant cell lines. During the course of this work a

marked morphological heterogeneity was observed in both the parental cell line and the multidrug resistant subline used for these experiments. This heterogeneity stimulated a study of clonal populations derived from these cell lines. Chapter 4 describes differences in the stability of P-glycoprotein expression and the drug resistance phenotype for some of these clones.

It was clear that drug sensitivity could be affected by changes in intracellular drug distribution. This suggested that it might be possible to alter drug sensitivity by changing the composition of the cell membranes. Furthermore, Timmer-Bosscha *et al* (1989) have shown that docosahexaenoic acid can sensitise a cisplatin resistant cell line to cisplatin and demonstrated that the fatty acid was incorporated into the cell membrane. Chapter 5 describes an attempt to modulate both doxorubicin and cisplatin sensitivities with two polyunsaturated fatty acids, gamma linolenic acid and eicosapentaenoic acid. Although modulation was not observed, these agents were found to exhibit interesting additive toxicity which could be explored in the clinic.

CHAPTER 2

General methods and characterisation of verapamil as a resistance modulator

Introduction

Although verapamil was the first agent identified that can overcome multidrug resistance associated with P-glycoprotein overexpression (Tsuruo *et al*, 1981), a number of other compounds have since been identified that share this property (Stewart & Evans, 1989). However, verapamil remains one of the most interesting resistance modulators in the laboratory since it has also been shown to be active in cell lines that express the multidrug resistance phenotype in terms of cross resistance patterns but do not express P-glycoprotein (Cole *et al*, 1989; Coley *et al*, 1991a). This property of verapamil has been ascribed either to effects on membrane trafficking (Sehested *et al*, 1987a & b) or to the presence of an alternative drug efflux pump (Reeve *et al*, 1990). It is not known whether verapamil is unique in this activity since there have been few comparative studies of resistance modulators.

One of the major aims of this thesis is to characterise the activity of verapamil in multidrug resistant cell lines in order to determine whether

sensitisation is due solely to effects on drug accumulation. Three pairs of cell lines were available within the Department of Medical Oncology for use in these studies. Only two of these were chosen for use since the third, a human small-cell lung cancer cell line NCI-H69 and a doxorubicin resistant subline H69LX10 are non-adherent cell lines with indistinct cytoplasm. This latter pair is clearly not suitable for studies of intracellular drug distribution. The human ovarian cancer cell line A2780 and the *in vitro* derived doxorubicin resistant subline were selected for use in the majority of experiments. The latter cell line, 2780AD, expresses high levels of P-glycoprotein (Sugawara *et al*, 1988) and has been shown to be sensitised to doxorubicin by verapamil (Plumb *et al*, 1990; Rogan *et al*, 1984). In contrast the parent cell line A2780 does not express P-glycoprotein (Sugawara *et al*, 1988; R. Brown, personal communication) and is not sensitised by verapamil (Plumb *et al*, 1990). The human breast cancer cell line MCF7 and the doxorubicin resistant subline MCF7/Adr were also used for comparison. These cell lines share similar properties to those described for the ovarian cell lines, including their P-glycoprotein status, except that MCF7/Adr is highly resistant (ID_{50} for doxorubicin 21 μM, Plumb *et al*, 1990) and thus requires large quantities of drug for cytotoxicity assays.

The aim of the experiments described in this chapter was to characterise the effects of verapamil on drug sensitivity and drug accumulation in the two pairs of multidrug resistant cell lines. Both doxorubicin and daunorubicin were used. Although doxorubicin was the main drug of interest in view of its wide range of antitumour activity in the clinic, supplies of radioactively labelled doxorubicin

were limited and expensive. Daunorubicin was therefore included in order to determine whether this anthracycline could be used to answer the same questions. In addition four non-small cell lung cancer cell lines were included in the study. Previously, a number of human non-small cell lung cancer cell lines were shown to demonstrate a drug sensitivity profile similar to that of multidrug resistant cell lines (Merry *et al*, 1987). None of these cell lines had been exposed to cytotoxic drugs *in vitro* and it was not known whether any of them express P-glycoprotein. However, this form of lung cancer is usually resistant to chemotherapy at presentation (Minna *et al*, 1985). Four of the non-small cell lung cancer cell lines have now been characterised for P-glycoprotein expression by detection of mRNA in the Department of Medical Oncology. One of the cell lines WIL has low levels of *mdr*1 mRNA but the other three have very low if any expression of *mdr*1 mRNA (R. Brown, personal communication). Since the analysis was carried out by Northern blots it is not possible to state that these lines do not express *mdr*1 mRNA as a more sensitive technique such as the polymerase chain reaction could possibly detect the mRNA (Barrand & Twentyman, 1992). Furthermore, P-glycoprotein could not be detected by immunocytochemistry in any of the four non-small cell lung cancer cell lines. Merry *et al* (1987) had shown that some of these cell lines could be sensitised to etoposide, vincristine and doxorubicin by verapamil. These cell lines were therefore included in the study in order to determine whether the activity of verapamil was related to the level of expression of P-glycoprotein. Since it was possible that verapamil was not acting via P-glycoprotein in 3 of the non-small cell lung cancer cell lines two other resistance

modulators were included for comparison. Quinidine was used since it has been shown that plasma levels of 6 μM can be achieved in patients and is therefore a potential candidate for clinical trials (Jones *et al*, 1990; Tsuruo *et al*, 1984). Whilst verapamil is a calcium channel blocker quinidine is thought to exhibit cardiological activity through a general membrane perturbation (Surewicz & Jozwaik, 1983). Bepridil is a calcium channel blocker like verapamil and has been shown to modulate drug resistance (Schuurhuis *et al*, 1987). Although it is not in clinical use in this country it has been used in France and plasma levels of 4 μM can be achieved in patients (S.B. Kaye, personal communication).

In these experiments and throughout this work drug sensitivity was determined by a tetrazolium dye based microtitration assay. This assay was developed and characterised with the Department of Medical Oncology and has been shown to give identical results to a standard clonogenic assay (Plumb *et al*, 1989). Cell lines A2780 and 2780AD have very poor plating efficiencies in clonogenic assays (about 15%) and produce very small colonies. Both perform well in the microtitration assay. A second advantage of the latter assay is that large numbers of cytotoxicity tests can be performed at any one time under standardised conditions. This is important where direct comparisons are made between cell lines and in experiments where modulators are used.

In addition to presenting results on the characterisation of verapamil and to a lesser extent certain other agents as resistance modifiers in various cells, this chapter also gives details of the general methods used throughout the thesis.

Materials and Methods

Cell lines

The human ovarian cancer cell line A2780 and the subline made resistant *in vitro* to doxorubicin (2780AD), were obtained from Dr. R. F. Ozols (Fox Chase Cancer Centre, Philadelphia, U.S.A.). The human breast cancer cell line MCF7 and its doxorubicin resistant subline MCF7/Adr were kindly provided by Dr. K. Cowan (National Cancer Institute, Bethesda, U.S.A.). Both 2780AD and MCF7/Adr express high levels of P-glycoprotein (Fairchild *et al*, 1987; Sugawara *et al*, 1988).

The two of four human non-small cell lung cancer cell lines used, a bronchiolo-alveolar carcinoma cell line A549 and a squamous carcinoma cell line SK-MES, were from the American Type Tissue Culture Collection (ATCC, U.S.A.). The adenocarcinoma cell line WIL was obtained from the Ludwig Institute (Sutton, U.K.) and the squamous lung carcinoma cell line L-DAN was established in the CRC Department of Medical Oncology, University of Glasgow, U.K.). Cell lines A549, L-DAN and SK-MES express very low levels of *mdr*1 mRNA by Northern blot analysis. The levels in WIL are about 4 times higher than in the 3 other non-small cell lung cancer lines but are only one tenth of the levels observed in the human small cell lung cancer line H69LX10 which is 60 fold resistant to doxorubicin (Plumb *et al*, 1990; R. Brown, personal communication). P-glycoprotein cannot be detected by immunocytochemistry in

any of the 4 non-small cell lung cancer cell lines.

Culture media

The ovarian and breast cancer cell lines were grown in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Northumbria Biologicals, Cramlington, Northumberland, U.K.) supplemented with glutamine (2 mM) and foetal calf serum (10%). For the ovarian cell lines insulin (0.25 units/ml) was also added.

The non-small cell lung cancer cell lines were grown in a mixture (50:50) of Ham's F10 (Northumbria Biologicals, Cramlington, Northumberland, U.K.) and Dulbecco's Modified Eagles Medium (Life Technologies Ltd., Paisley, U.K.) supplemented with glutamine (2 mM) and foetal calf serum (10%).

The media were prepared from 10 × concentrated stock solutions. It was buffered by addition of sodium bicarbonate (0.075%) and equilibrated with CO_2 (2% in air).

Culture conditions

All the cell lines grew as monolayer cultures. They were maintained in exponential phase of growth in 75cm^2-flasks (J. Bibby Science Products Ltd,

Staffordshire, U.K.) or in 25cm^2-flasks (Nunclon, Life Technologies Ltd., Paisley, U.K.). Cells were subcultured at one week intervals. The monolayer of cells was exposed to trypsin (0.25%, Life Technologies Ltd., Paisley, U.K.) in the presence of ethylenediaminetetraacetic acid (EDTA, 1 mM, BDH, Poole, U.K.) in Ca^{2+} and Mg^{2+} free phosphate buffered saline (Dulbecco's A, PBS) for 30 seconds. The solution was removed and the monolayer incubated at 37°C for 5 minutes. Trypsin was inactivated and the cells collected by addition of 10 ml of culture medium. Cell number was determined with an electronic counter (Coulter Model ZB1, Coulter Electronics Ltd., Luton, Beds, U.K.) and cells were plated into new flasks at a density of 5×10^5/25cm^2 flask or 2×10^6/75cm^2 flask. Flasks were equilibrated with CO_2 (2% in air) and sealed. Cultures were fed with fresh medium at intervals of 2-3 days.

For the doxorubicin resistant cell lines resistance was maintained by regular exposure to the selecting agent. Cell line 2780AD was grown constantly in the presence of doxorubicin (2 μM). Cell line MCF/Adr was exposed to doxorubicin (10 μM) for 24 hours once every six weeks. Both cell lines were grown in the absence of drug for at least 5 days prior to experiments so that cellular doxorubicin content was at minimal levels.

Mycoplasma testing

All the cell lines were examined once a month for mycoplasma

contamination by the Hoechst staining method (Chen, 1977).

Maintenance of cell stocks

Stocks of all cell lines were held in liquid nitrogen. Cells were maintained in culture for 3 months and then replaced with fresh cells from the frozen stocks. This reduced variability due to maintenance in continuous culture and reduced the risk of cross contamination of cell lines.

Monolayer cultures were harvested with trypsin, collected into a sterile universal container (Bibby Sterilin Ltd., Stone, Staffs, U.K.) and centrifuged for 5 minutes at 200 *g*. The pellet was resuspended at a density of about 5×10^6/ml in culture medium containing dimethyl sulphoxide (10%, Merck, Thornlibank, Glasgow, U.K.). This suspension was divided into 1 ml cryotubes (Nunclon, Life Technologies Ltd., Paisley, U.K.) and frozen at a rate of 1°C per minute in a insulated container in a freezer at -70°C. After at least 4 hours they were transferred into liquid nitrogen.

When required, an ampoule was removed and placed in warm water (37°C). The cell suspension was transferred into a 25 cm^2 flask and 5 ml of culture medium added. The cells were equilibrated with CO_2 (2%), the flasks sealed and incubated at 37°C for 24 hours. The medium was then replaced with fresh medium and the cells allowed to grow. They were passaged twice before use in experiments.

Measurement of cell volume

When necessary cell size was determined with an electronic particle counter. Calibration of the Coulter counter (Model Industrial D; Coulter Electronics Ltd., Luton, Beds, U.K.) for cell sizing was carried out with standard polystyrene divinyl benzene latex beads (P.D.V.B. Latex, Coulter Electronics Ltd., Luton, Beds, U.K.) using a half-count technique (Section 6.1, Reference Manual for the Coulter Counter Model D, issue A: 1984). The threshold (t), the attenuation (A) and the aperture (I) were determined for a given size of latex beads. The calibration constant (K_d) was calculated by the following equation:

$$K_d = d / (t \times I \times A)^{1/3}$$

where **d** is the known mean diameter of the P.D.V.B. particles in μm.

The cell sizing was done by determining **t**, **I** and **A** for each cell line. The mean diameter of a single cell was derived from the above formula and the volume of a cell assuming a spherical shape was calculated as $1/6\ \pi d^3$ in μm^3.

Determination of doubling time

The doubling time of a cell line was determined from cell counts of cells in optimal culture conditions. Cells were seeded in 1 ml of culture medium into 24-well plates (Nunclon, Life Technologies Ltd., Paisley, U.K.) at a density of 10^4 cells per well and grown at 37°C in a humidified atmosphere of 2% CO_2 in air.

Cells in 3 wells were trypsinised and counted daily and the medium was replaced daily. Cell doubling time was determined from the slope of the exponential growth phase obtained from a plot of log cell number against time in days.

Cytotoxicity assay

Drug sensitivity was determined by a tetrazolium dye based microtitration assay (Mosmann, 1983) which was further developed and optimised in the department (Plumb *et al*, 1989). In this assay cells in the exponential phase of growth are exposed to drug for a fixed time period and then allowed to grow in drug free medium for 2-3 cell doublings. Finally, cell number is determined indirectly by the reduction of tetrazolium dye, 3-(4,5-dimethylthiazo(-2-yl)-2,5-di-phenyletetrazolium bromide (MTT, Sigma Chemical Company, Poole, U.K.), by live but not dead cells to a water-insoluble purple formazan product. Under appropriate conditions the absorbance of the reduced dye is proportional to the biochemically viable cell number. It is a rapid and reproducible assay which is ideally suited to this type of project and the results agree well with those of a standard clonogenic assay (Plumb *et al*, 1989).

Cells were plated out into 96-well microtitre plates (12 $\times$ 8 wells, Linbro, ICN Biomedicals Ltd., High Wycombe, Bucks, U.K.) in 200 µl of medium at a concentration of 5 $\times 10^2$ to 1 $\times 10^3$ cells per well and grown at 37°C for 2-3 days in a humidified atmosphere of 2% CO_2 in air. The first and last rows of 8 wells

on the plate contained medium only. Cells were then exposed to drug for 24 hours unless stated otherwise. A serial dilution of drug was prepared (usually 1:4) to give a total of 8 concentrations such that the ID_{50} of the cell line was in the middle of the range. Four replicate wells were used for each drug concentration and cells in the second and eleventh rows of eight wells were fed with drug-free medium only. When a resistance modulator was used the drug was prepared at twice the final concentration and diluted 1:1 with medium or medium containing the modulator at twice the final concentration before addition to the cells. For these experiments the plate was divided into 2 such that the top 4 wells received drug alone while the bottom 4 contained drug plus modulator. Modulator alone was added to the second and eleventh rows in the bottom half of the plate. After 24 hours, the medium and drugs were removed by needle aspiration and cells fed with drug free medium daily for 3 days. They were then fed with 200 µl of fresh medium containing Hepes buffer (10 mM, Life Technologies Ltd., Paisley, U.K.) and MTT (50 µl, 5 mg/ml in PBS) was added to all 96 wells. Plates were incubated in the dark at 37°C, in an atmosphere of 2% CO_2 in air for 4 hours. The medium and MTT was removed and the formazan crystals dissolved by addition of 200 µl of dimethyl sulphoxide to each well followed by 25 µl of Sorensen's glycine buffer (0.1 M glycine, 0.1 M NaCl adjusted to pH 10.5 with NaOH).

The absorbance was measured with a microplate reader (Bio-Rad Model 3550, Bio-rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K.) at a wavelength of 570 nm. The first row of the plate that had contained medium and

MTT but no cells was used as a blank for the plate reader. The absorbance values were plotted versus drug concentrations on semilog graph paper, and drug sensitivity was expressed as the ID_{50} defined as the drug concentration required to reduce the absorbance value to 50% of that of the control untreated cells.

Conditions were optimised such that cells underwent at least 2 population doublings during the period between removal of drug and the end of the experiment. In addition absorbance was shown to be linear with viable cell number in the range used for these experiments.

Cytotoxic drugs

Doxorubicin was obtained as a sterile powder (Farmitalia, St. Albans, U.K.). It was prepared as a stock solution in distilled water at 2 mg/ml and stored at -20°C in aliquots of 100 or 200 µl. Daunorubicin (Sigma Chemical Company, Poole, U.K.) was prepared as a stock solution at 2 mg/ml in PBS, filter sterilised (pore size 0.22 µm, Millex-GS, Millipore Ltd., Watford, Herts, U.K.) and stored at -20°C in aliquots for up to 2 months. The resistance modulators verapamil and quinidine sulphate (Sigma Chemical Company, Poole, U.K.) were dissolved in PBS and filter sterilised just before use. Bepridil (Sigma Chemical Company, Poole, U.K.) was made up as a stock solution just before use at a concentration 2 mM by addition of 200 µl of polyethylene glycol 200 (Merck, Thornlibank, Glasgow, U.K.) to 4.03 mg of bepridil followed by addition of 4.8

ml of PBS.

Estimation of cellular drug accumulation

Cellular drug accumulation was determined according to the method described by Plumb *et al* (1990). Cells were trypsinised, counted and plated out into 6-well plates (Nunclon, Life Technologies Ltd., Paisley, U.K.) at a density of 10^6 cells per well in 5 ml of medium and grown at 37°C in a humidified atmosphere of 2% CO_2 for 24 hours. The medium was removed and the cells were fed with 4.5 ml of fresh medium and allowed to equilibrate for 1 hour at 37°C, 2% CO_2.

Daunorubicin and ^{3}H-daunorubicin (3.2 Ci/mmol, New England Nuclear, Stevenage, Herts, U.K.) was added as a 10× concentrated stock (0.5 ml per well) to give a final concentration of 1 μM and 0.01 μCi/ml. In some experiments doxorubicin and ^{14}C-doxorubicin (76.6 mCi/mmol, Amersham International, Amersham, Bucks, U.K.) was added to give the same final concentrations. When resistance modulators were used, cells were fed with 4 ml of medium and the modulator was added (0.5 ml/well) as a 10× concentrated solution.

At specified times plates were placed on ice and the medium removed. Cells were washed twice with 5 ml of ice cold PBS and trypsin/EDTA (0.25%/1mM, 0.5 ml/well) was added. Plates were incubated at 37°C for 5 minutes to remove cells and wells were washed twice with PBS (0.5 ml) and the

cells plus washes transferred to scintillation vials. Scintillation fluid (10 ml, Ecoscint, National Diagnostics, B.S. & S. Ltd., Edinburgh, U.K.) was added to the vial and the contents mixed. Radioactivity was determined in a scintillation counter (Packard Liquid Scintillation Spectrometer, Model 3255, Canberra, Packard Ltd., Pangbourne, Berks, U.K.). A sample (25 μl) of the concentrated drug stock was also counted in order to determine the specific activity of the drug. This value was used to convert cpm to pmoles of drug. Cell counts were determined in 3 additional wells and drug accumulation is expressed as pmoles/10^6 cells. In some cases accumulation is expressed as a concentration based on the estimated volume of the cell.

Estimation of drug efflux

Drug efflux was determined by the method described by Fojo *et al* (1985). Cells were plated into 6-well plates as for the drug accumulation assay. They were incubated for 1 hour at 37°C with daunorubicin and ^{3}H-daunorubicin (1 μM, 0.01 μCi/ml) in 5 ml of glucose free Hank's Balanced Salt Solution containing 0.075% sodium bicarbonate and sodium azide (10 mM, Sigma Chemical Company, Poole, U.K.). The azide was included in the glucose-free medium to fully inhibit energy dependent efflux during the drug loading period. Thus more or less equalising drug accumulation in the parent and resistant cells. In some experiments cells were incubated with drug in culture medium containing

verapamil (6.6 or 50 μM). Medium and drug were then removed and the cells washed twice with ice cold PBS. The drug loaded cells were incubated in drug and azide-free culture medium at 37°C and at specified times the medium was removed, the cells washed with ice cold PBS and total intracellular daunorubicin determined as for the drug accumulation studies.

Statistical analysis

Where necessary the results were subject to non-paired Student *t* test using Minitab Data Analysis Software (Release 7.1--Standard version, Minitab, Inc., 1989).

Results

Effect of resistance modulators on the sensitivity to doxorubicin in a panel of human cell lines

Table I shows the sensitivities of a number of cell lines to doxorubicin either alone or when exposed to doxorubicin in the presence of one of 3 resistance modulators. The two multidrug resistant cell lines where resistance was derived *in vitro* were highly resistant to doxorubicin with resistance factors (drug sensitivity of the resistant cell line divided by that of the sensitive parent cell line) of 10950 (ovarian line 2780AD) and 580 (breast line MCF7/Adr). The four non-small cell lung cancer cell lines showed similar sensitivities to doxorubicin to the breast parent line MCF7 whereas the ovarian parent line A2780 was about 50 fold more sensitive to doxorubicin. Verapamil (6.6 μM) sensitised 6 of the 8 cell lines to doxorubicin. For the MDR cell lines sensitivities were increased by 13 (2780AD) and 15 (MCF7/Adr) fold whereas verapamil had no effect on the sensitivity of the parental cell lines (Fig. 1A). A much smaller but significant (2-3 fold, $P < 0.05$ & $P < 0.01$) increase in sensitivity was observed for the 4 non-small cell lung cancer cell lines. Both bepridil (6.6 μM) and quinidine (6.6 μM) sensitised the MDR cell lines to doxorubicin to a similar degree as observed with verapamil (6.6 μM). In contrast, bepridil and quinidine had no effect on the sensitivity of the 4 non-small cell lung cancer cell lines to doxorubicin ($P > 0.20$). In no experiment did this concentration of modulators alone significantly alter the

growth or viability of the cell lines (Table II).

Effect of verapamil on the sensitivity of the MDR cell lines to daunorubicin

The sensitivity to daunorubicin of the two multidrug resistant cell lines and the respective parental cell lines is shown in Table III. Also shown is the effect of various concentrations of verapamil on the sensitivity of the cells to daunorubicin. Both multidrug resistant cell lines were cross resistant to daunorubicin with resistance factors of 860 (2780AD) and 370 (MCF7/Adr), comparable to those for doxorubicin (Table I). Verapamil (6.6 μM) sensitised both resistant cell lines to daunorubicin (Fig. 1B) but the degree of sensitisation was greater for 2780AD (35 fold) and smaller for MCF7/Adr (11 fold) than that observed with doxorubicin. For 2780AD sensitisation with verapamil was dose dependent (Table III). Verapamil had no effect on the sensitivity of parent MCF7 or A2780 cells to daunorubicin.

ID_{50} (nM)				
Cell line	DOX	DOX+Vp	DOX+Bp	DOX+Qn
A2780	0.95±0.34 (1.0)	0.71±0.29 (1.3)	1.32±0.38 (0.8)	0.59±0.23 (1.6)
2780AD	10400±1390 (1.0)	800±46 (13.0*)	1320±60 (7.9*)	1500±220 (6.9*)
MCF7	78.0±13.0 (1.0)	72.0±10.0 (1.1)	ND	ND
MCF7/Adr	44870±1070 (1.0)	2900±215 (15.5**)	6700±700 (6.7**)	7000±400 (6.4**)
A549	48.3±2.3 (1.0)	12.6±1.7 (3.8**)	42.6±7.3 (1.1)	35.7±5.2 (1.4)
WIL	88.2±1.7 (1.0)	28.7±2.7 (3.1**)	83.3±6.9 (1.1)	76.5±10.1 (1.2)
SK-MES	53.7±8.0 (1.0)	18.0±2.4 (3.0*)	50.3±5.8 (1.1)	54.7±5.5 (1.0)
L-DAN	55.5±3.1 (1.0)	22.7±1.3 (2.4**)	60.2±7.8 (0.9)	59.8±4.7 (0.9)

Table I Doxorubicin (DOX) sensitivities of a panel of cell lines determined in the presence and absence of resistance modulators by the MTT assay. Cells were exposed to the drug for 24 hours and the modulators, verapamil (Vp), quinidine (Qn) and bepridil (Bp) were all used at 6.6 μM. Values are the mean ± standard error of 3 independent determinations and the ID_{50} is defined as the drug concentration required to reduce the absorbance to 50% of that of the control, untreated wells. The ratio of the ID_{50} in the absence of modulator to that in the presence of modulator is shown in parentheses and the asterisks denote the significance of any difference (* $P < 0.05$, ** $P < 0.01$; ND, not done).

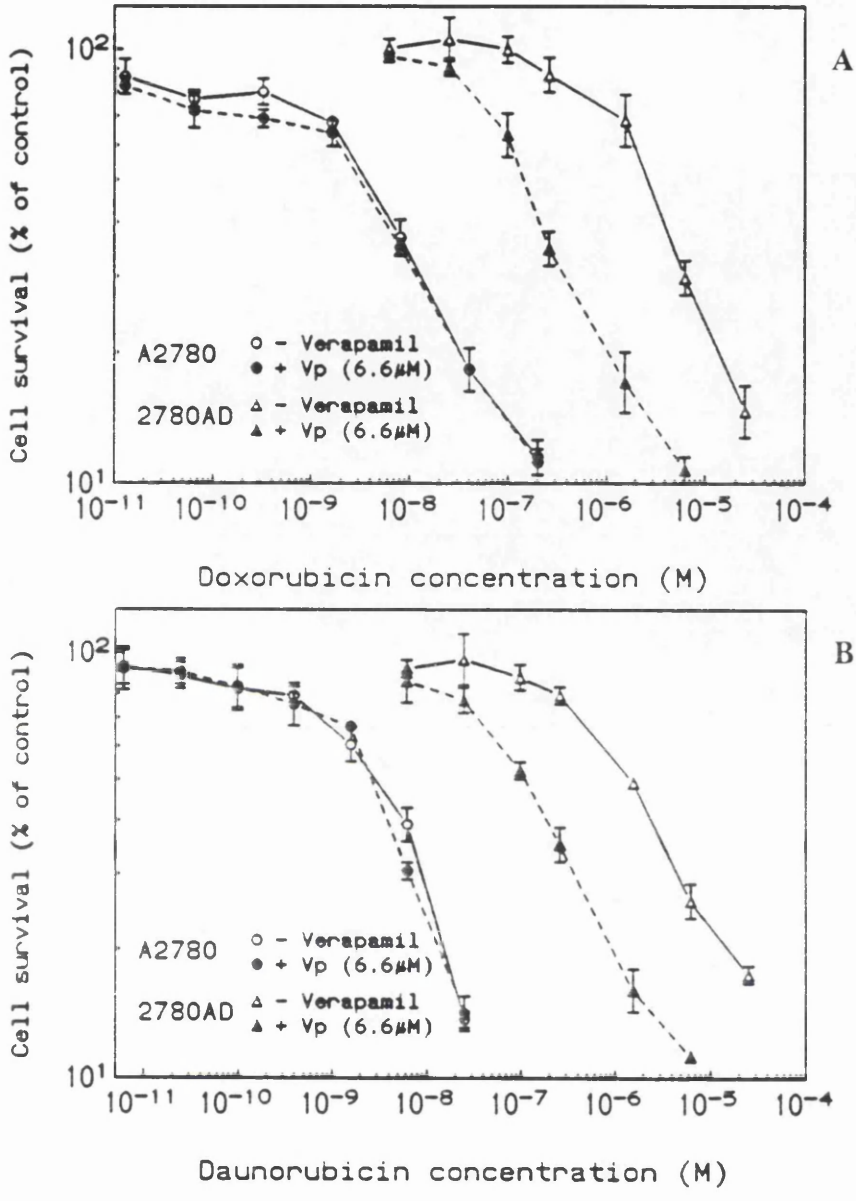


Figure 1 Dose response curves for human ovarian multidrug resistant cell line 2780AD and its drug sensitive counterpart A2780 to doxorubicin (A) and daunorubicin (B). Cells were exposed to drug for 24 hours in the presence and absence of verapamil (6.6 μM) and sensitivity was determined by the MTT assay. For the estimation of cell survival absorbance values are expressed as a percentage of the absorbance of the control, untreated wells and values are the mean ± standard error of triplicates from one representative experiment.

Absorbance (% of control)			
Cell line	Verapamil	Bepridil	Quinidine
A2780	102.0 ± 9.8	98.8 ± 2.5	101.1 ± 1.5
2780AD	105.4 ± 1.2	99.0 ± 2.3	98.6 ± 3.6
MCF7	101.7 ± 2.6	102.3 ± 3.0	99.2 ± 1.7
MCF7/Adr	97.9 ± 2.4	100.2 ± 1.3	98.7 ± 2.3
A549	95.4 ± 4.8	95.9 ± 3.7	100.6 ± 1.9
WIL	99.5 ± 2.1	97.9 ± 3.5	98.4 ± 1.7
SK-MES	101.9 ± 5.1	99.6 ± 2.2	96.2 ± 3.6
L-DAN	99.0 ± 4.9	90.2 ± 5.3	93.9 ± 5.6

Table II Effect of the three resistance modulators on cell survival. Results are the mean absorbance (± standard error) of the wells exposed to modulator alone, expressed as a percent of the absorbance of the control, untreated wells and are from the plates used in the MTT assays from Table I.

Anthracycline accumulation in the presence and absence of verapamil

Figure 2 shows the total amount of anthracycline accumulated with time when cells (A2780 & 2780AD) were incubated with either doxorubicin or daunorubicin in the presence and absence of verapamil (6.6 μM). Drug accumulation increased with time for A2780 and had not reached a plateau after incubation for 5 hours. At all time points the amount of daunorubicin per cell was twice that of doxorubicin. Cell line 2780AD accumulated much less drug than A2780 and for both daunorubicin and doxorubicin accumulation was maximal after incubation for 1 hour. At this time the cells had accumulated about half as much doxorubicin and one sixth as much daunorubicin as the parental cell line. After 5 hours, A2780 had accumulated 4 times more doxorubicin and 16 times more daunorubicin than 2780AD.

Verapamil (6.6 μM) increased the amount of both doxorubicin and daunorubicin accumulation by 2780AD by about 2 fold. Accumulation in A2780 was not affected by verapamil (Fig. 2). For doxorubicin in the presence of verapamil the points are identical to those for doxorubicin alone and these data are not shown for clarity. Thus in the presence of verapamil (6.6 μM) A2780 accumulated 2 times more doxorubicin and 8 times more daunorubicin than 2780AD after 5 hours.

Similar results were obtained with cell lines MCF7 and MCF7/Adr (Fig. 3). After 5 hours MCF7 accumulated 14 times as much doxorubicin as did MCF7/Adr.

Effect of energy deprivation and verapamil on daunorubicin accumulation

Figure 4 shows the total amount of daunorubicin accumulated by A2780 and 2780AD when incubated for 1 hour in daunorubicin (1 μM). Cells were incubated with daunorubicin either in culture medium alone, in medium containing verapamil (6.6 μM) or in glucose-free Hank's balanced salt solution containing sodium azide (10 mM). In regular culture medium, A2780 accumulated about 11 times more daunorubicin than 2780AD. However when incubated in the absence of an energy supply there was no significant difference ($P > 0.05$) between the total amount of drug accumulation in the two cell lines and the levels were similar to the amount accumulated by A2780 in the presence of an energy supply. Addition of verapamil (6.6 μM) to the culture medium had no effect on daunorubicin accumulation in A2780. Verapamil (6.6 μM) doubled accumulation of daunorubicin in 2780AD.

Daunorubicin efflux

Figure 5 shows the amount of daunorubicin retained by cells when loaded with daunorubicin in glucose-free Hank's balanced salt solution in the presence of sodium azide and then transferred to drug free culture medium. Very little of the accumulated drug was lost from the parental A2780 line. After 30 minutes 82% of the drug was retained by the cells. In contrast, 83% of the accumulated

drug was lost from the resistant 2780AD line in the first 5 minutes after transfer into drug free medium. The cells continued to lose drug at a slower rate such that after 30 minutes only 7 % of the initial accumulated drug remained.

ID_{50} (nM)				
Cell line	DNR	+ Vp (1.65 μM)	+ Vp (3.3 μM)	+ Vp (6.6 μM)
A2780	4.6±0.1 (1.0)	4.3±0.3 (1.1)	5.9±1.6 (0.8)	4.8±0.2 (1.0)
2780AD	3970±260 (1.0)	496.0±105.7 (8.0**)	239.0±61.0 (16.6**)	112.0±9.3 (35.4**)
MCF7	29.0±0.8 (1.0)	ND	ND	24.0±1.6 (1.2)
MCF7/Adr	10720±710 (1.0)	ND	ND	940.0±76.0 (11.4**)

Table III Daunorubicin (DNR) sensitivities of P-glycoprotein-expressing multidrug resistant cell lines (2780AD & MCF7/Adr) and drug sensitive counterparts (A2780 & MCF7) in the presence and absence of verapamil determined by the MTT assay (see Table I legend). Values are the mean ± standard error of 3 observations.

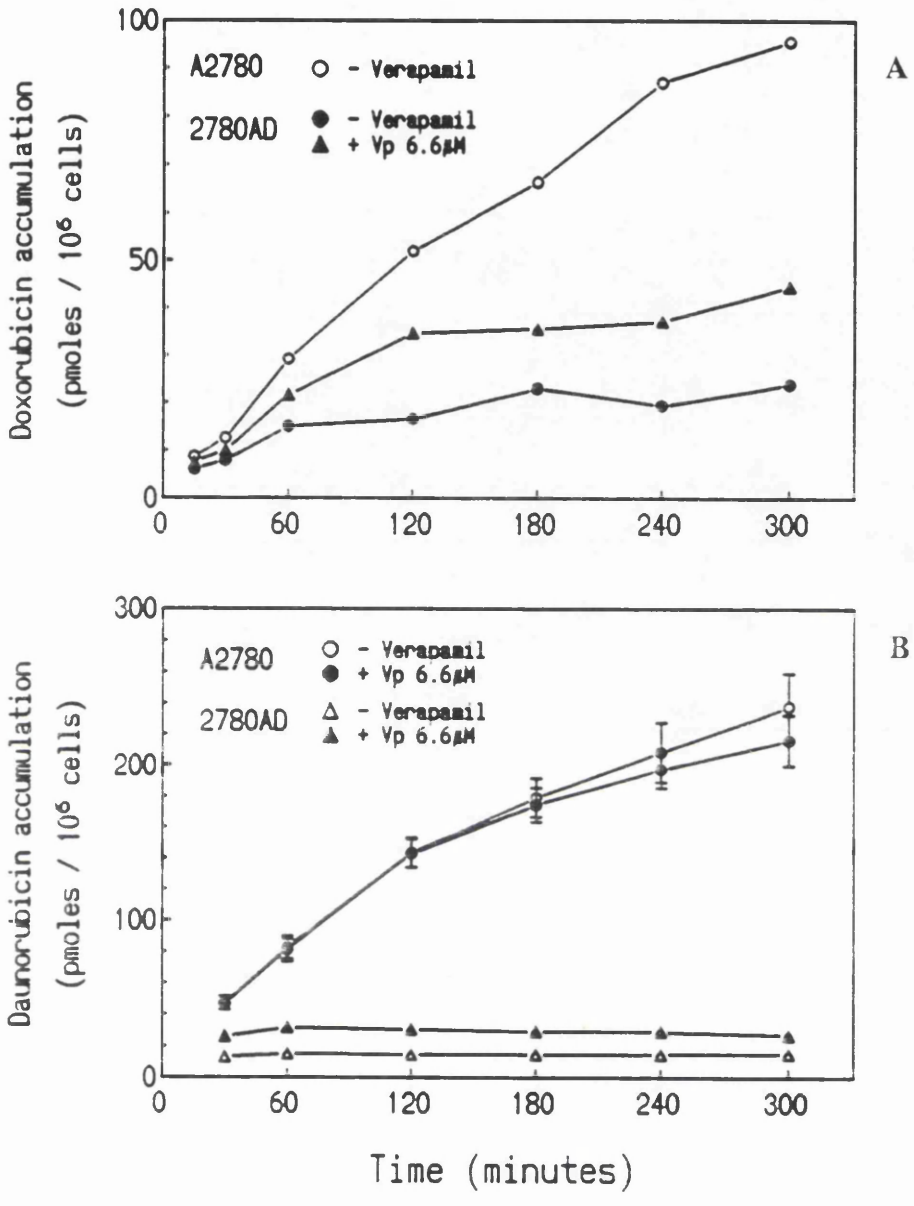


Figure 2 Total cellular doxorubicin (A) and daunorubicin (B) accumulation by A2780 and 2780AD assessed using liquid scintillation counting. Cells were incubated for various times in drug (1 μM, 0.01 μCi/ml) in the presence and absence of verapamil (6.6 μM) at 37°C. Results are the mean of 3 observations and standard error bars are shown when larger than the symbols.

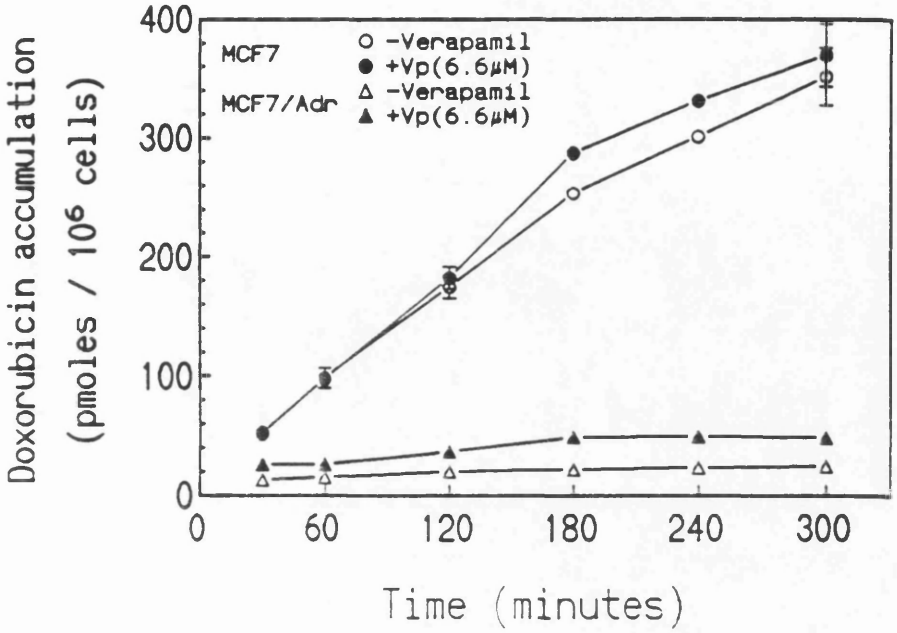


Figure 3 Total cellular doxorubicin accumulation in cell line MCF7/Adr and its drug sensitive parent line MCF7. See Figure 2 legend for details.

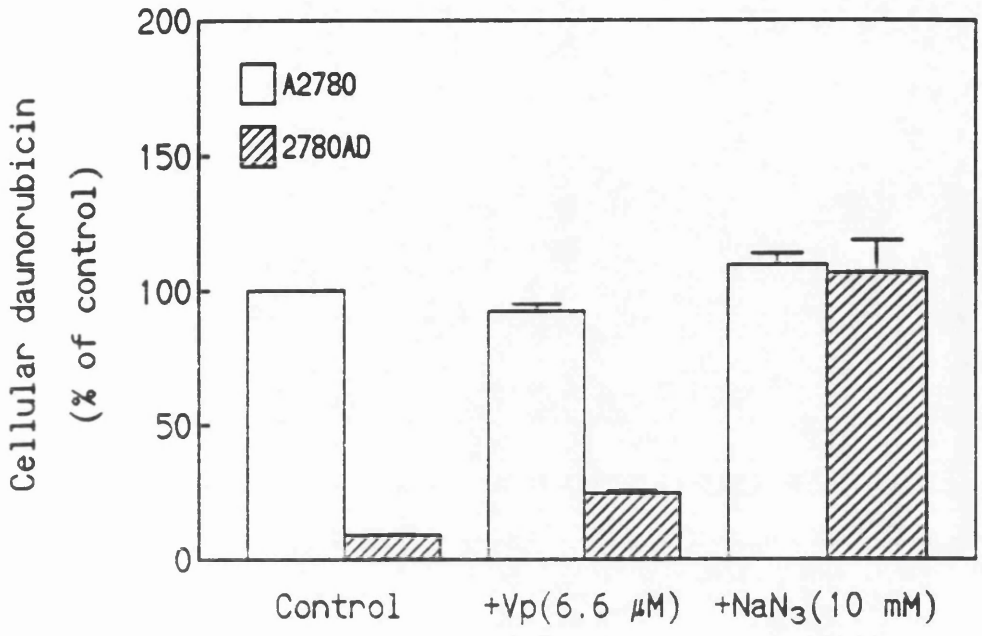


Figure 4 Effect of verapamil and energy deprivation on daunorubicin accumulation by cell lines A2780 and 2780AD following an 1 hour incubation with drug (1 μM, 0.01 μCi/ml) either in culture medium containing verapamil (6.6 μM) or in glucose-free Hank's balanced salt solution containing sodium azide (10 mM). Total cellular drug (pmoles/10^6 cells) was measured using liquid scintillation counting and presented as percentages of control (A2780, incubated with daunorubicin only) for comparisons. Bars are the mean $\pm$ standard error of 3 determinations.

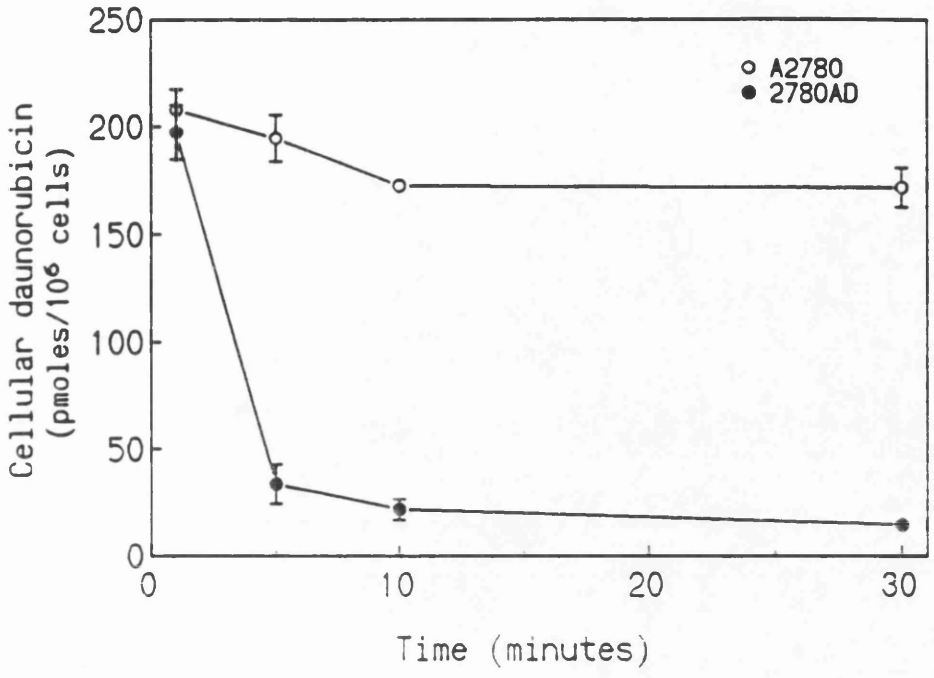


Figure 5 Daunorubicin efflux from cell lines A2780 and 2780AD determined by liquid scintillation counting. Cells were loaded with drug (1 μM, 0.01 μCi/ml) in glucose-free Hank's balanced salt solution containing sodium azide (10 mM) for 1 hr at 37°C, washed and transferred to culture medium. Cells were collected at specified times and radioactivity measured. Results are the mean ± standard error of triplicates from one representative of 2 observations showing similar results.

Discussion

These results show clearly that verapamil can sensitise the two *in vitro* derived human multidrug resistant cell lines to both doxorubicin and daunorubicin. In both cell line sensitisation is associated with decreased drug accumulation and a rapid drug efflux mechanism. Quinidine and bepridil also sensitised both cell lines to doxorubicin but the degree of sensitisation was smaller than that seen with an equimolar concentration of verapamil. In contrast, verapamil but not quinidine or bepridil sensitised the four non-small cell lung cancer cell lines to doxorubicin.

Verapamil (6.6 μM) increased the doxorubicin sensitivity of the human ovarian multidrug resistant line 2780AD by 13 fold and that of the human breast multidrug resistant line MCF7/Adr by 15 fold. This confirms previous observations (Ford *et al*, 1991; Plumb *et al*, 1990; Rogan *et al*, 1984). The degree of sensitisation is small compared with the resistance factors of the cell lines. This discrepancy can be accounted for in part by the presence of other mechanisms of drug resistance within these cell lines such as altered topoisomerase II expression (Sinha *et al*, 1988) and changes in the activities of drug detoxification pathways (Batist *et al*, 1991; Hamilton *et al*, 1985; Ozols *et al*, 1988). Both the 2780AD and MCF7/Adr were cross-resistant to daunorubicin and the resistance factors when compared to the drug sensitive parental cell lines were similar to those obtained for doxorubicin. The effect of verapamil (6.6 μM) on the daunorubicin sensitivity of 2780AD (35 fold) was greater than that

observed with doxorubicin. In contrast, the effect on the daunorubicin sensitivity of MCF7/Adr (11 fold) was slightly smaller than the effect on doxorubicin sensitivity (Tables I & III). Previously, it has been shown that verapamil sensitises both multidrug resistant cell lines to doxorubicin in a dose-dependent manner (Plumb *et al*, 1990). This finding is consistent with the dose-dependent effect of verapamil in the daunorubicin sensitivity of cell line 2780AD (Table III).

In both 2780AD and MCF7/Adr, resistance to doxorubicin is associated with a marked reduction in overall drug accumulation (Figs. 2 & 3; Louie *et al*, 1986; Schuurhuis *et al*, 1987). Sensitisation by verapamil (6.6 μM) is accompanied by a two fold increase in drug accumulation in the two cell lines. Whilst verapamil has no effect on the drug sensitivity of the parental cell lines A2780 and MCF7, it does produce a small though not significant ($P > 0.05$) increase in doxorubicin accumulation in MCF7. This effect might be related to the high concentration of drug used for the accumulation studies. Whilst 1 μM doxorubicin is well below the ID_{50} concentration for cell line MCF7/Adr it is about 40 times the ID_{50} for cell line MCF7. This concentration of drug was however necessary in order to detect drug accumulation in the drug resistant cell lines.

Accumulation of doxorubicin and daunorubicin were compared in the ovarian cell lines. Cell line 2780AD also showed a marked reduction in daunorubicin accumulation (Fig. 2) consistent with the observation that it is cross resistant to this drug. However, cell line A2780 accumulated twice as much daunorubicin as doxorubicin. This may be related to the increased lipopholicity

of daunorubicin when compared with doxorubicin which facilitates entry into the cell (Arcamone, 1985; Gewirtz & Yanovich, 1987). The magnitude of the difference in drug accumulation between A2780 and 2780AD was much greater (16 fold) for daunorubicin than for doxorubicin (4 fold). It is therefore surprising that verapamil (6.6 μM) only doubled daunorubicin accumulation in 2780AD, the same factor as observed for doxorubicin, yet sensitisation was greater with daunorubicin than doxorubicin. This observation does, however, indicate that the potential effects of verapamil on drug sensitivity, distinct from the direct effects on P-glycoprotein, are more apparent with daunorubicin than with doxorubicin. If so then daunorubicin is more suitable drug for studies of the effects of verapamil on intracellular drug distribution.

Decreased drug accumulation in cell line 2780AD appears to be accounted for by an energy dependent drug efflux mechanism. Incubation of cells for 1 hour in drug in the presence of sodium azide and in the absence of an exogenous energy supply results in identical drug accumulation in cell lines A2780 and 2780AD (Fig. 4). If the cells are then transferred to drug-free medium in the presence of an energy supply 93% of the accumulated drug is lost from cell line 2780AD in the first 30 minutes. In contrast very little drug is lost from cell line A2780 in this same period (Fig. 5). Whether the entire efflux mechanism is due to the presence of P-glycoprotein is not clear. Verapamil was only able to inhibit a fraction of the drug efflux from cell line 2780AD at a concentration that has maximal effects on drug sensitivity (Plumb *et al*, 1990). However, it cannot be assumed that verapamil is capable of complete inhibition of the activity of P-

glycoprotein. Perhaps the use of a specific blocking antibody would be able to answer this question (Broxterman *et al*, 1988; Pearson *et al*, 1991; Rittmann-Grauer *et al*, 1990 & 1992).

The two other resistance modulators included in this study, quinidine and bepridil, were also able to sensitise both 2780AD and MCF7/Adr to doxorubicin. However, the modulation factors were smaller than those achieved with verapamil (Table I). Sehested *et al* (1989) have shown that even though quinidine was able to inhibit vincristine binding to plasma membrane vesicles from drug resistant cells more effectively than verapamil the effect of verapamil on drug sensitivity was greater than that of quinidine. Schuurhuis *et al* (1987) reported that bepridil was equally effective when compared with verapamil at 4 μM in sensitising cell line 2780AD to doxorubicin. However, they noted that at lower concentrations verapamil was more effective than bepridil and they did not compare the modulators at concentrations above 4 μM. As with verapamil neither bepridil nor quinidine altered the sensitivity of the drug sensitive parental cell lines A2780 and MCF7.

Of the four non-small cell lung cancer cell lines studied only WIL shows significant expression of P-glycoprotein and this is only detectable at the level of mRNA. Significantly this cell line is the most resistant to doxorubicin of the 4 non-small cell lung cancer cell lines and it is also more resistant than A2780 and MCF7. Surprisingly verapamil was able to sensitise, albeit modestly (2-3 fold), the doxorubicin sensitivity of all four non-small cell lung cancer cell lines (Table I). Sensitisation by verapamil was not greater for WIL than for the other cell

lines. In contrast to the results for verapamil, bepridil and quinidine had no effect on the drug sensitivity of the four non-small cell lung cancer cell lines. Although sensitisation by verapamil in WIL can possibly be explained by effects on P-glycoprotein this is unlikely to explain modulation in the three cell lines with undetectable levels of P-glycoprotein (A549, SK-MES & L-DAN). Furthermore, if the effects on WIL are due to P-glycoprotein it is surprising that neither bepridil or quinidine had any effect on drug sensitivity. These observations are consistent with a previous report that has shown modulation of drug sensitivity by verapamil but not other modulators in P-glycoprotein negative cell lines (Cole *et al*, 1989). Furthermore, this supports suggestions that verapamil may have effects on intracellular drug distribution independent of effects on the drug efflux mechanism associated with P-glycoprotein (Bass *et al*, 1990; Coley *et al*, 1991; Hindenburg *et al*, 1989; Schuurhuis *et al*, 1991; Slapak *et al*, 1990).

In summary, verapamil is a potent modulator of drug sensitivity in cell lines that overexpress P-glycoprotein. The effects on drug sensitivity in both MCF7/Adr and 2780AD are of greater magnitude than the effects on drug accumulation. Furthermore, verapamil but not other resistance modulators can sensitise cell lines with low or negligible expression of P-glycoprotein. These observations clearly indicate the potential of verapamil as a resistance modulator of great mechanistic interest at least *in vitro*.

CHAPTER 3

Effect of verapamil on intracellular drug distribution

Introduction

In the previous chapter two multidrug resistant cell lines, 2780AD and MCF7/Adr, were shown to accumulate less drug than their respective drug sensitive parent cell lines. Under normal growth conditions, the steady-state cellular drug content of the MDR cell lines was about 10% of that of their drug sensitive counterparts. These two drug resistant variants are known to express P-glycoprotein and the activity of this drug efflux pump probably accounts, at least in part, for the drug resistance. Consistent with this is the observation that verapamil (6.6 μM) increases the overall drug accumulation and drug sensitivity of the two cell lines. It was apparent, however, that whilst drug sensitivity was increased by 10-30 fold by verapamil (6.6 μM) drug accumulation was increased only by 2 fold (Chapter 2 Table III & Fig. 2). Furthermore, Gervasoni *et al* (1991) have shown that HL-60/AR cells are 100-200 fold resistant to daunorubicin but drug accumulation was only 50-71% of that in the parental cell line HL-60S from which they were derived. This cell line does not express P-glycoprotein. Although there is not necessarily a one to one relationship between

these two parameters, the size of the discrepancy suggested that verapamil may have effects other than just via inhibition of P-glycoprotein. In addition verapamil was shown to sensitise cell lines that had little or no expression of P-glycoprotein (Chapter 2 and Cole *et al*, 1989; Coley *et al*, 1991a, Nygren *et al*, 1991).

Recently, several drug resistant cell lines that do not express P-glycoprotein have been shown to accumulate less drug than the cell line from which they were derived (Cole *et al*, 1989; Coley *et al*, 1991; Nygren *et al*, 1991). Decreased drug accumulation was found to precede increased expression of P-glycoprotein during induction of resistance in murine erythroleukaemia cells (Slapak *et al*, 1990). Furthermore, Bass *et al* (1990) showed that induction of drug resistance occurred prior to expression of P-glycoprotein in human lung cancer cells. Thus decreased drug accumulation and development of drug resistance is not always related to P-glycoprotein activity.

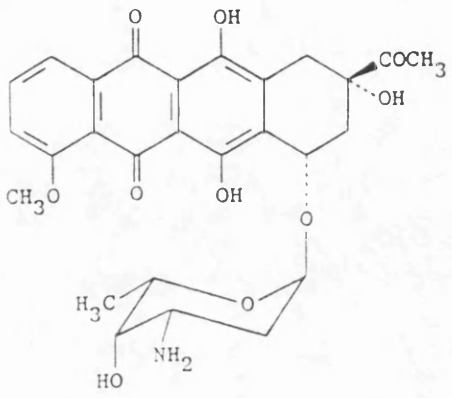
An increase in membrane traffic and enhanced exocytosis was observed in drug resistant P388 leukaemia cells and Ehrlich ascites tumour cell lines and this was associated with enhanced drug extrusion from the cells (Sehested *et al*, 1987a, b). Moreover, vinblastine resistant human lymphoblastic leukaemic CEM cells had lower levels of lysosomal enzymes, such as N-acetylglucosaminidase and β-galactosidase, and a greater rate of secretion of these enzymes than did the drug sensitive CEM cells (Warren *et al*, 1991). Whether the exocytosis is linked to the expression of P-glycoprotein in these cells is not known but any drug present within the lysosomes would be more rapidly removed from the cell. A number of cytotoxic drugs have been shown to distribute into subcellular vesicles and

organelles (Gervasoni *et al*, 1991; Hindenburg *et al*, 1989). This may represent an alternative mechanism for drug resistant cell lines that exhibit a drug accumulation deficiency but not necessarily associated with P-glycoprotein expression (Beck, 1987). It is interesting to note that verapamil can inhibit degradation of low density lipoproteins in HeLa cells. The low density lipoproteins are degraded in the lysosomes and verapamil was shown to decrease the activity of a number of lysosomal enzymes including cathepsin B, β-hexosaminidase and acid phosphatase (Akiyama *et al*, 1985).

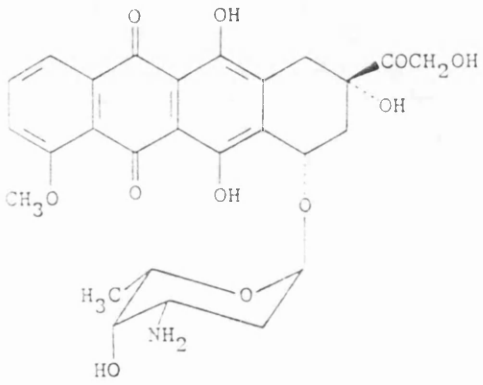
The aim of the experiments described in this chapter was to determine whether multidrug resistant cell lines demonstrate altered intracellular drug distribution and if so whether verapamil alters the distribution. There are several methods available that could be used to study drug distribution. The simplest technique for drugs such as the anthracyclines which are inherently fluorescent is to observe live cells with the fluorescence microscope. This technique has the advantage of speed since no preparation of the cells is required before observation and with the added discriminatory powers of a laser-scanning confocal microscope can give detailed information on the location of fluorescence (Gervasoni *et al*, 1991). A major disadvantage of the method is that of fluorescence quenching, particularly of drug bound to DNA or other cellular components. The use of a radioactively labelled drug in association with autoradiography can overcome the problem of fluorescence quenching but the technique is obviously much more time consuming. Subcellular fractionation has been used to determine the drug content of cellular organelles (Londos-Gagliardi

et al, 1980). However, the processing time involved is of the order of hours and it is quite possible that much of the unbound drug will be lost during preparation of the fractions.

Initial studies were carried out by fluorescence but in the absence of the precision of the confocal microscope only limited information was obtained. The technique of autoradiography was therefore developed for detailed studies of intracellular drug distribution. Although the two pairs of MDR cell lines (A2780 : 2780AD and MCF7 : MCF7/Adr) were both selected with doxorubicin the experiments were performed with the closely related anthracycline daunorubicin (Fig. 1). These studies required the low energy β particles and hence the short path length of tritium in order that the grains appear as close as possible to the site of origin of the radioactivity. Doxorubicin is only available with a carbon 14 label and the energy and hence path length of β particles from this radionuclide is too long for grains to remain associated with the site of origin of the radioactivity (Aherne *et al*, 1977).



Daunorubicin



Doxorubicin

Figure 1 The structures of the anthracyclines, daunorubicin and doxorubicin. There is a difference of only a hydroxyl group between these two drugs at the C-14 site on the C-9 side chain.

Materials and Methods

Cell lines

Two P-glycoprotein-expressing MDR cell lines, 2780AD and MCF7/Adr, and the respective wild type parental cell lines, A2780 and MCF7, were used in these studies. The growth conditions for these cell lines have been presented in Chapter 2.

Cellular fluorescence

Cells (3×10^4) were seeded into 35-mm plastic tissue culture petri dishes (Nunclon, Life Technologies Ltd., Paisley, U.K.) in 3 ml of medium and were grown at 37°C for 3 days. The medium was then replaced with 3 ml of fresh medium and cells were incubated for 1 hr to allow equilibration of pH and temperature. Daunorubicin was then added to give a final concentration of 2 μM or 10 μM. In some dishes verapamil was added at the same time to give a final concentration 6.6 μM.

At various times dishes were placed on ice, the drug and medium removed and the cells washed twice with ice cold PBS. Cells were mounted in PBS with a glass coverslip and viewed immediately under oil immersion with a fluorescence microscope (Polyvar, Leica UK Ltd., Milton Keynes, U.K.) fitted with a blue (475-

495 nm) filter.

Autoradiography

Glass coverslips were cleaned and sterilised before use as summarised below.

95% Ethanol	5 min
1 N Hydrochloride acid	5 min
Deionised distilled water	5 min
Deionised distilled water	4 × rinses
95% Ethanol	2 ×
100% Ethanol	2 ×
Oven 160°C	2 hr

Exponentially growing cells weredisaggregatedwith trypsin and plated onto sterile glass coverslips in 6-well plates at a density of 2.5 $\times 10^4$ cells/well (MCF7 & MCF7/Adr) or 7.5 $\times 10^4$ cells/well (A2780 & 2780AD) in 5 ml of medium. The cells were allowed to settle and grow at 37°C for 3 days.

The cells were fed with 5 ml of fresh medium and allowed to equilibrate for 1 hr. Daunorubicin and ^{3}H-daunorubicin were added to give a final concentration of 1.0 μM and 0.1 μCi/ml. In some wells verapamil (3.3 or 6.6 μM final concentration) was added with the daunorubicin.

At specified times, plates were placed on ice, the medium and drug removed and the cells washed three times with ice-cold PBS. The cells were then fixed for 5 min at 0-4°C in 1% paraformaldehyde (w/v in PBS, pH 7.0). The coverslips rinsed in PBS and left to dry at room temperature. Coverslips were mounted with the cells on the top surface onto clean microscope slides in DPX mountant (BDH, poole, U.K.) and allowed to air dry overnight.

Slides were coated with Kodak K-2 emulsion (Kodak Ltd., T.M. Kodak, Herts, U.K.) in the darkroom with a red safelight when necessary.

An appropriate amount of the emulsion was placed into a clean glass beaker and melted in a waterbath at about 40°C for 30 minutes with occasional gentle stirring with a clean glass rod. The melted emulsion was diluted with an equal volume of prewarmed deionised distilled water and transferred into a dipping vessel (Lab-Tek Cyto-Mailer, Life Technologies Ltd., Paisley, U.K.).

Slides were dipped one by one into the emulsion for about two seconds, removed slowly, excess emulsion allowed to drain off and then placed on a flat surface to dry. The dried slides were arranged into slide holders and kept in light-tight boxes with a pack of desiccant. The boxes were sealed with vinyl tape and stored at 4°C for six days.

Slides were then developed in the dark as follows:

Developer (Kodak D19, Kodak Ltd., Herts, U.K.)	2.5 min
Deionised distilled water	10 sec
Fixative (Ilford fixer, Ilford Ltd., Essex, U.K.)	7 min

Deionised distilled water	10 sec
Hypocleaning agent (Kodak unifix, Kodak Ltd., Herts, U.K.)	1 min
Running tap water	20 min
Deionised distilled water	1 min
Air dry	2 days

Cells were stained with undiluted Giemsa (BDH, Poole, U.K.) for 1 min, and with diluted Giemsa (1 part Giemsa, 2 parts PBS) for 2 min followed by a two minute destain in PBS and a brief rinse with tap water.

Examination was carried out under oil immersion with a 100× objective lens. Grain counts were performed with respect to the whole cell, the cytoplasm only and the nucleus only. In each case 20 cells per slide were assessed. Background counts were obtained by counting the grains in cell free areas similar in size to the cells. In all cases, the background count was less than 10% of the counts in the cells, and this was subtracted from the total grain counts.

Statistical analysis

All the data from autoradiography were analysed statistically using analysis of variance with Minitab Data Analysis Software (Release 7.1--Standard version, Minitab, Inc., 1989).

Results

Intracellular localisation of daunorubicin fluorescence in the ovarian cancer cell lines

Plate 1 shows the distribution of daunorubicin fluorescence in the sensitive parental cell line A2780 (Plate 1A & 1B) and in the drug resistant cell line 2780AD (Plate 2A & 2B).[a] Cells were incubated with daunorubicin (DNR, 10 μM) for 1 hour. For A2780 cells there is an abundance of DNR fluorescence in both the nucleus and cytoplasm and the fluorescence in the cytoplasm is punctate. In contrast, very little fluorescence is apparent in the cytoplasm of cells from 2780AD and almost none is visible in the nucleus (Plate 2A). However, when cells were incubated with DNR in the presence of verapamil (6.6 μM) bright fluorescence was apparent throughout both the nucleus and cytoplasm of cells from the resistant line, 2780AD (Plate 2B). Furthermore, fluorescence was also clearly associated with the chromatin and the nuclear membrane.

Verapamil had no effect on the fluorescence intensity or distribution in A2780. Similar results were obtained when cells were incubated with DNR (2 μM) although the intensity of the fluorescence was much less in both cell lines (results not shown).

[a] For photography cells were selected and focused under phase contrast.

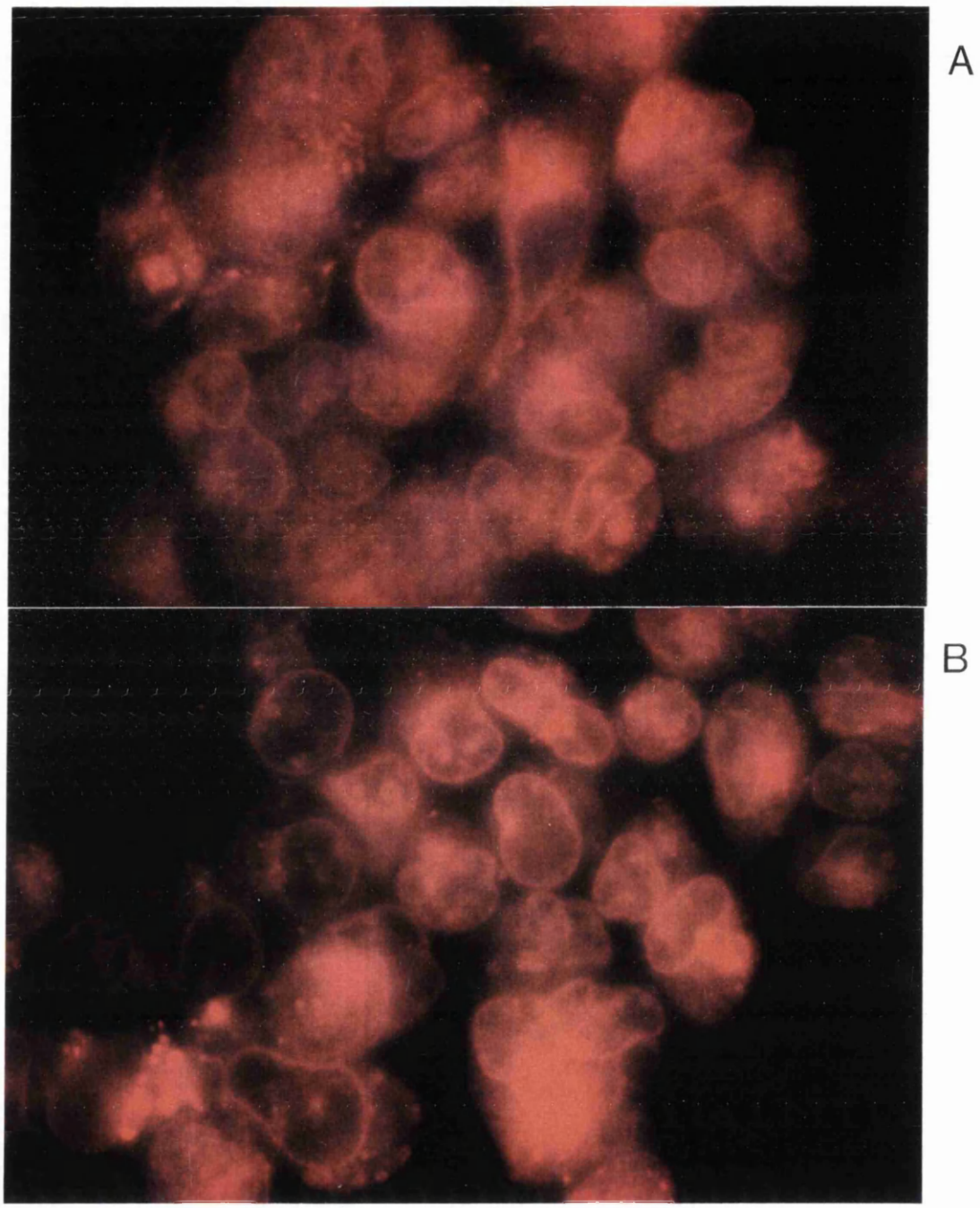


Plate 1 Daunorubicin fluorescence distribution in A2780. Cells grown in 35 mm petri dishes for 3 days were incubated with daunorubicin (10 μM) in the presence (B) and absence (A) of verapamil (6.6 μM) for 30 minutes, washed and viewed under oil immersion with a fluorescence microscope fitted with a blue (475-495 nm) filter. Photomicrographs were taken with Kodak P800/1600 film with an objective magnification of 100×.

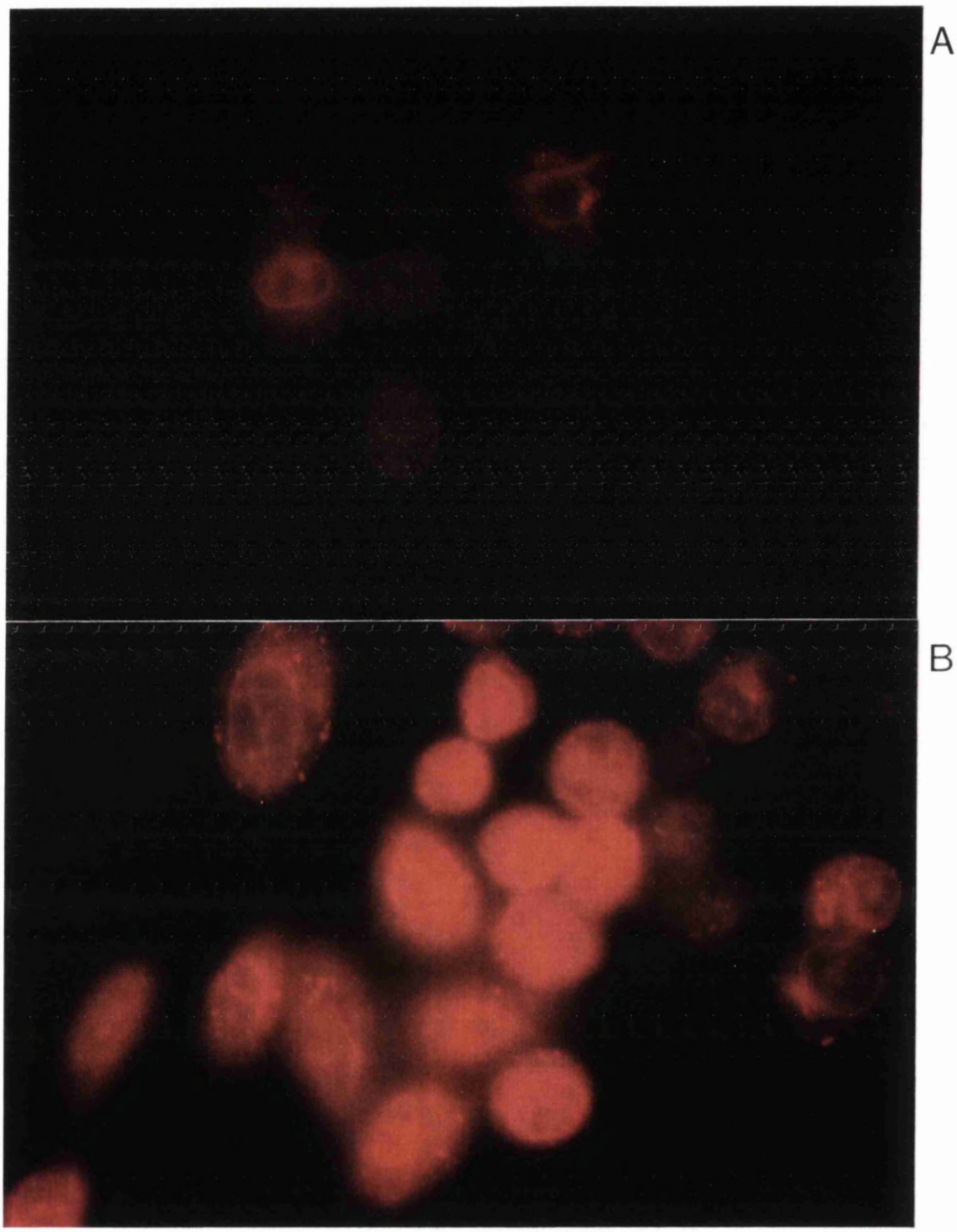


Plate 2 Daunorubicin fluorescence distribution in 2780AD (see Plate 1 legend for details). (A) in the absence of verapamil (B) in the presence of verapamil.

Accumulation of ^{3}H-daunorubicin in the ovarian cancer cell lines

Both A2780 and 2780AD showed a marked morphological heterogeneity. Two major cell types were apparent in these cell lines. The lightly stained large cells have a distinct nucleus and cytoplasm whereas the small heavily stained cells have no distinguishable cytoplasm (Plate 3A, 3B & 3C). ^{3}H-daunorubicin accumulation was determined by autoradiography over an 8 hour time course. For A2780 both cell types showed a similar pattern of daunorubicin accumulation with a rapid increase in grain count up to 60 minutes after which no further increase was observed (Fig. 2). The large cells contained about 3 times as many grains as the small cells at all time points studied.

In contrast, very few grains were apparent in either cell type throughout the 8 hour time course for cell line 2780AD (Fig. 3). The zero time point was obtained by addition of ^{3}H-daunorubicin followed by rapid removal and washing the cells. In these few seconds daunorubicin accumulation appears to have reached a maximum.

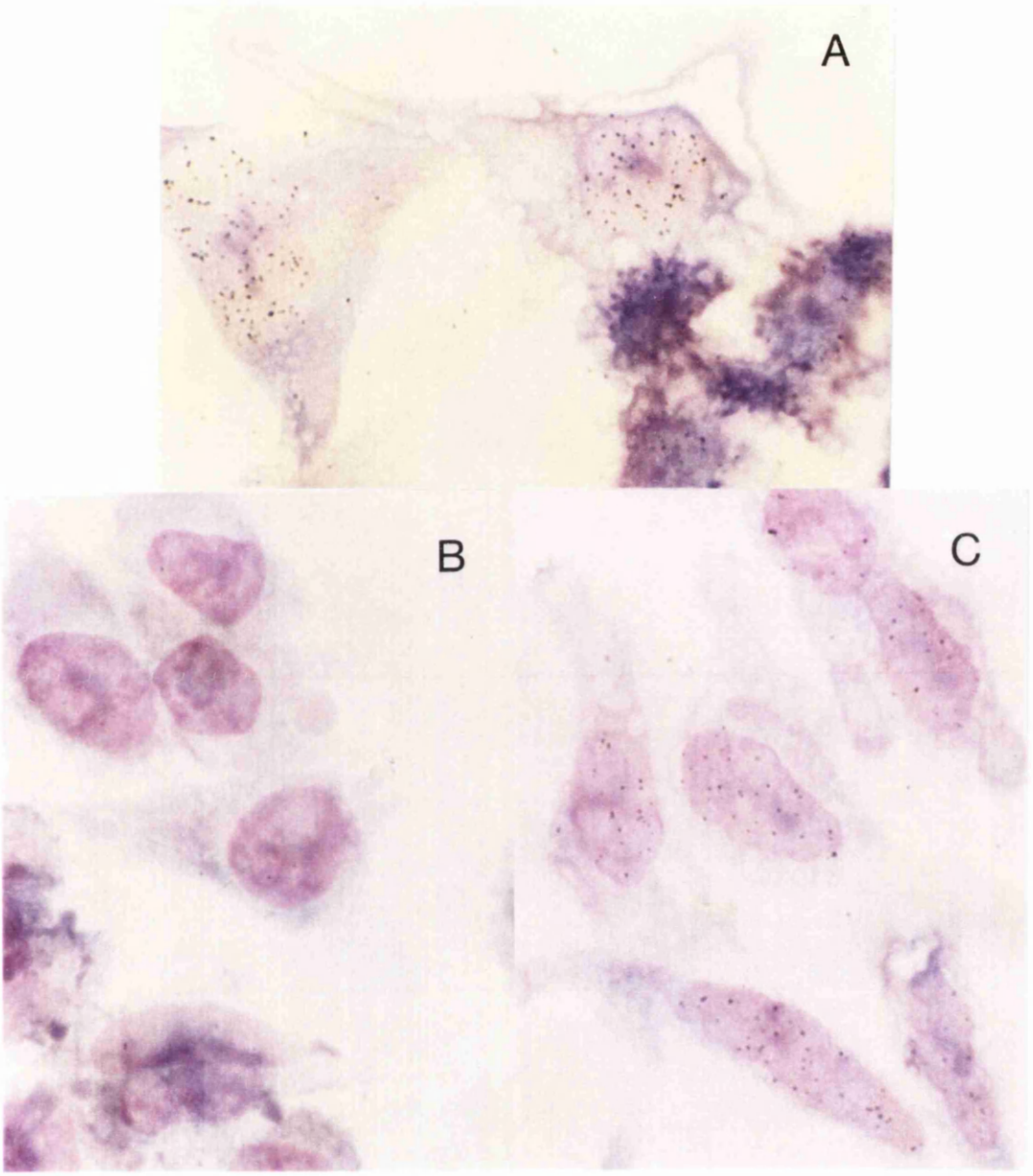


Plate 3 Distribution of grains in cell lines A2780 and 2780AD. Cells grown on glass coverslips for 3 days were incubated with daunorubicin (1 μM, 0.1 μCi/ml) for 30 minutes, washed and fixed in paraformaldehyde (1%). The slides were coated with Kodak K-2 emulsion, exposed, developed and stained with Giemsa. Photomicrographs were taken with an objective magnification of 100×.
(A) A2780 (B) 2780AD (C) 2780AD incubated with daunorubicin in the presence of verapamil (6.6 μM).

For photography cells were selected and focused under phase contrast.

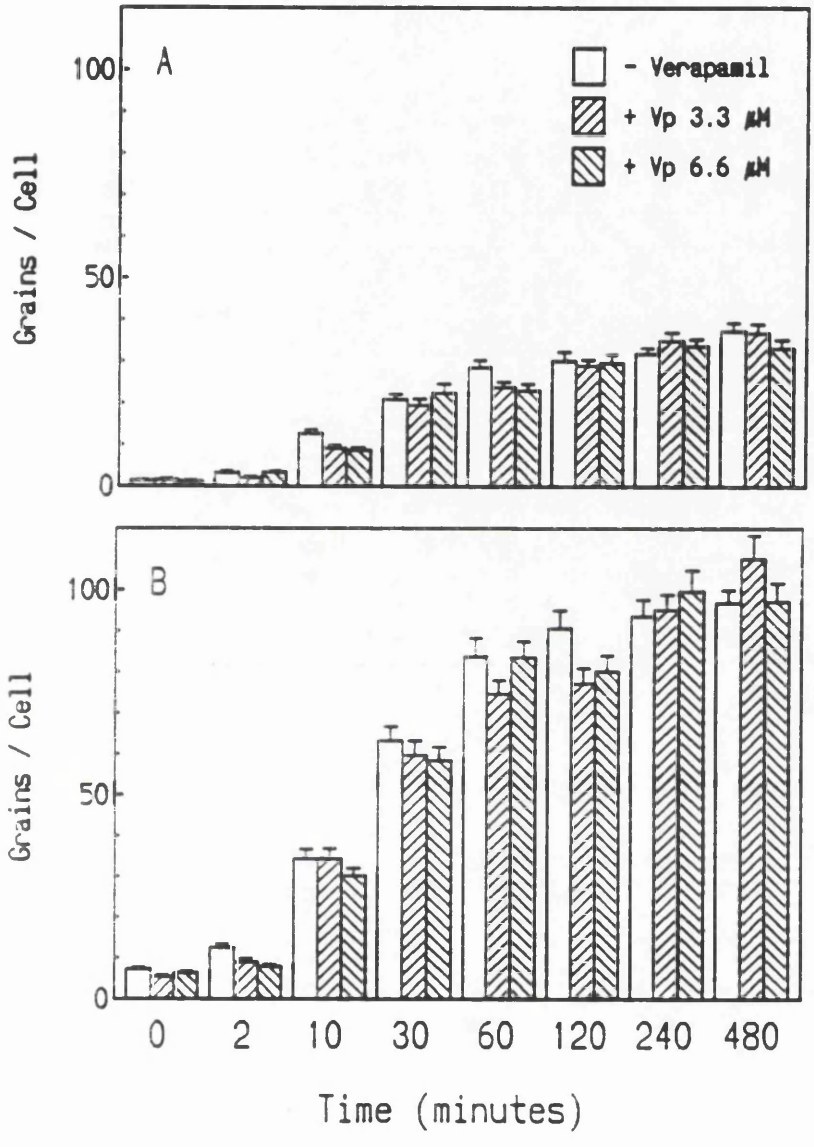


Figure 2 Total grain counts in small (A) and large (B) cells of the parental cell line A2780 following incubation of the cells with drug in the presence or absence of verapamil for specified times. Bars are the mean plus standard error of grain counts of 20 cells and results are from one representative of 4 similar observations. Background was less than 10% of the grain count and this was deducted from the total grain counts.

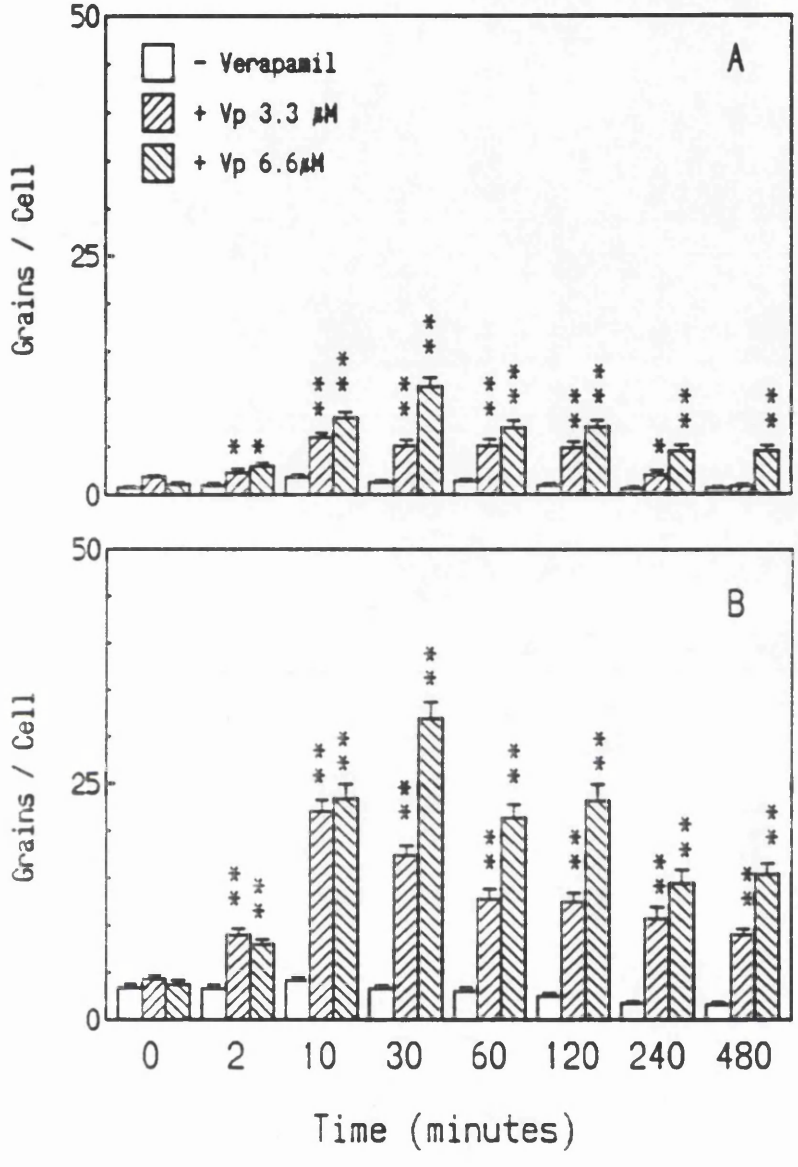


Figure 3 Total grain counts in small (A) and large (B) cells of the resistant cell line 2780AD. See legend for Figure 2 for details. Asterisks represent statistically significant difference (Two-tailed Student's *t* test, * $P < 0.01$; ** $P < 0.001$) in grain count between cells incubated in the presence and absence of verapamil.

Effect of verapamil on ^{3}H-daunorubicin accumulation

Verapamil had no effect on DNR accumulation in cell line A2780 (Fig. 2). In contrast, a marked increase in grain counts was observed at all time points when 2780AD was incubated with DNR in the presence of verapamil (3.3 or 6.6 μM). Up to 10 minutes the increase was independent of the concentration of verapamil. However, by 30 minutes the increase was greater for the higher concentration of verapamil (Fig. 3). In both the small and large cells accumulation was maximal at 30 minutes and decreased with longer exposures.

Distribution of grains between the nucleus and cytoplasm

Differential grain counts in the nucleus and cytoplasm were carried out on the large cells of both A2780 and 2780AD. For both cell lines over 80% of the DNR was present in the nucleus at all time points (Figs. 4 & 5; Table I). Verapamil had no significant effect ($P > 0.05$) on DNR distribution in A2780. For cell line 2780AD verapamil increased the grain count in both compartments but the increase was greater in the nucleus than in the cytoplasm (Fig. 5). Thus, the nucleus to cytoplasm ratio increased significantly ($P < 0.01$) in the presence of verapamil (6.6 μM) (Table II).

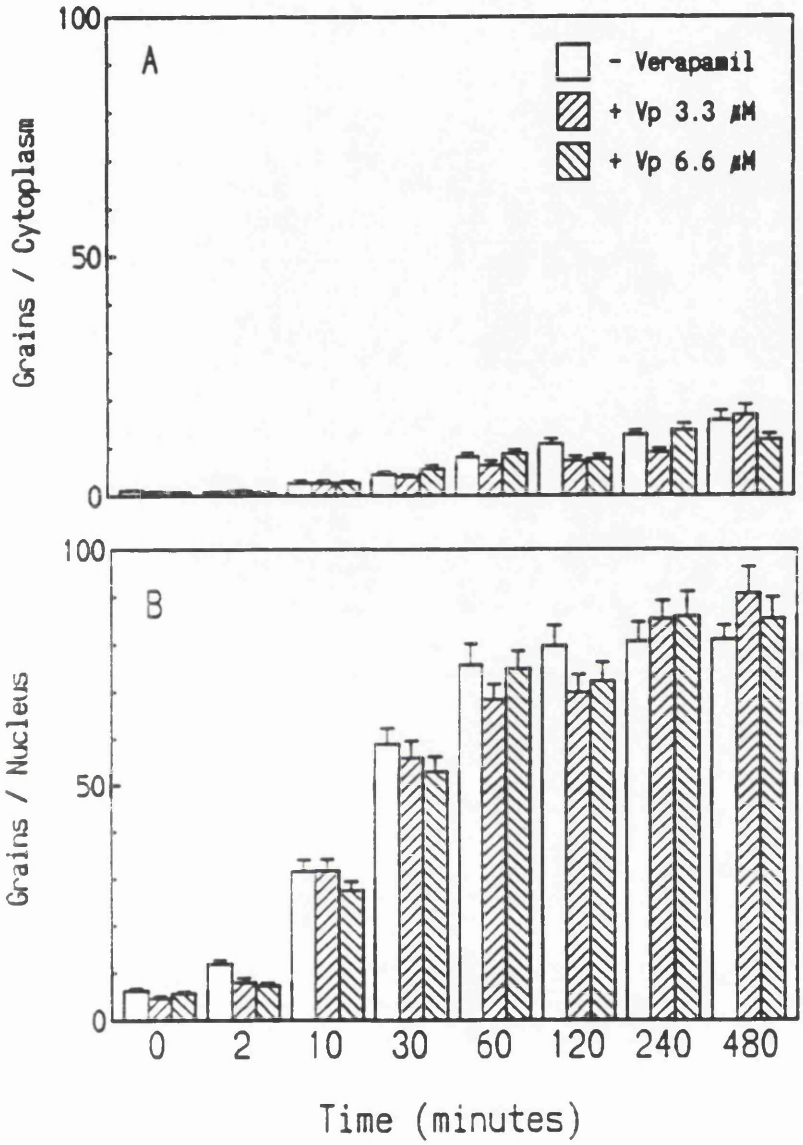


Figure 4 Grain counts in cytoplasm (A) and nucleus (B) of the large cells of A2780. See Figure 2 legend for details.

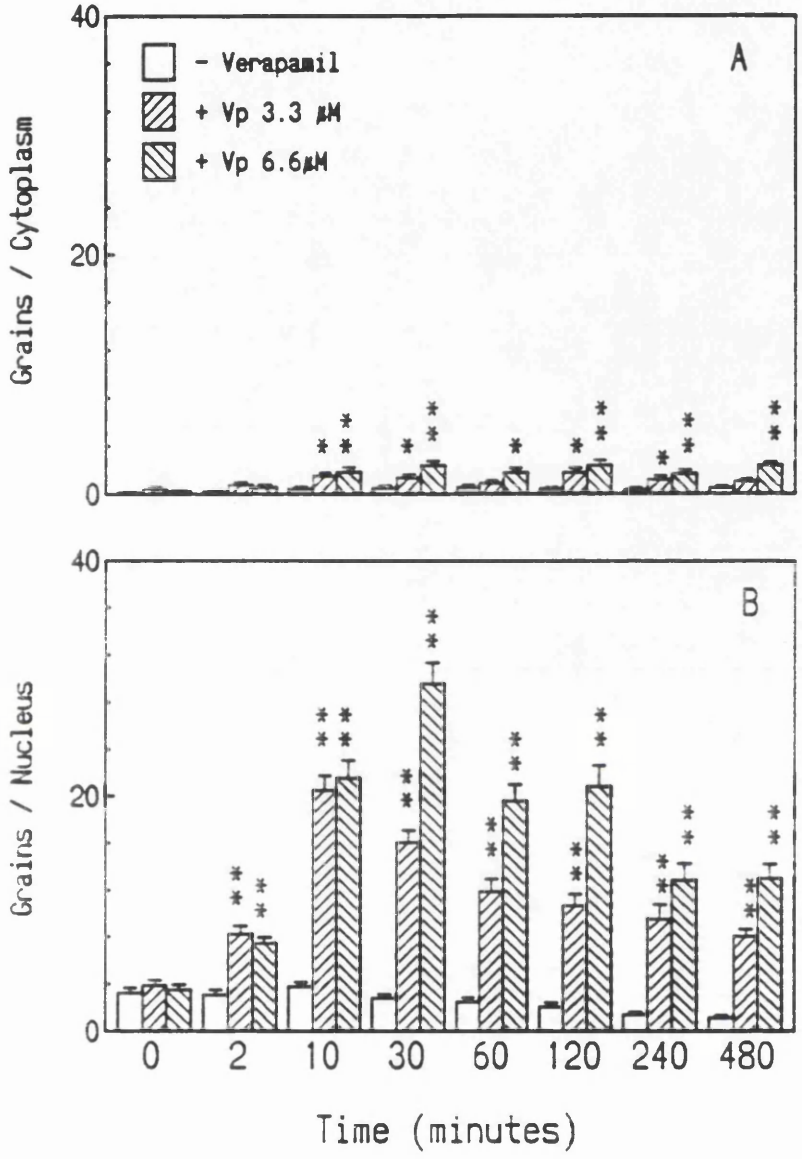


Figure 5 Grain counts in cytoplasm (A) and nucleus (B) of the large cells of 2780AD. See legend for Figure 3 for details.

Grain count (% of total)						
	- Verapamil		+ Vp (3.3 μM)		+ Vp (6.6 μM)	
Time (min)	Nucleus Cytoplasm	N/C	Nucleus Cytoplasm	N/C	Nucleus Cytoplasm	N/C
10	92.1 7.9	11.7	92.2 7.8	11.8	91.2 8.8	10.4
30	92.9 7.1	13.1	93.5 6.5	14.4	90.5 9.5	9.5
60	90.3 9.7	9.3	91.6 8.4	10.9	89.5 10.5	8.5
120	88.0 12.0	7.3	91.0 9.0	10.5	90.3 9.7	9.3
240	86.4 13.6	6.4	89.9 10.1	8.9	86.3 13.7	6.3
480	83.7 16.3	5.1	84.3 15.7	5.4	88.0 12.0	7.3

Table I Percentage of total intracellular grain count in the nucleus and cytoplasm and the ratio of nuclear to cytoplasmic (N/C) grain counts. Results are shown for large cells examined following incubation of the parental line A2780 with daunorubicin (1.0 μM) and ^{3}H-daunorubicin (0.1 μCi/ml) in the presence or absence of verapamil (3.3 or 6.6 μM) for the period of time specified. Values are mean of 20 cells and are representative of 4 independent observations showing similar results.

N/C = Grain count in nucleus / Grain count in cytoplasm

Grain count (% of total)						
	- Verapamil		+ Vp (3.3 μM)		+ Vp (6.6 μM)	
Time (min)	Nucleus Cytoplasm	N/C	Nucleus Cytoplasm	N/C	Nucleus Cytoplasm	N/C
10	90.5 9.5	9.5	93.2 6.8	13.7	92.1 7.9	11.7
30	84.8 15.2	5.6	92.2 7.8	11.8	92.6 7.4	12.5
60	82.0 18.0	4.6	93.0 7.0	13.3	91.6 8.4	10.9
120	84.0 16.0	5.3	85.6 14.4	5.9	89.8 10.2	8.8
240	82.4 17.6	4.7	88.8 11.2	7.9	88.6 11.4	7.8
480	68.8 31.2	2.2	89.0 11.0	8.1	90.3 9.7	9.3

Table II Percentage of total intracellular grain count in the nucleus and cytoplasm and the ratio of nuclear to cytoplasmic (N/C) grain count for the resistant line 2780AD. See Table I legend for details. The ratio of N/C grain count are significantly higher in cells incubated with verapamil (6.6 μM) than those in cells incubated in the absence of verapamil ($P < 0.01$, analysis of variance). Values are mean of 20 large cells for individual groups and are representative of 4 independent observations showing similar results.

Accumulation of ^{3}H-daunorubicin in the breast cancer cell lines

^{3}H-daunorubicin accumulation was studied over a 5 hour period for the parental breast cancer cell line MCF7 and its resistant counterpart MCF7/Adr. For MCF7 grain counts showed a gradual increase throughout the time course (Fig. 6B). In contrast, grain counts in cell line MCF7/Adr reached a maximum after 1 hour and the total counts per cell were about 8% of that for cell line MCF7 (Fig. 6A). As for the ovarian cell lines the majority of the grains were present in the nucleus of the cells (Plate 4A, 4B & 4C; Figs. 7 & 8 and Tables III & IV). However, for MCF7/Adr the nuclear grain count was only about twice that in the cytoplasm such that the nuclear to cytoplasmic ratio was much smaller than that for the parental cell line MCF7 (Tables III & IV).

Effect of verapamil on ^{3}H-daunorubicin accumulation in the breast cancer cell lines

Verapamil had no effect on ^{3}H-daunorubicin accumulation in MCF7 except after incubation for 3 and 5 hours when the grain count was significantly ($P < 0.01$) increased in the cytoplasm in the presence of verapamil (Fig. 7). For cell line MCF7/Adr the grain count in both the cytoplasm and nucleus was increased in the presence of verapamil (6.6 μM) and the increase was greatest in the nucleus (Fig. 8; Plate 4C). Thus in the presence of verapamil the nuclear to

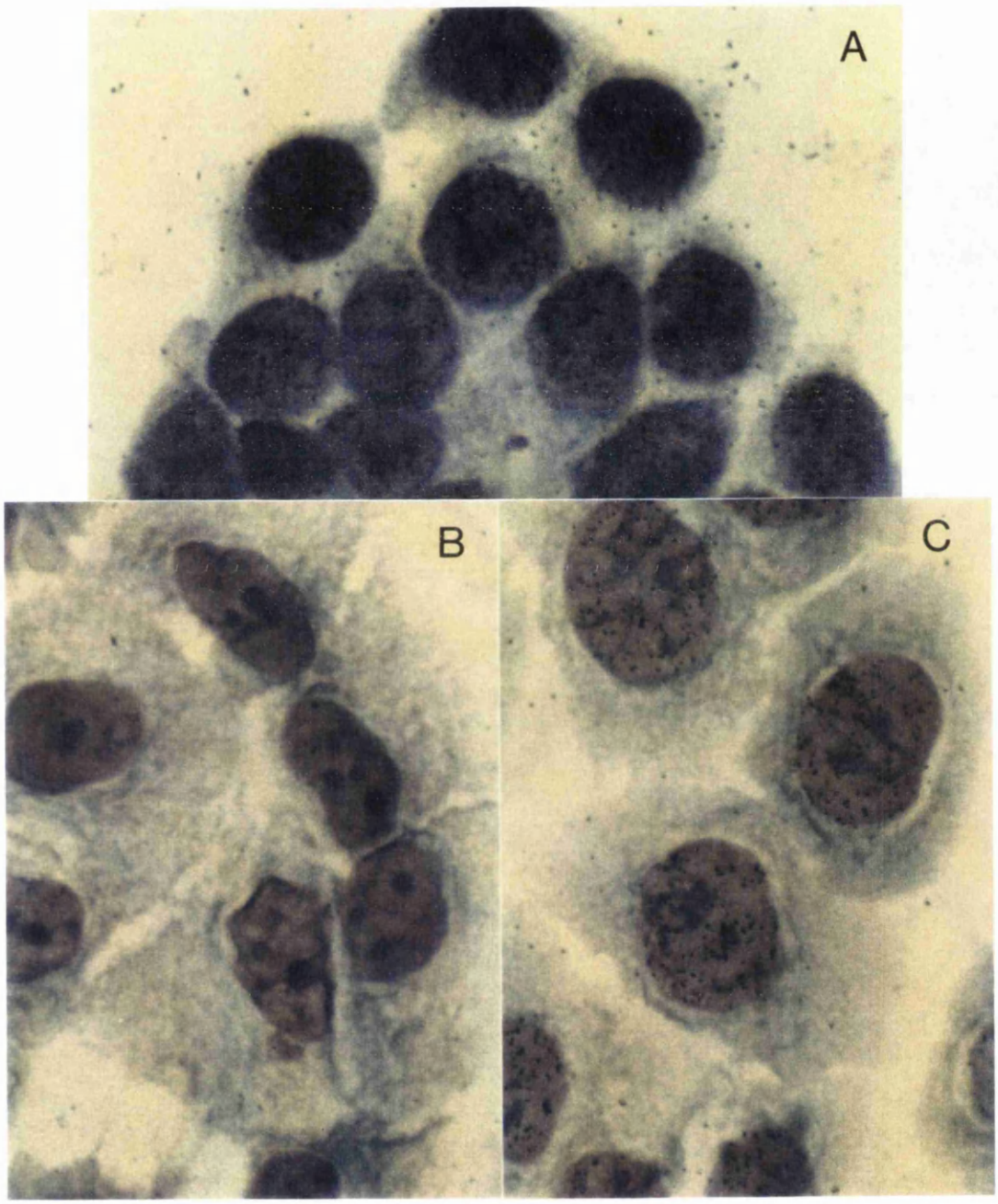


Plate 4 Distribution of grains in cell lines MCF7 and MCF7/Adr. Cells were incubated as described in the Legend for Plate 3. (A) MCF7 (B) MCF7/Adr (C) MCF7/Adr incubated with daunorubicin in the presence of verapamil (6.6 μM). For photography cells were selected and focused under phase contrast.

cytoplasmic ratio of grain counts in MCF/Adr approached that of the parental cell line MCF7 (Tables III & IV).

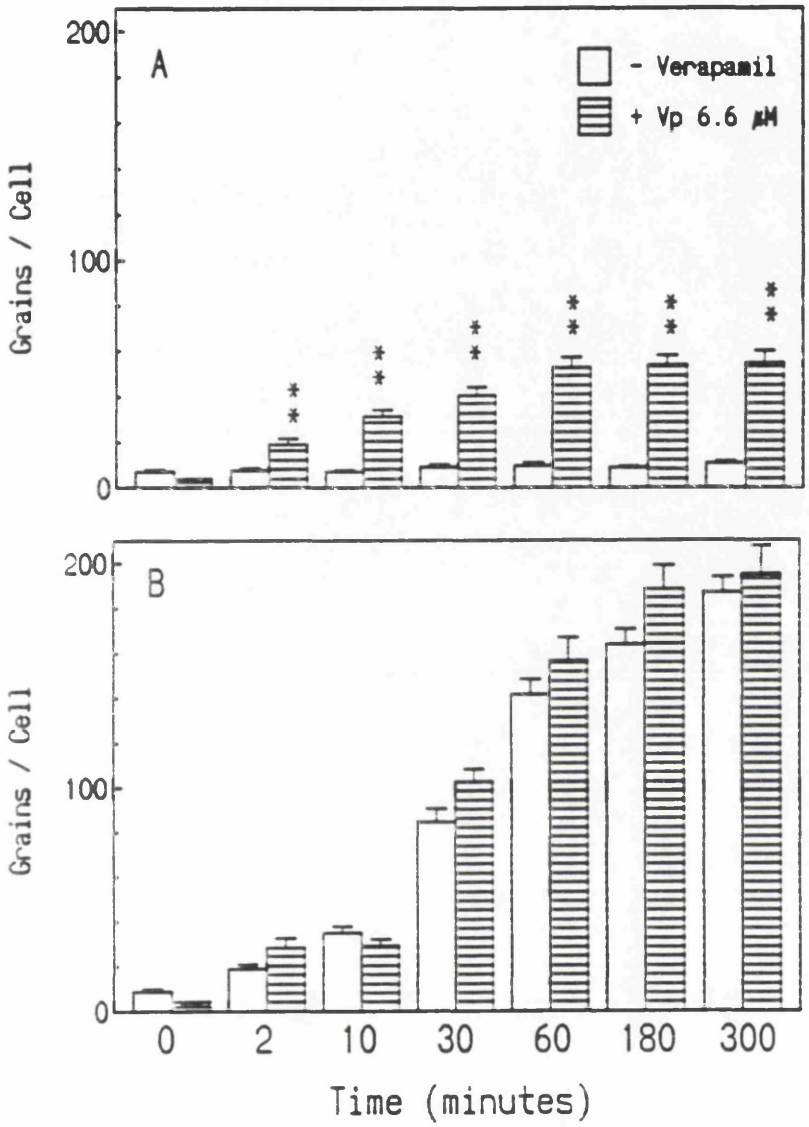


Figure 6 Total grain counts in the resistant line MCF7/Adr (A) and the parental line MCF7 (B) following incubation of the cells with drug in the presence or absence of verapamil for specified times. Bars are mean plus standard error of 20 cells examined and results are from one of 2 similar observations. See Figure 3 legend for statistics.

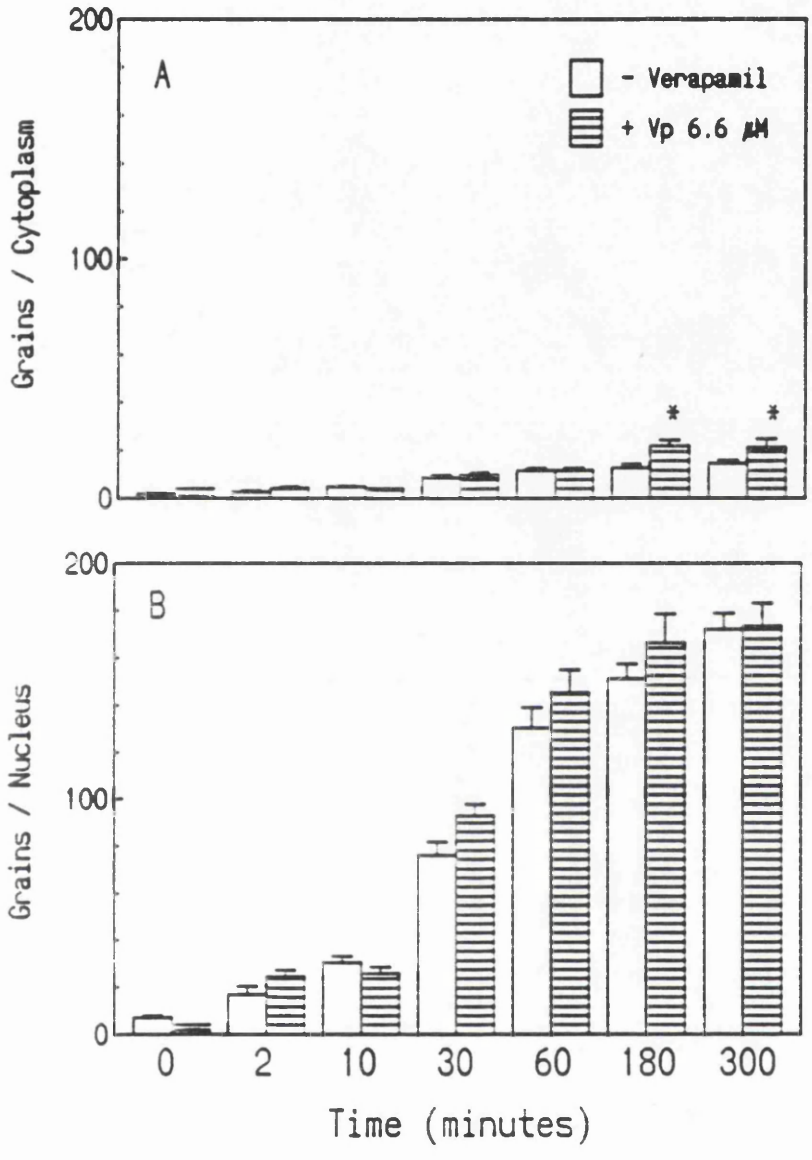


Figure 7 Grain counts in cytoplasm (A) and nucleus (B) of the MCF7 cells. See Figure 6 legend for details.

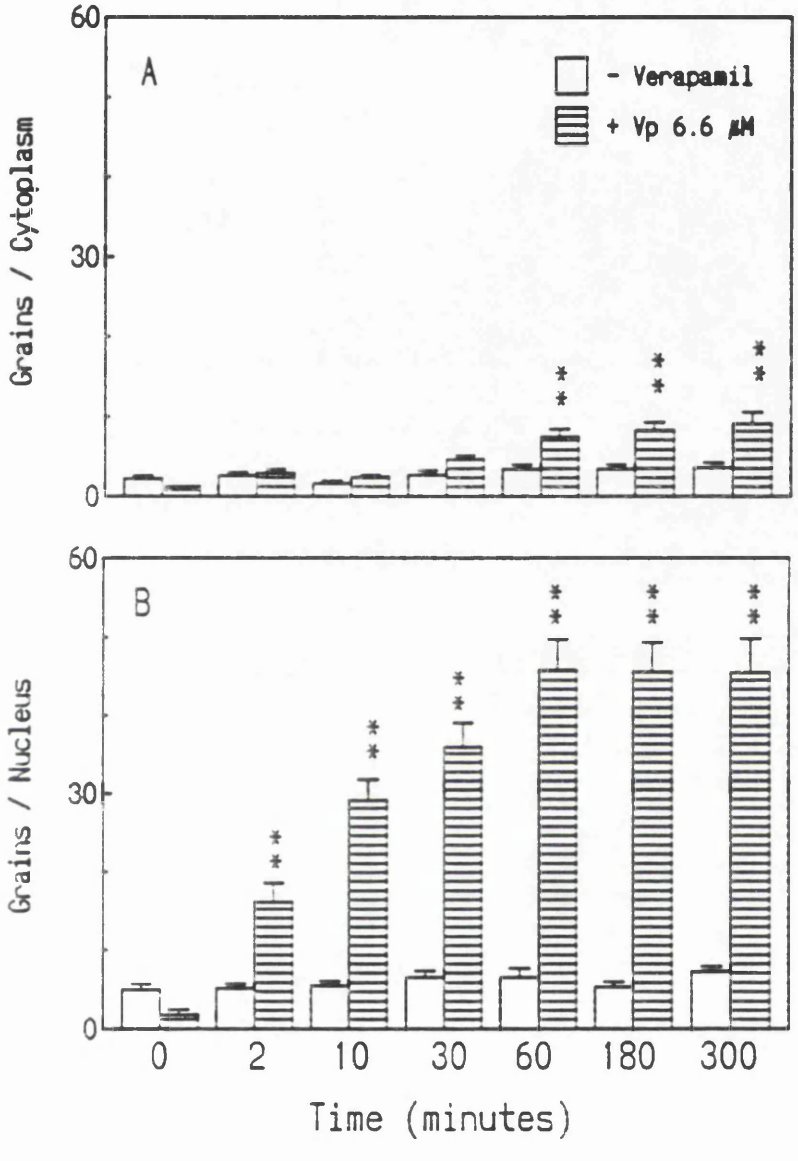


Figure 8 Grain counts in cytoplasm (A) and nucleus (B) of the MCF7/Adr cells. See legend for Figure 6 for details.

Grain count (% of total)				
	- Verapamil		+ Verapamil (6.6 μM)	
Time (min)	Nucleus Cytoplasm	N/C	Nucleus Cytoplasm	N/C
10	86.6 13.4	6.5	88.0 12.0	7.3
30	89.7 10.3	8.7	90.4 9.6	9.4
60	91.6 8.4	10.9	92.7 7.3	12.7
180	92.2 7.8	11.8	88.4 11.6	7.6
300	92.1 7.9	11.7	89.0 11.0	8.1

Table III Percentage of total intracellular grain count in the nucleus and cytoplasm and the ratio of nuclear to cytoplasm (N/C) grain count for the parental line MCF7. See Table I legend for details. Values are mean of 20 cells for individual groups and are representative of 2 independent observations showing similar results.

$$\text{N/C ratio} = \frac{\text{Grain count in nucleus}}{\text{Grain count in cytoplasm}}$$

Grain count (% of total)				
	- Verapamil		+ Verapamil (6.6 μM)	
Time (min)	Nucleus / Cytoplasm	N/C	Nucleus / Cytoplasm	N/C
10	77.1 / 22.9	3.4	92.4 / 7.6	12.2
30	70.7 / 29.3	2.4	88.7 / 11.3	7.8
60	65.7 / 34.3	1.9	85.9 / 14.1	6.1
180	60.9 / 39.1	1.6	84.6 / 15.4	5.5
300	66.7 / 33.3	2.0	83.3 / 16.7	5.0

Table IV Percentage of total intracellular grain count in the nucleus and cytoplasm and the ratio of nuclear to cytoplasmic (N/C) grain count for the resistant line MCF7/Adr. See Table I legend for details. Values are mean of 20 cells and are representative of 2 independent observations showing similar results. The ratio of N/C grain count in the presence of verapamil was significantly larger than in the absence of verapamil ($P < 0.01$, analysis of variance).

Discussion

Decreased drug accumulation in the ovarian cancer cell line 2780AD was clearly demonstrated both by daunorubicin autofluorescence and by autoradiography. By autoradiography it was possible to quantify the difference. Most of the drug was present in the nucleus of both sensitive and resistant cell lines but the ratio of the grain counts in the nucleus and cytoplasm was smaller in both resistant cell lines (2780AD/MCF7/Adr). Verapamil increased the grain count in both nucleus and cytoplasm of the resistant cells but the increase was greatest in the nucleus such that the nucleus to cytoplasm grain count ratio approached that of the drug sensitive parental cell lines. Furthermore, the magnitude of the effects of verapamil on the grain count in the nucleus closely resembled that of the effects of verapamil on drug sensitivity.

Daunorubicin autofluorescence was used successfully to demonstrate decreased drug accumulation in cell line 2780AD. Whilst bright fluorescence was apparent throughout both the nucleus and cytoplasm of A2780 the fluorescence in 2780AD was close to the limit of detection by this technique (Plates 1 & 2). In the sensitive cell line, A2780, daunorubicin is associated with the chromatin and nuclear envelope. There are also defined areas of fluorescence in the cytoplasm suggesting that the drug may be associated with a subcellular compartment. This pattern of distribution is also seen in the drug resistant cell line 2780AD when the cells are incubated with daunorubicin in the presence of verapamil (6.6 μM, Plate 2B). However, based on cellular fluorescence verapamil

appears to restore drug accumulation in 2780AD to the same level as observed in A2780. It has already been demonstrated by the use of scintillation counting that even in the presence of verapamil accumulation in 2780AD is only about 20% of that observed in A2780 (Chapter 2 Fig. 2). These results differ from the fluorescence distribution of daunorubicin in A2780 reported by Broxterman *et al* (1987). They observed that all fluorescence was nuclear when cells were incubated with daunorubicin (2 μM) but do not state for how long. Although the fluorescence distribution shown in Plate 1 was obtained with a daunorubicin concentration of 10 μM a similar distribution was seen at a concentration of 2 μM (results not shown). Since they do not illustrate their fluorescence study it is difficult to compare results and the difference is possibly indicative of the subjective nature of this technique in the absence of a method to quantify fluorescence. A further problem encountered with this technique is that of fluorescence quenching that arises when the drug binds to DNA or protein (Chaires *et al*, 1982; Rhodes *et al*, 1992; Schuurhuis *et al*, 1991). Thus the amount of drug in the nucleus, the proposed site of action of daunorubicin, is underestimated.

A punctate pattern of daunorubicin fluorescence in the cytoplasm of both sensitive and resistant cell lines has been reported previously (Gervasoni *et al*, 1991). They hypothesised that daunorubicin distributes to the prelysosomal sorting compartment which is localised in the perinuclear region. This compartment is the site for plasma membrane and receptor recycling and sorting of vesicles to lysosomes or plasma membrane (Kornfeld, 1987). Localisation to

such a compartment is consistent with a mechanism of drug removal via lysosomal trafficking.

The distribution of ^{3}H-daunorubicin observed by autoradiography differed from that seen by autofluorescence of the drug. For both A2780 and 2780AD the majority of grains were located in the nucleus (Plate 2). Analysis of the grain counts was complicated by the presence of two cell types in roughly equal proportions in the two cell lines. The small cells with indistinguishable cytoplasm contained about a third as many grains as the large cells with clearly defined nucleus and cytoplasm (Figs. 2 & 3; Plate 3). For the small cells it was only possible to count total grains per cell whilst for the large cells differential grain counts between nucleus and cytoplasm were performed. After accumulation for 1 hour total grain counts were about 30 fold lower in 2780AD than in A2780 (Fig. 2) and about 90% of the grains were present in the nucleus of A2780 compared with 82% in 2780AD (Tables I & II). When cells were incubated with daunorubicin in the presence of verapamil the total grain count per cell was unchanged in A2780 but was increased by about 8 fold in 2780AD (Fig. 3). Furthermore, the proportion of the drug present in the nucleus in 2780AD increased to 92% in the presence of verapamil, comparable to that seen in A2780 (Table II). Thus autoradiography has a major advantage over fluorescence drug distribution in that it allows quantification of the drug distribution. However, Willingham *et al* (1986) were able to quantify their fluorescence distribution and also reported that about 90% of the drug was present in the nucleus of drug sensitive KB3-1 cells. A similar proportion was found in the nucleus by cell

fractionation (Londos-Gagliardi *et al*, 1980).

Clearly, verapamil not only increased the total amount of drug taken up by the resistant line but also caused a redistribution of drug between nucleus and cytoplasm such that the proportion present in nucleus increased whilst that in the cytoplasm decreased. This effect was more marked in the multidrug resistant breast cell line MCF7/Adr. Total grain counts in MCF7/Adr were about 18 fold lower than those in MCF7 even after incubation with daunorubicin for 5 hours. Again about 90% of the grains were present in the nucleus of MCF7 cells but only about 70% of the grains were present in the nucleus of MCF7/Adr cells. Addition of verapamil (6.6 μM) during incubation of the cells with daunorubicin increased the total grain count in MCF7/Adr by about 5 fold and increased the proportion of drug present in the nucleus to about 85-90%. Verapamil caused a significant increase in daunorubicin accumulation in MCF7 after incubation for 3 hours. Since verapamil had no effect on the drug sensitivity of this cell line this increase may be an artifact caused by the high concentration of daunorubicin used (1 μM) relative to the ID_{50} of this cell line (0.029 μM, Chapter 2 Table III). For both drug resistant cell lines the increase in the grain count in the nucleus caused by verapamil is closer to the degree of sensitisation observed. Verapamil (6.6 μM) increased the sensitivity of 2780AD by 35 fold (Chapter 2 Table III) and MCF7/Adr by 11 fold (Chapter 2 Table III). By autoradiography verapamil increased the total grain count per cell by 8 fold and 5 fold and the nuclear grain count by 11 fold and 7 fold for 2780AD and MCF7/Adr, respectively. In contrast total drug accumulation determined by scintillation counting was increased by only

2 fold in both cell lines.

The discrepancy between these two techniques is probably due to loss of unbound drug during fixation and processing for autoradiography. Although the fixative used, paraformaldehyde, was chosen since it caused least loss of drug from the cells it has been reported that the majority of the cytoplasmic fluorescence is lost when cells loaded with daunorubicin are fixed in formaldehyde (Willingham *et al*, 1986). Thus the results obtained by autoradiography, particularly in terms of the ratio of grain counts between the nucleus and cytoplasm should be interpreted with caution. However, since the magnitude of the changes induced by verapamil agreeswith the magnitude of the effects on drug sensitivity and, since the nucleus is a prime target for the cytotoxic activity of daunorubicin it could be argued that bound drug represents active drug in terms of cytotoxicity (Ganapathi *et al*, 1984; Gigli *et al*, 1989; Noel *et al*, 1978; Terasaki *et al*, 1984).

The ability of verapamil to cause a redistribution of drug on multidrug resistant cells has been observed previously (Keizer *et al*, 1989; Schuurhuis *et al*, 1989 & 1991). However, Schuurhuis *et al* (1991) reported that the nuclear to cytoplasm drug ratio, as measured by quantitative fluorescence, was reduced in a number of multidrug resistant cell lines but only in those that express P-glycoprotein could verapamil partially restore the ratio. These observations suggest that intracellular redistribution is a mechanism of drug resistance. It is not clear how this redistribution occurs although it has been hypothesised that a pump other than P-glycoprotein exists in intracellular vesicles (Schuurhuis *et al*, 1991). Even in P-glycoprotein positive cell lines the protein has been widely

reported to be present in the cell membrane but only one report suggests it is present in an intracellular membrane, the Golgi stack (Willingham *et al*, 1987). The multidrug resistant cell line HL-60/AR does not express P-glycoprotein but has been shown to accumulate less drug than the parent HL-60S cells when incubated in the same concentration of daunorubicin (Hindenburg *et al*, 1989). Fluorescence was shown to localise in the Golgi apparatus initially but then to relocate to the lysosomes and the mitochondria. When cells were incubated with drug in the presence of sodium azide and deoxyglucose the drug remained in the Golgi apparatus, suggesting that relocation is an energy dependent process. A number of reports have suggested that the membrane composition of drug resistant cells differs from that of the parental cell line from which they were derived (Cornwell *et al*, 1986a; Gervasoni *et al*, 1991; Mann *et al*, 1988; Perterson *et al*, 1983; Ramu *et al*, 1983a). Since anthracyclines such as daunorubicin have a high affinity for the membranes (Gottesman & Pastan, 1990; Raviv *et al*, 1990), these differences may account for changes in the rate of clearance of drug via pathways responsible for membrane recycling (Pagano & Sleight, 1985).

Thus there is evidence both from the present study and from the literature, that drug resistance can result not only from reduced drug accumulation but also from redistribution of drug within the cell. The results of this study indicate that verapamil not only increases the overall amount of drug accumulated but also enhances the proportion of the drug associated with the nucleus. There have been a number of attempts to synthesise novel anthracyclines that are not substrates for P-glycoprotein (Mross, 1991). Clearly, any change in the structure

of the anthracycline will have the potential to alter the lipophilicity of the drug and hence influence the distribution pattern of the drug within the cell. Thus a compound which is a poor substrate for the efflux pump may not necessarily locate preferentially to the nucleus once in the cell (Rhodes *et al*, 1992). In this situation both total drug accumulation measurements and fluorescent drug distribution studies are of value. Whilst autoradiography can give valuable information with regard to the nuclear accumulation of the drug it is not suited to studies of cytoplasmic drug distribution due to loss of drug during fixation.

Finally, an interesting observation that arose from the autoradiography study was the morphological heterogeneity present in the ovarian cell lines. Two distinct populations that accumulated different amounts of drug were clearly apparent. Whilst some of the difference could be accounted for by differences in cell size the difference was largely associated with the nucleus. This observation suggested that populations with differing phenotypes in terms of drug sensitivity were present and this possibility is addressed in the next chapter.

Part of this work was presented at the British Association for Cancer Research meeting in Brighton in March 1990 (Luo *et al*, 1990).

CHAPTER 4

Heterogeneity in the stability of the MDR phenotype between subpopulations of the ovarian cell line 2780AD

Introduction

During the analysis of drug accumulation by autoradiography described in the previous chapter (Chapter 3) a marked morphological heterogeneity was observed in the ovarian cell lines A2780 and 2780AD. A clear distinction could be made between densely stained cells with little cytoplasm and lighter staining cells with substantial cytoplasm. However, marked heterogeneity was apparent even within these two subgroups. Interestingly the heterogeneity was also apparent in cellular drug accumulation. Since most of the drug detected by autoradiography (about 90%) was associated with the nucleus the differences in drug accumulation between cells of different morphologies did not appear to be due simply to differences in cell size. These observations suggested that there are subpopulations within the two cell lines that differ in their handling of cytotoxic drugs.

Cell lines with the multidrug resistance phenotype are usually obtained by chronic exposure of cells to agents, such as doxorubicin, vincristine and colchicine,

over a prolonged period of time (Beck, 1983; Ling & Thompson, 1974; Twentyman *et al*, 1986b). It is generally accepted that a single step selection does not give rise to P-glycoprotein positive variants. The time required indicates that some cells can adapt to the presence of the drug rather than resistance arising from selection of a preexisting stable population within the parental cell line. Adaptation probably involves genetic changes that are unstable (Courtenay & Robins, 1972). This is supported by the fact that most P-glycoprotein positive multidrug resistant cell lines need to be maintained in the presence of the selecting agent in order to retain their resistance phenotype (Biedler *et al*, 1983; Yang & Trujillo, 1990; Yusa & Tsuruo, 1989). Selection of a stable preexisting population should result in a stable resistant population even in the absence of the selecting agent. It is clear that a given cell line can give rise to a number of resistant variants. In some cases this can be explained by different selection protocols (Yang & Trujillo, 1990). However, in some cases similar protocols can give rise to very different drug resistant sublines. For example, Twentyman *et al* (1986b) developed a number of P-glycoprotein positive MDR cell lines from the small cell lung cancer cell line NCI-H69 by chronic exposure to doxorubicin. In contrast, Mirski *et al* (1987) used a similar selection procedure to derive a P-glycoprotein negative MDR cell line from NCI-H69. Observations such as these suggest that there are a number of mechanisms by which drug resistance can develop. Some multidrug resistant cells that do not express P-glycoprotein have been shown to have decreased levels of topoisomerase II, an enzyme involved in DNA unwinding and a target for a number of anticancer agents including etoposide and

doxorubicin (Beck, 1987; Gewirtz, 1991; Morrow & Cowan, 1990; Sinha *et al*, 1988). Whether these mechanisms represent different stages along a common pathway or distinct pathways is not clear. However, a number of P-glycoprotein positive multidrug resistant cell lines also express decreased levels of topoisomerase II (Deffie *et al*, 1989; Liu, 1989; Moscow & Cowan, 1988).

A number of tumour cell lines have been cloned and this has given rise to subpopulations of cells with differing biological properties. Clones derived from a malignant fibrous histiocytoma cell lines showed markedly different morphologies and biological properties *in vitro* (Kanzaki *et al*, 1991). However, when grown *in vivo* in nude mice all clones gave rise to tumours with histology similar to that of the original tumour. Furthermore, these phenotypic differences were lost after culture of the clones for 15 passages. In contrast, clones derived from a human bladder cancer cell line maintained differences *in vitro* for more than 40 passages and each gave rise to tumours of unique histologies (Brown *et al*, 1990). When drug resistant cell lines are compared with the parental cell line from which they were derived it is often the case that a number of possible mechanisms of resistance are identified (Batist *et al*, 1986; Beck *et al*, 1989; Danks *et al*, 1987; Ozols *et al*, 1988; Reeve *et al*, 1990; Sehested *et al*, 1987a & b; Sinha *et al*, 1989). This could well be explained by multiple changes occurring within the cells. However, it is also possible that the drug resistant cell line contains subpopulations, each of which has developed resistance via a different pathway. Provided that the growth requirements and doubling times do not differ significantly the populations will coexist. This suggestion is supported by the work

of the Goldenberg group who have isolated cloned cell lines from the P388 leukaemia cell line and from a doxorubicin resistant variant P388/ADR (Deffie *et al*, 1988; Goldenberg *et al*, 1986). Clones from the resistant cell line differed in a number of aspects including drug sensitivity, drug uptake, glutathione-S-transferase activity, and susceptibility to DNA strand breaks.

Clearly, if a drug resistant cell line contains subpopulations with differing resistance phenotypes attempts to characterise the cell line in terms of resistance mechanisms will be complicated. Since it was apparent that subpopulations exist within the multidrug resistant ovarian cell line 2780AD this line was cloned along with the drug sensitive parental cell line A2780. The original aim was to isolate a pure population from each line to allow detailed studies of the effects of verapamil on intracellular drug distribution. However when the clones derived from cell line 2780AD were tested for sensitivity to daunorubicin it became apparent that the range of sensitivities observed depended on when the clones were last exposed to the selecting agent, doxorubicin. This chapter, therefore, describes the phenotypic instability of some, but not all subpopulations derived from the multidrug resistant ovarian cell line 2780AD. Clones were isolated by limiting dilution and drug sensitivity and drug accumulation determined over a period of 20 weeks during which time cells were grown in the absence of the selecting agent.

Materials and Methods

Isolation and establishment of clones

Exponentially growing cultures of cell lines A2780 and 2780AD were trypsinised and suspended at a density of 4 cells/ml. They were plated out into flat-bottomed 96-well plates (200 μl/well) so that each well should contain not more than one cell. Wells containing a single cell were identified under a light microscope and marked within a few hours after plating. The cells were allowed to grow in drug-free medium to form colonies at 37°C in a humidified 2% CO_2 atmosphere without changing the medium. Clones were harvested 2 or 3 weeks later, depending upon colony size. After microscopical examination to ensure that there was only a single colony formed in a given well, individual cell colonies were dispersed by trypsinisation and transferred to 24-well plates and subsequently to 25-cm^2 flasks. Stocks were stored in liquid nitrogen. The clones were designated according to the parental cell line, followed by the appropriate clone numbers, *e.g.*, A2780/clone-1 or 2780AD/clone-2. These are sometimes abbreviated as A-1 or AD-2 etc.

Clones were grown in the absence of drug unless otherwise stated. Population doubling times were determined for each clone as described previously (Chapter 2 page 23). Photomicrographs were taken with an inverted phase contrast microscope (Olympus CK2; Optical CO., Ltd., Tokyo, Japan) with a camera attached (Olympus OM System; Optical CO., Ltd., Tokyo, Japan) and

using Kodak film (Ektachrome 160T; Kodak Ltd., Herts, England).

Chemosensitivity and drug accumulation

Drug sensitivity was determined by the microtitration assay described in Chapter 2 (page 24). The sensitivity for each clone was expressed as the ID_{50} and comparisons of sensitivity were made on this basis. Drug accumulation was determined by liquid scintillation counting as described previously (Chapter 2, page 27). These measurements were repeated after growth of the clones in the absence of drug for various times.

Drug efflux

Drug efflux was determined as described in Chapter 2 (page 28). Clones from 2780AD were maintained in drug free medium for 12 weeks and drug efflux was determined before and 3 days after a single exposure to doxorubicin (2 μM) for 24 hours. The original parental cell lines (A2780 & 2780AD) were also included for comparison.

Cell sizing

Measurement of cell size was performed as described in Chapter 2 (page 23). Cell size was expressed as a volume (μm^3) determined by assuming that a single cell in suspension in isotonic saline is spherical.

Chromosome analysis

Cells grown in the absence of drug for 12 weeks were exposed to colchicine (Demecolcine 0.1 μM; Sigma Chemical Company, Poole, U.K.) at 37°C for 4 hr. The drug and medium were removed and the cells were incubated for 20 min in a hypotonic solution (KCl, 0.04 M; sodium citrate, 0.025 M). In order to obtain a high proportion of cells in metaphase, cells were harvested by gentle shaking. The cell suspension was centrifuged at 100 *g* for 3 min and the supernatant discarded. Cells were fixed by addition of ice cold acetic-methanol (1 part glacial acetic acid + 3 parts methanol) with constant mixing. The suspensions were centrifuged at 100 *g* for 3 min and the resultant pellets were dispersed by using a vortex mixer. Fresh fixative solution was then added while mixing continuously. The process of cell fixation was completed by repeating this procedure three times. The final pellet of fixed cells was resuspended in 0.5 ml of fixative. Approximately 50 μl of this suspension was dropped onto a clean glass slide, air dried at room temperature, stained with Giemsa and examined under a light

microscope (Polyvar, Leica UK Ltd., Milton Keynes, U.K.).

The chromosome number was determined for each spread and 50 spreads were examined for each clone. The results are presented as a frequency distribution.

Immunocytochemistry

Fixation For immunocytochemistry cells were grown on eight chamber glass slides (Lab-Tek, Life Technologies Ltd., Paisley, U.K.). The cells were allowed to grow at 37°C for 3 days before staining.

Cytospin preparations of cell line H69LX10 were used as a positive control and the wild type cell line A2780 was used as one of the negative controls. Non-adherent H69LX10 cells were centrifuged at 200 *g* for 5 min, dispersed and resuspended in PBS. The cells were then spun onto clean microscopic glass slides in a cytospin centrifuge (Shandon Scientific Co. Ltd., London, U.K.) to give a monolayer of cells.

Slides were washed three times in PBS (pH 7.4), fixed in acetone for 10 min, and air dried at room temperature. The slides were then immersed in Tris saline (0.05 M, pH7.6) for 5 min.

Antibodies A mouse monoclonal antibody C219 (CIS UK Ltd., High Wycombe, Bucks, U.K.) which recognises an internal epitope of P-glycoprotein was used as

the primary antibody at a optimal working dilution of 10 μg/ml. A rabbit anti-mouse immunoglobulin conjugated to alkaline phosphatase (Dako, High Wycombe, Bucks, U.K.) was used as the second antibody, diluted 1/20 in filtered normal human serum. As a negative control, C219 was replaced by an irrelevant monoclonal antibody (Clonab LN-C, Biotest, U.K.). All subsequent dilutions of antibodies were made with Tris buffer (0.05 M, pH 7.6).

Alkaline phosphate substrate solutions Two and half milligrams of Naphthol AS B1 phosphate (Sigma Chemical Company, Poole, U.K.) was dissolved in 50 μl of dimethyl formamide (Sigma, U.K.), in vertical laminar flow conditions, until the solution turned clear (Solution A). Solution B was prepared by solubilising 2.5 mg Fast Red TR salt in 5 ml of Veronal Acetate Buffer in a universal container. The buffer was made up as follows:

Sodium Acetate (3 H_2O; BDH Chemicals, U.K.)	0.9715 g
Sodium Barbitone (BDH Chemicals, U.K.)	1.4715 g
Distilled Water	247.5 ml

The buffer solution was adjusted to pH 9.2 with 0.1 N hydrochloric acid (approx. 2.5 ml) and stored at 4°C away from light.

Solution A and B were mixed together and 100 μl of levamisole (Sigma Chemical Company, Poole, U.K.), which is known to inhibit the activity of endogenous alkaline phosphatase, was added. The resulting solution was filtered

(Whatman No 1 filter).

Staining Immunocytochemical staining was performed at room temperature in a humidified container. The cells were incubated for 2 hr with the primary antibody C219, washed in Tris saline for 5 min and then incubated for 45 min with the second antibody conjugated to alkaline phosphatase. They were then washed in Tris saline for 5 min and incubated with the alkaline phosphate substrate solution for 30 min. Slides were counterstained in Haemotoxylin solution for 15 seconds, washed briefly (about 20 seconds) in tap water and mounted in a water-based mounting medium, glycergel (Dako, High Wycombe, Bucks, U.K.).

For specific blocking of C219 immunostaining, a 15 amino acid peptide (VVQEALDKAREGTC), kindly donated by Dr Elias Georges (Ontario Cancer Research Institute, Canada), was used to block the binding site for C219 on P-glycoprotein (Georges *et al*, 1990). Both H69LX10 and 2780AD cells were incubated for one hour under the conditions specified above, with C219 (10 μg/ml) and the peptide (670 μM, 1000 fold molar excess of C219).

Results

Drug sensitivity of the clones

Sensitivity to daunorubicin was determined immediately after establishment of clones. At this time they had been grown in the absence of drug for almost 4-5 weeks. Whereas a slight (less than 2 fold) difference existed between clones of A2780, clones from 2780AD varied in sensitivity by up to 5 fold (Table I). Six resistant clones and 2 from the parental cell line were selected for detailed studies.

Morphology of the clones

There were marked differences in the morphologies of the clones (Plate 1). For example, whereas small rounded cells were characteristic of clone AD-3 (Plate 1C), slender and spindle shaped cells were characteristic of AD-2 (Plate 1B). Clone A-10 consisted of long bipolar cells closely aligned with long axes parallel to each other (Plate 1A). While a triangular or rectangular shape was a feature of clone AD-8, a mixture of rounded and irregular shaped cells was characteristic of clones AD-6, AD-10 and AD-16 (Plate 1D). In general, clones from A2780 tended to grow in tight cell-to-cell contact whereas clones from 2780AD grew in loose contact. All the clones retained their characteristic

	ID_{50} (nM)						
	Experiment				Experiment		
Clone	1	2	3	Clone	1	2	3
A-1	7.3	3.5	11.6	AD-1	5400	4460	
A-2	6.2	4.6	10.0	AD-2*	2625	1625	2700
A-3	7.2	4.6	8.0	AD-3*	5700	4850	8130
A-4	10.2	6.7	10.7	AD-4	1310	1170	
A-5*	6.6	5.5	6.7	AD-5	4800	4260	
A-6	6.2		10.4	AD-6*	3000	2220	2590
A-7	6.5		7.7	AD-7	5250	3770	
A-10*	7.5		10.7	AD-8*	3350	2090	3050
A-11	7.5		10.3	AD-10*	3075	1695	4080
				AD-14	2355	2910	
				AD-15	5950	4920	
				AD-16*	7000	5295	5240
				AD-17	1710		
				AD-18	3415	3025	

Table I Daunorubicin sensitivities of clones from A2780 (A-) and 2780AD (AD-) determined using the MTT assay at within 4 weeks of the establishment of the clonal populations. Results are shown for three independent experiments and each value is the mean of triplicate plates. Clones selected for detailed studies are indicated by an asterisk.

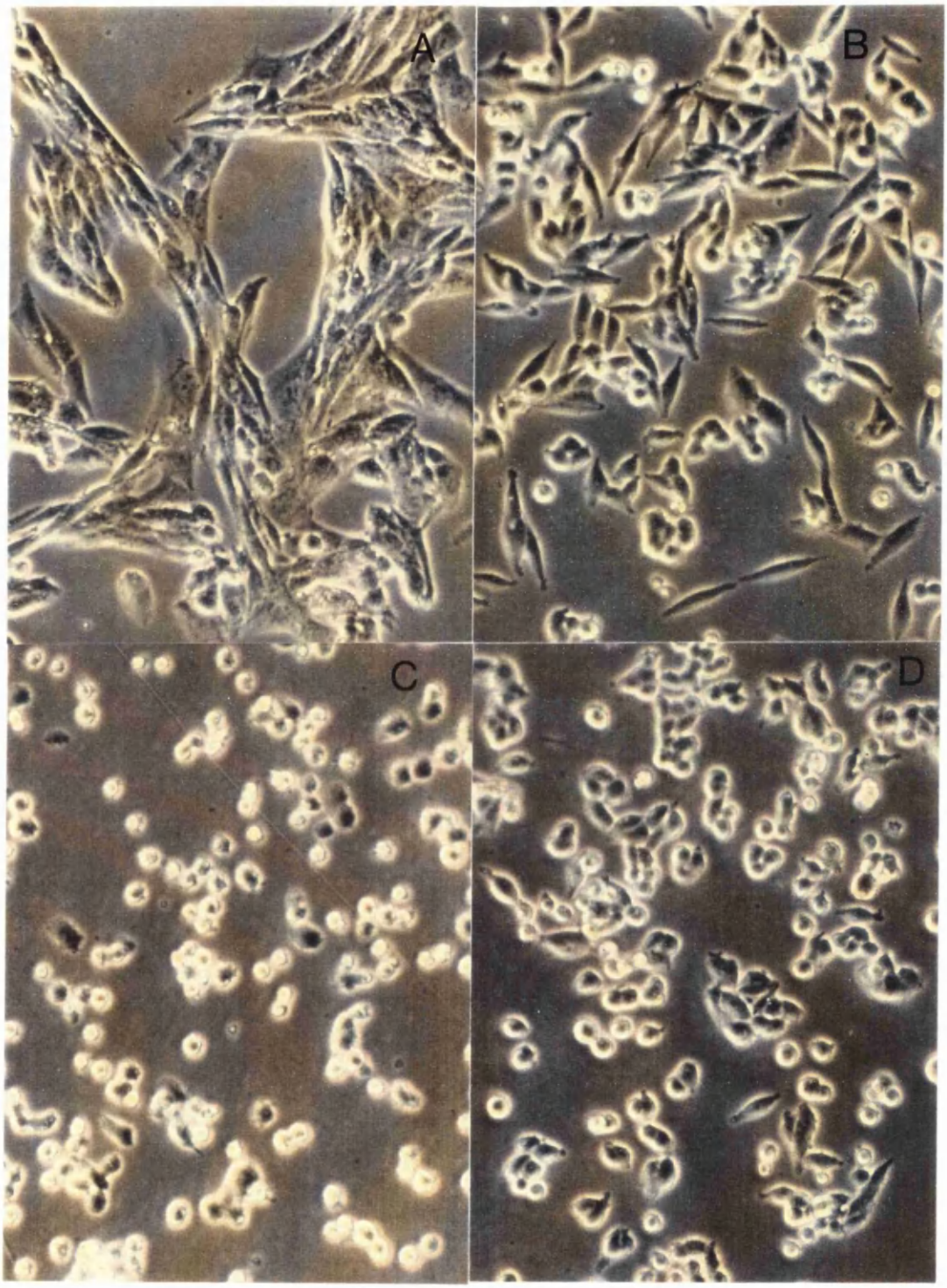


Plate 1 Morphology of clonal cell lines grown in drug free medium for 12 weeks. Photomicrographs were taken with Kodak film (Kodak Ektachrome 160T) at 10× objective 3 days after passaging of clones A-10 (A), AD-2 (B), AD-3 (C) and AD-16 (D).

morphology when grown in drug free medium for at least 20 weeks.

Doubling times and cell volumes

Population doubling times for cell lines A2780 and 2780AD and their cloned subpopulations are shown in Table II. Cell line 2780AD and clones derived from this line showed similar doubling times and volumes. The parental cell line A2780 had a similar doubling time to 2780AD but was smaller. Two clones derived from cell line A2780 differed by a factor of 2 in both cell volumes and doubling times.

Modal chromosome count

Figure 1 shows the frequency distribution of chromosome numbers for the various clones. The number of chromosomes per spread varied from 43 to 49 with a modal number of 46 for clones AD-2, AD-6, AD-8 and AD-10. However, one of the clones (AD-16) comprised mainly cells with a modal chromosome number of 47 and AD-3 was largely composed of a mixture of almost equal number of cells having 45, 46 or 47 chromosomes.

Cell line	Volume (μm^3)	Doubling time (hr)
A-5	640.9	18.5
A-10	1314.7	34.5
AD-2	920.3	29.0
AD-3	854.5	24.5
AD-6	1281.8	27.5
AD-8	1230.0	27.5
AD-10	1314.7	25.0
AD-16	1216.1	27.0
A2780	723.1	24.0
2780AD	1022.7	26.0

Table II Characterisation of clones from A2780 (A-) and 2780AD (AD-). Cells were dispersed with trypsin and sized in isotonic saline using an electronic particle counter. Cell population doubling time was calculated from daily cell counts in triplicate of exponential phase cultures. Cell volume are single measurements estimated with a suspension of almost 10^5 cells/ml. Also shown are results for A2780 and 2780AD for comparison.

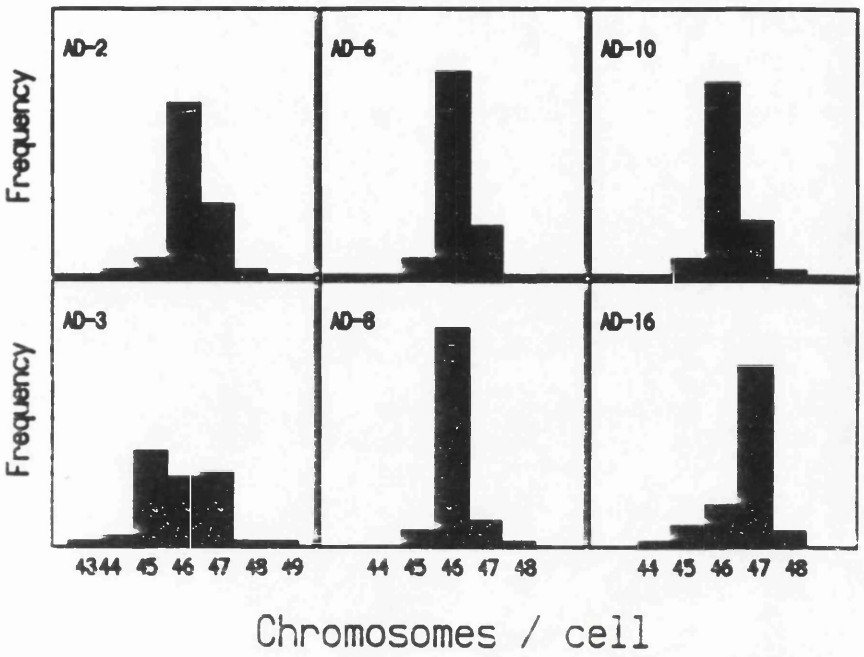


Figure 1 Frequency distribution of chromosome counts for clones isolated from cell line 2780AD. Fifty chromosome spreads were examined for individual clones.

Effect of growth in drug-free medium on daunorubicin sensitivity of the clones

When drug sensitivities were estimated two weeks after exposure to doxorubicin, clones derived from cell line 2780AD all showed similar sensitivities to daunorubicin (Fig. 2). A gradual and significant increase ($P < 0.001$; analysis of variance) in drug sensitivity was observed for 4 clones (AD-2, AD-6, AD-8 & AD-10) when grown in drug-free medium for up to 12 weeks. In contrast, no significant difference ($P > 0.05$) in overall drug sensitivity of clones AD-3 and AD-16 was observed even after 20 weeks growth in drug-free medium. Clones AD-2, AD-10 and AD-8 showed a further increase in sensitivity between 12 and 20 weeks such that the ID_{50}s for these 3 clones were similar to that of clone A-5 derived from the drug sensitive cell line A2780. At week 20 only 3 of the clones derived from 2780AD (AD-3, AD-6 & AD-16) showed an increase in sensitivity to daunorubicin when exposed to drug in the presence of verapamil (6.6 μM, Table III).

Drug accumulation

Drug accumulation was determined by incubation of cells in daunorubicin (1 μM) for one hour. The accumulation was estimated after growth of the clones in drug-free medium for various times. All six clones derived from 2780AD showed markedly reduced daunorubicin accumulation when compared with the

original A2780 (Fig. 3). However, when grown in drug-free medium for longer than 2 weeks, the amount of drug accumulated increased significantly ($P < 0.01$) with increased time of growth in nonselective conditions for 4 of the clones. In contrast, AD-3 and AD-16 showed no change in drug accumulation throughout the period of study. There was a 6 - 8 fold difference in drug accumulation between clones after growth for 12 weeks in the absence of doxorubicin. This difference between clones remains apparent when drug accumulation was determined over a 5 hour time course (Fig. 4).

Drug efflux

Daunorubicin efflux was measured in the resistant clones grown for 12 weeks in the absence of drug. The results are shown in Figure 5 together with the results from parental cell lines A2780 and 2780AD for comparison. Cell line 2780AD had been maintained routinely in the presence of drug, until 3 days before use. After the 1 hour loading period in the absence of an energy source, cell lines A2780 and 2780AD accumulated similar amounts of daunorubicin. Five of the resistant clones showed similar accumulation levels but one clone, AD-16, accumulated much less drug. Drug accumulation in clone AD-16 could be increased to that of the drug sensitive parental cell line A2780 by incubation of the cells with daunorubicin in the presence of an energy source and verapamil (50 μM, Fig. 6).

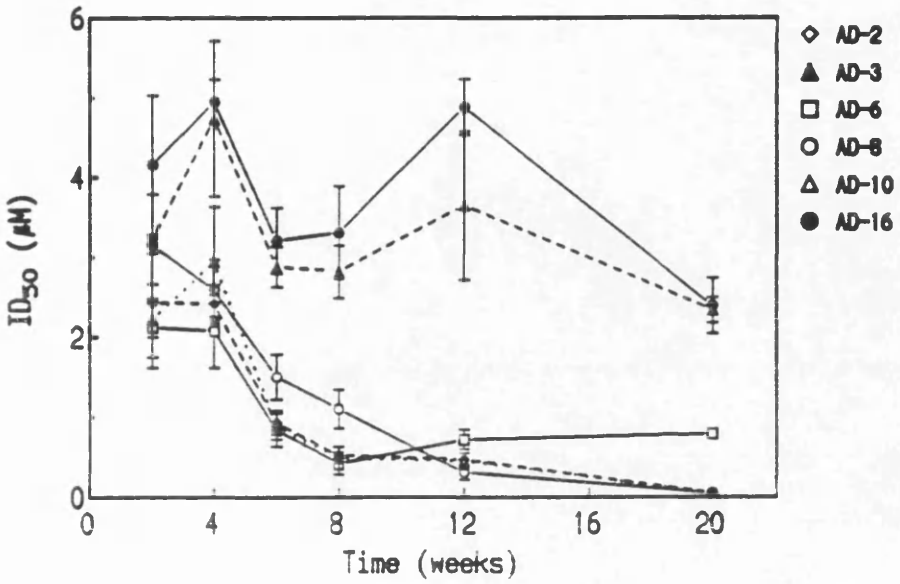


Figure 2 Daunorubicin sensitivity of the clones determined at various times after exposure to doxorubicin for 24 hours (week 0). Sensitivity was assessed by the MTT assay. Points are the mean ± standard error of 3 observations.

	ID_{50} (nM)			
Clone	DNR	DNR + Vp	MF	*P*
AD-2	12.0 ± 5.3	9.6 ± 2.2	0.8	> 0.05
AD-3	2340 ± 156	173 ± 22	13.5	< 0.001
AD-6	793 ± 6.9	43 ± 2.2	18.4	< 0.001
AD-8	3.4 ± 0.2	4.5 ± 0.10	0.8	> 0.05
AD-10	5.7 ± 1.2	7.0 ± 1.0	0.8	> 0.05
AD-16	2390 ± 202	169 ± 6.9	14.1	< 0.001
A-5	5.4 ± 1.1	5.9 ± 0.7	0.9	> 0.05

Table III Drug sensitivities of clones when exposed to daunorubicin in the presence and absence of verapamil (6.6 μM) determined by the MTT assay. Clones had been grown in drug free medium for 20 weeks. Values are the mean ± standard error of 3 determinations. Also shown is the sensitivity of clone A-5 (from A2780) for comparison.

$$\text{Modulation Factor (MF)} = \frac{ID_{50} \text{ DNR in the absence of verapamil}}{ID_{50} \text{ DNR in the presence of verapamil}}$$

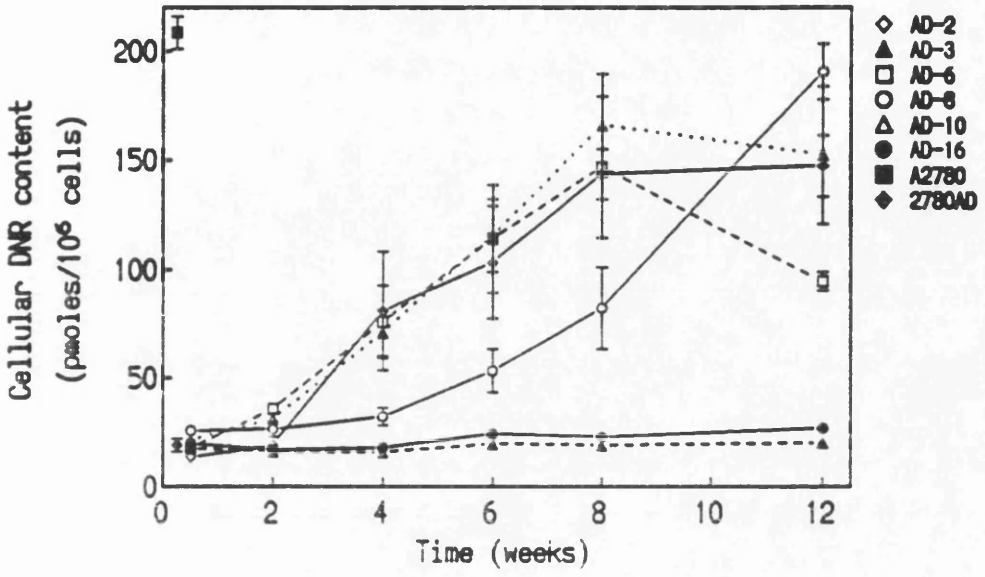


Figure 3 Drug accumulation determined at various times after exposure of the clones to doxorubicin for 24 hours (week 0). Cells were incubated in ^{3}H-daunorubicin (1 μM) for 1 hour and total cellular drug accumulation was determined by scintillation counting. Results for cell lines A2780 and 2780AD are shown for comparison. Points are the mean ± standard error of 3 observations.

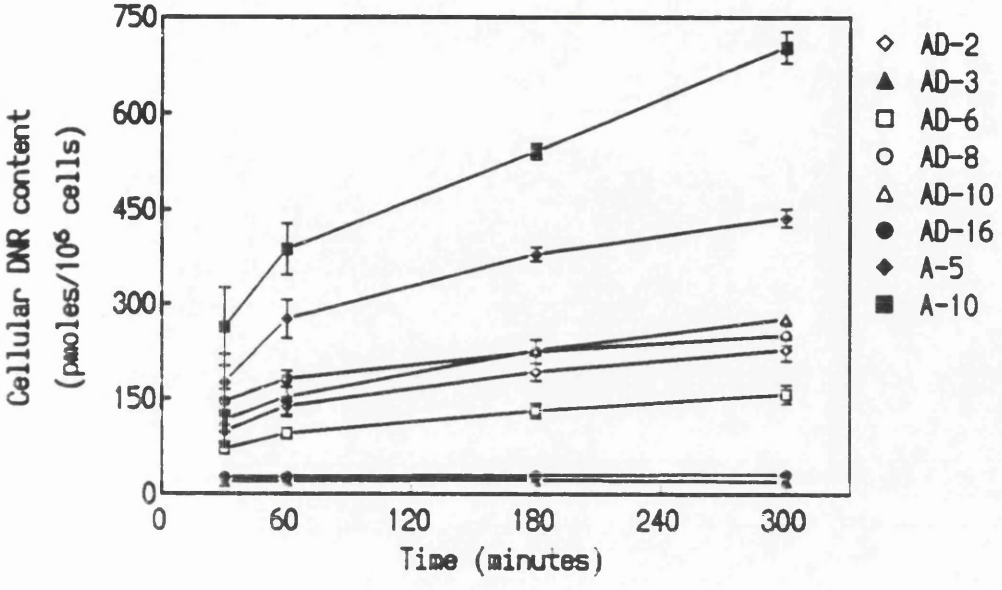


Figure 4 Total cellular daunorubicin content of the clones after incubation with ^{3}H-daunorubicin (1 μM) for various times. Clones from cell line 2780AD were grown in the absence of drug for 12 weeks before estimation of drug accumulation. Results are the mean ± standard error of 3 observations.

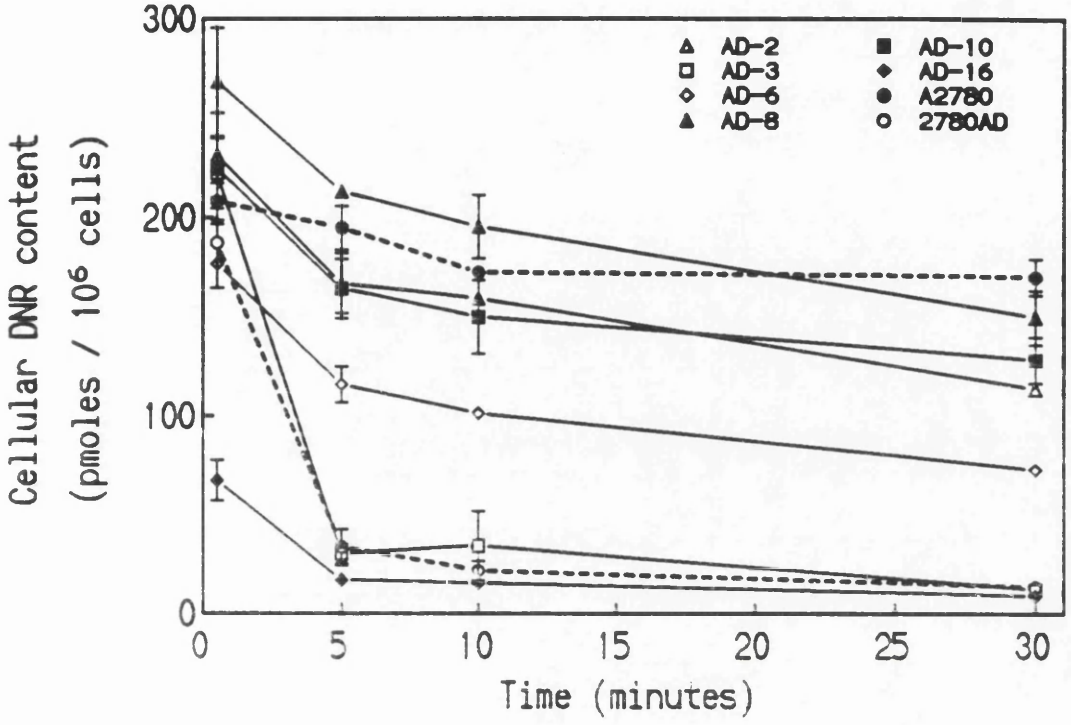


Figure 5 Drug efflux for clones from 2780AD estimated after growth in drug free medium for 12 weeks. Cells were loaded with daunorubicin in glucose free Hank's Balanced Salt Solution containing sodium azide (10 mM) for an hour and were then transferred into standard culture medium. Cellular drug content was measured using liquid scintillation counting. Results are the mean $\pm$ standard error of 3 observations.

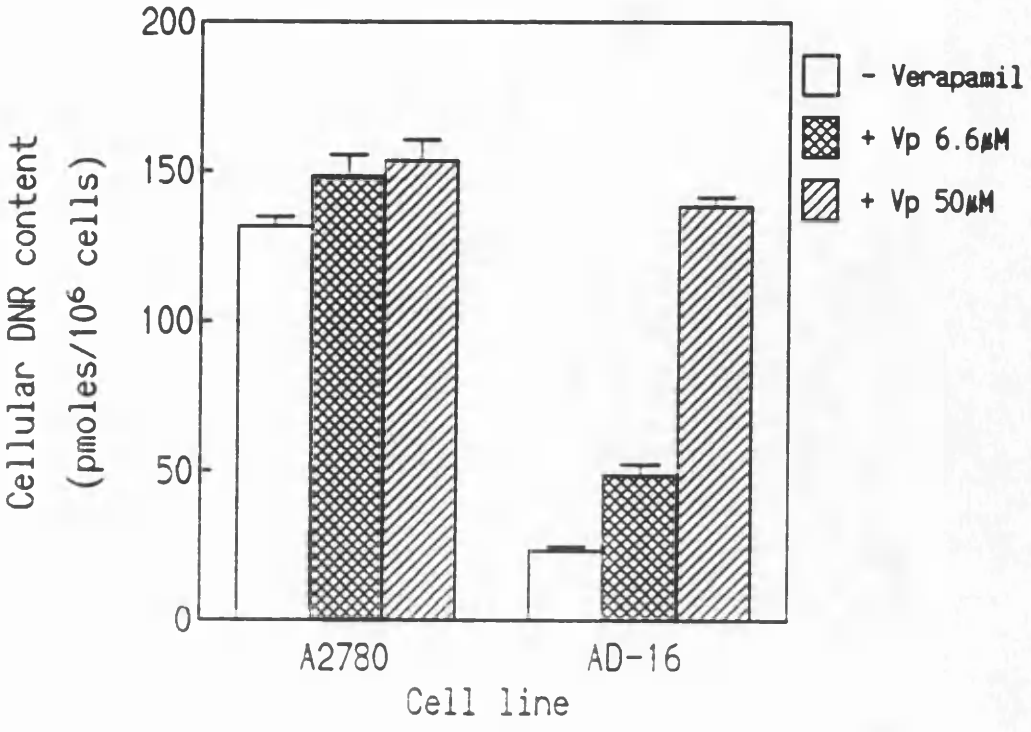


Figure 6 Total cellular drug accumulation for clone AD-16 following incubation of the cells with ^{3}H-daunorubicin (1.0 μM) for 1 hour in the presence or absence of verapamil. Results are the mean ± standard error of 3 determinations. Also shown are results for cell line A2780 for comparison.

Both 2780AD and clone AD-3 showed a marked decrease in accumulated drug when incubated for 5 min in the presence of an energy source but in the absence of daunorubicin, such that only 18% (2780AD) and 14% (AD-3) of the accumulated daunorubicin remained. The amount of drug released by the other 4 clones was much smaller such that 65 - 80% of the accumulated drug remained after 5 min, except for clone AD-16 where this represented 75% of the accumulated drug. Very little drug was released by A2780 over the duration of the study.

Effect of a single exposure to doxorubicin on drug sensitivity and accumulation of the resistant clones

Clones grown for 12 weeks in the absence of doxorubicin were exposed to doxorubicin for 24 hours. Measurements were made before and 3 days after drug exposure.

The effect of a single exposure to drug on daunorubicin sensitivity is shown in Table IV. For comparison, the sensitivity is expressed with respect to the sensitivity of a particular clone (A-5) from the sensitive parental cell line. After growth for 12 weeks in the absence of doxorubicin the drug sensitivities of 4 of the clones have increased such that the resistance factors for these clones had decreased by 8.7 fold (AD-2), 4.9 fold (AD-6), 17.3 fold (AD-8) and 8.5 fold (AD-10). For the other two clones drug sensitivity was not significantly altered ($P >$

0.05) and similarly the resistance factor was maintained. Exposure to doxorubicin for 24 hours decreased the drug sensitivity of all 6 clones such that the resistance factor was increased for all clones. For clones AD-3 and AD-16 this increase was modest (2.2 & 1.8 fold respectively). However, for the 4 clones that showed an increase in drug sensitivity with time in culture the resistance factors increased significantly by 14.5 (AD-2, $P < 0.001$), 7.0 fold (AD-6, $P < 0.01$), 16.1 fold (AD-8, $P < 0.001$) and 15.8 (AD-10, $P < 0.001$).

Exposure to doxorubicin had no effect on drug accumulation or drug efflux for clones AD-3 and AD-16 (Figs. 7 & 8). In contrast, drug accumulation was significantly decreased by 4.1-5.3 fold for the other 4 clones and drug efflux was markedly increased during the first 5 minutes of incubation in drug free medium (Fig. 8 cf Fig. 5 & Table V).

Relationship between daunorubicin sensitivity and intracellular drug content

Figure 9 shows the drug sensitivity of various clones derived from cell lines 2780AD and A2780 plotted against intracellular drug concentration. Clones from 2780AD had been grown in the absence of drug for 2, 4, 6 or 8 weeks. There is a clear relationship between drug sensitivity and intracellular drug concentration. For clones derived from cell line 2780AD the relationship is similar when intracellular drug is expressed as total amount per cell (Fig. 9A) or as a concentration calculated from measurements of cell volume (Fig. 9B). In

contrast, the 2 clones derived from A2780 show the same sensitivity to daunorubicin but one accumulates twice as much daunorubicin as the other (Fig. 9A). This difference is much less marked when the intracellular drug is expressed as a concentration (Fig. 9B) since clone A-10 (closed square) is twice the size of clone A-5 (closed circle).

Figure 10 shows the relationship between drug sensitivity and intracellular drug concentration for clones isolated from cell line 2780AD and grown for 4 weeks in the absence of drug. For each clone two measurements were made, one in the absence of verapamil (open symbols) and one in the presence of verapamil (6.6 μM, closed symbols). Verapamil clearly increases the drug sensitivity to a greater extent than drug accumulation (Fig. 10 cf Fig. 9).

P-glycoprotein expression

Plate 2 shows expression of P-glycoprotein in cell line 2780AD (A) but not in cell line A2780 (B). Staining was completely eliminated by the specific blocking peptide (Plate 3A & 3B). After growth for 12 weeks in the absence of drug 4 clones derived from the resistant line 2780AD (AD-2, AD-6, AD-8 & AD-10) showed patchy staining, with a small number of cells intensely stained while others were unstained (Plate 4A). Clones AD-3 and AD-16 showed intense staining in all cells (Plate 5A & 5B). After exposure of the clones to doxorubicin for 24 hours all clones showed uniform staining (Plate 4B).

	Initial[a]	12 weeks in drug free medium	24 hours exposure to DOX
Clone	RF	RF	RF
AD-2	695 ± 133	80 ± 18	1159 ± 138
AD-3	923 ± 159	618 ± 48	2035 ± 310
AD-6	605 ± 61	123 ± 15	867 ± 140
AD-8	899 ± 27	52 ± 6	835 ± 188
AD-10	637 ± 64	75 ± 9	1182 ± 227
AD-16	1189 ± 143	996 ± 154	2082 ± 502
A-5	1.0	1.0	1.0

Table IV Resistance factors (RF) for clones grown in drug free medium for 12 weeks and after exposure to doxorubicin (2 μM) for 24 hours. Drug sensitivity was measured using the MTT assay. Values are the mean ± standard error of 3 observations. Also shown for comparison are resistance factors derived from the first screen (initial) performed immediately after the establishment of the clones.

$$RF = \frac{ID_{50} \text{ of AD-clone}}{ID_{50} \text{ of A-5}}$$

[a] At this stage clones had been grown in drug-free medium for 4-5 weeks.

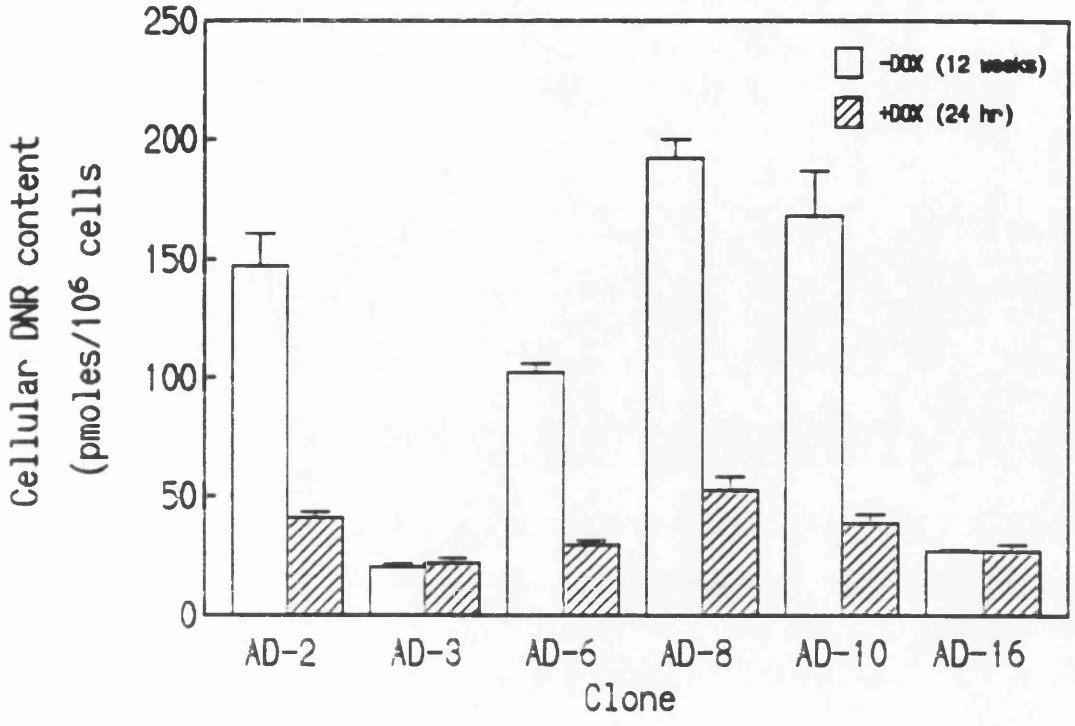


Figure 7 Cellular drug accumulation for clones determined after growth in drug free medium for 12 weeks and after exposure to doxorubicin (2 μM) for 24 hours. Drug content was determined after incubation for 1 hour in ^{3}H-daunorubicin (1 μM). Results are the mean ± standard error of 3 observations.

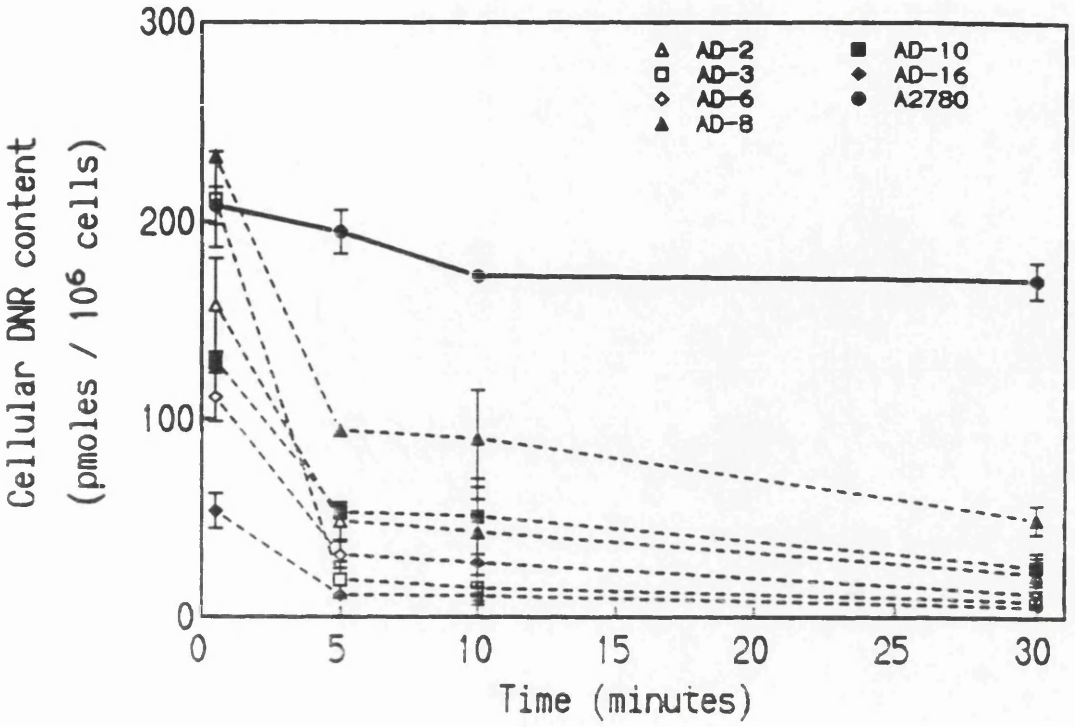


Figure 8 Drug efflux estimated in clones grown for 12 weeks in the absence of doxorubicin and then exposed to doxorubicin (2 μM) for 24 hours. The procedures were the same as for Figure 5. Results are the mean ± standard error of 3 observations.

	Drug efflux		
Cell line	12 weeks drug free	After exposure to DOX (24hr)	Fold increase
AD-2	27.7 ± 5.8	69.5 ± 1.3	2.5
AD-3	86.3 ± 1.1	91.1 ± 0.8	1.1
AD-6	34.5 ± 5.2	71.8 ± 2.9	2.1
AD-8	20.5 ± 6.3	59.5 ± 0.1	2.9
AD-10	26.8 ± 2.4	59.4 ± 1.3	2.2
AD-16	75.1 ± 7.1	79.4 ± 1.2	1.1
A2780	6.3 ± 3.7		
2780AD*	82.1 ± 2.7		

Table V Amount of drug (% of total loaded drug) lost during the first five minutes from clonal cell lines. Efflux was measured using liquid scintillation counting for clones grown in drug free medium for 12 weeks and after exposure to doxorubicin for 24 hours. Values are the mean ± standard error of 3 determinations.

* Resistant parental cell line was grown continuously in DOX-containing medium until 3 days prior to the assay.

Figure 9 Relationship between drug sensitivity (ID_{50}) and intracellular drug content expressed either as (A) total amount per cell (pmoles/10^6 cells) or (B) as the intracellular concentration (μM). Measurements were made after growth in drug free medium for 2, 4, 6 and 8 weeks and each symbol represents a particular clone derived from 2780AD. The two closed symbols are 2 clones derived from A2780. Cellular drug content was determined by incubation of the cells for 1 hour in the presence of ^{3}H-daunorubicin.

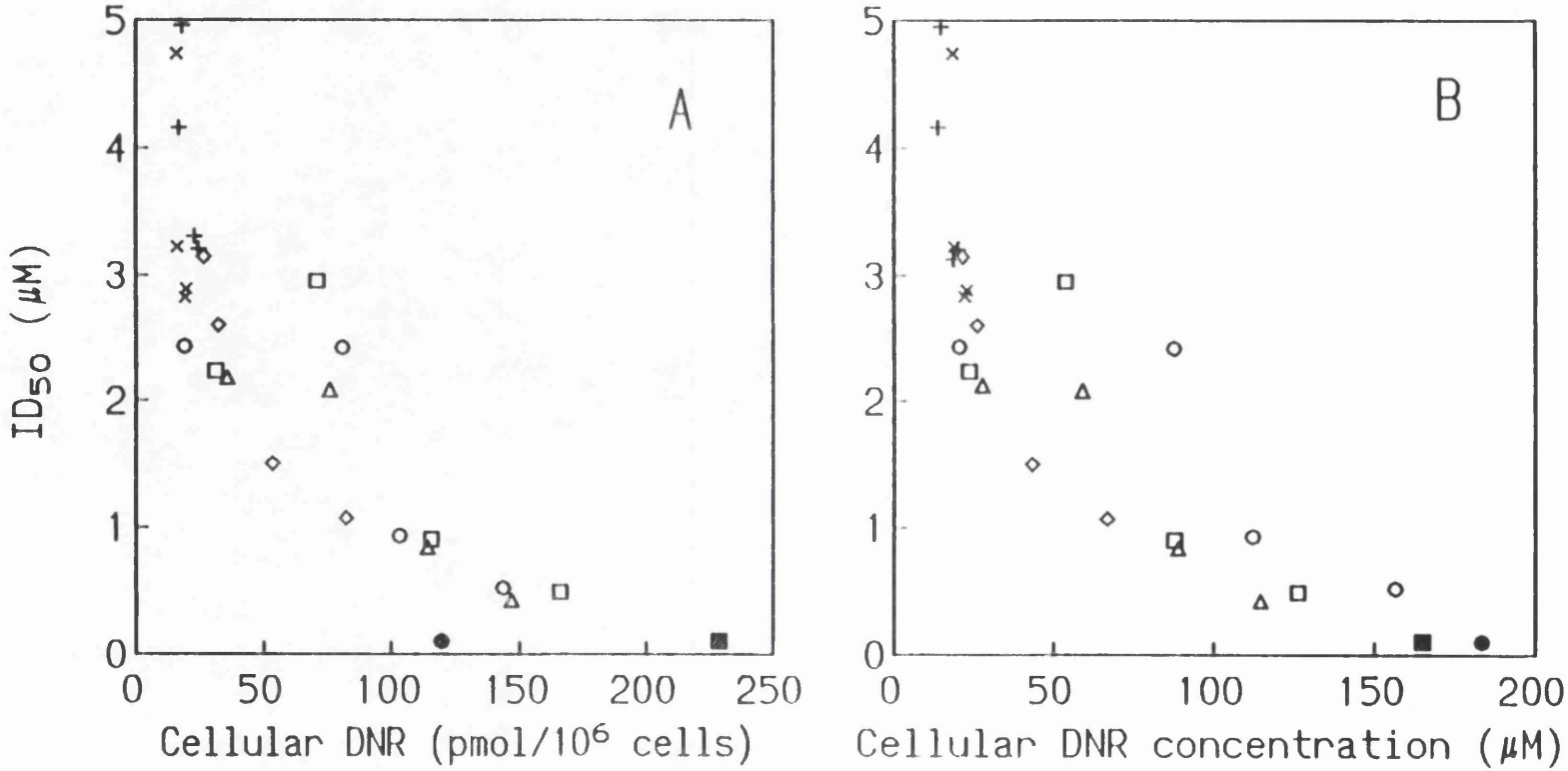
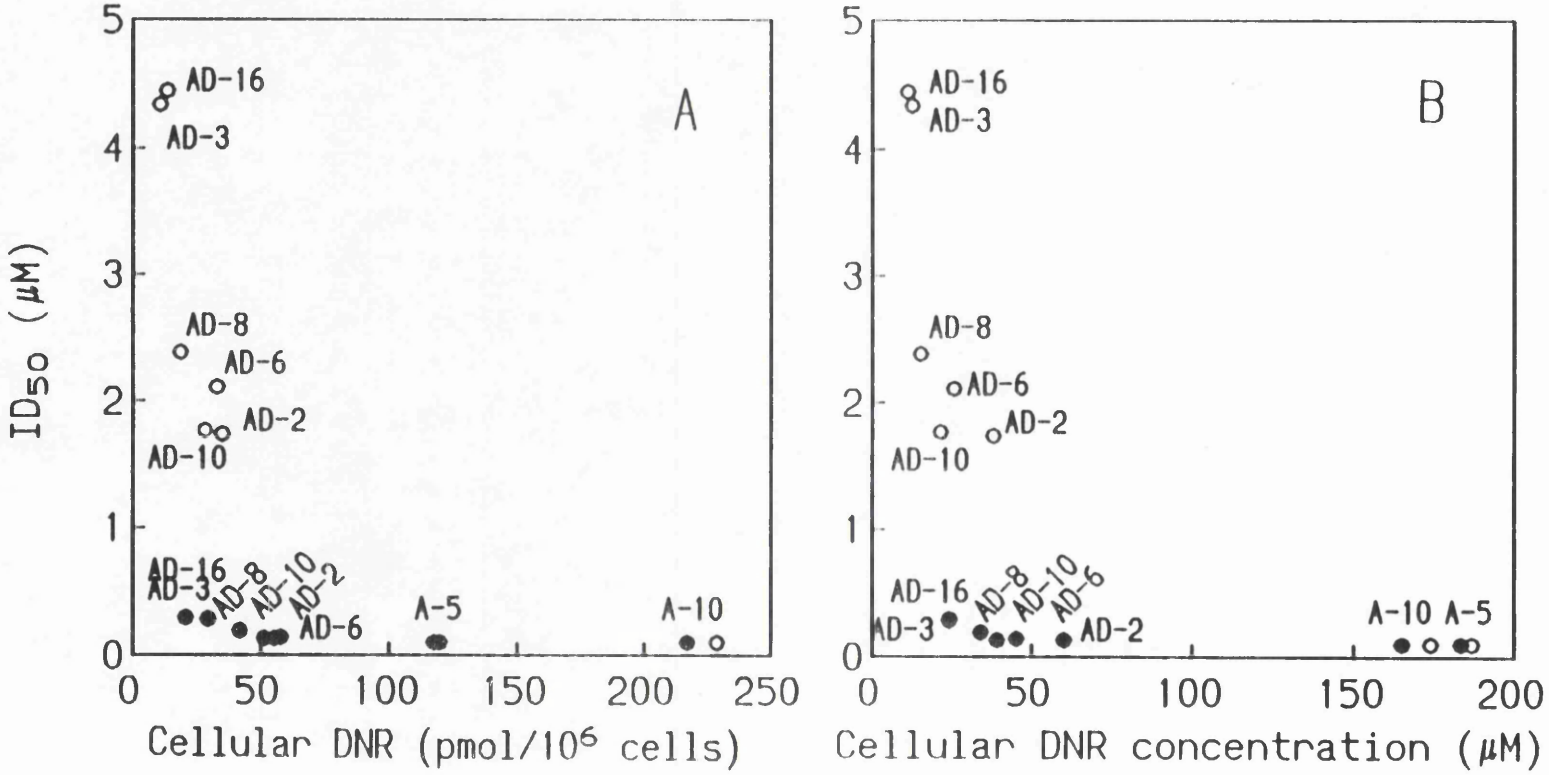

A
B
ID_{50} (μM)
Cellular DNR (pmol/10^6 cells)
Cellular DNR concentration (μM)
0
1
2
3
4
5
0
50
100
150
200
250

Figure 10 Relationship between drug sensitivity (ID_{50}) and intracellular drug content expressed either as (A) total amount per cell (pmoles/10^6 cells) or (B) as the intracellular concentration (μM). Measurements were made after growth in drug free medium for 4 weeks and cells were exposed to daunorubicin in the absence (open symbols) and presence (closed symbols) of verapamil (6.6 μM).


A
B
ID50 (μM)
Cellular DNR (pmol/10^6 cells)
Cellular DNR concentration (μM)
AD-16
AD-3
AD-8
AD-6
AD-2
AD-10
A-5
A-10
0
50
100
150
200
250
1
2
3
4
5

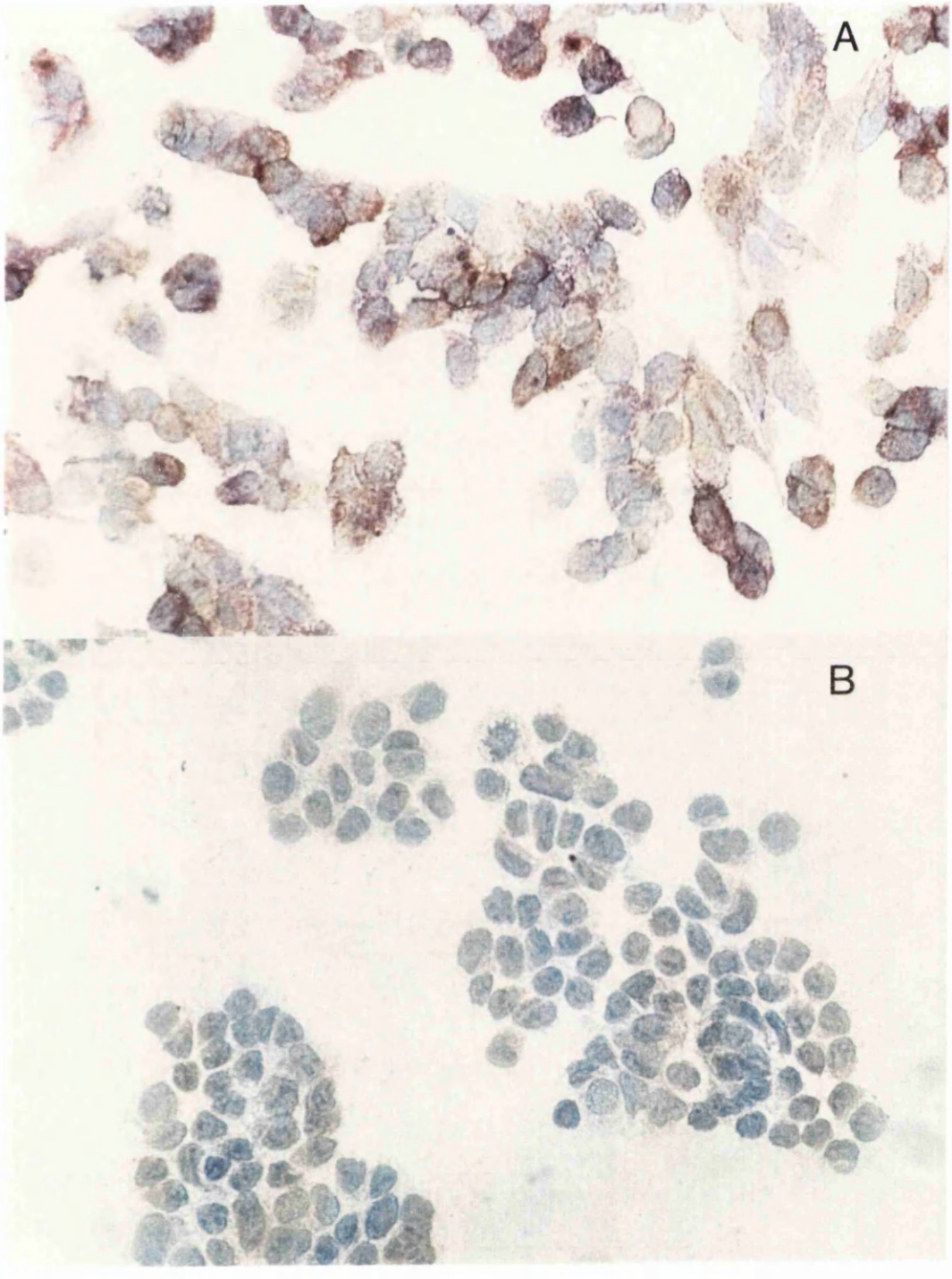


Plate 2 Immunocytochemical detection of P-glycoprotein with the monoclonal antibody C219. Cells were grown in multichamber slides for 3 days, washed in PBS, fixed in acetone and stained using an indirect alkaline phosphatase method. (A) 2780AD (B) A2780.

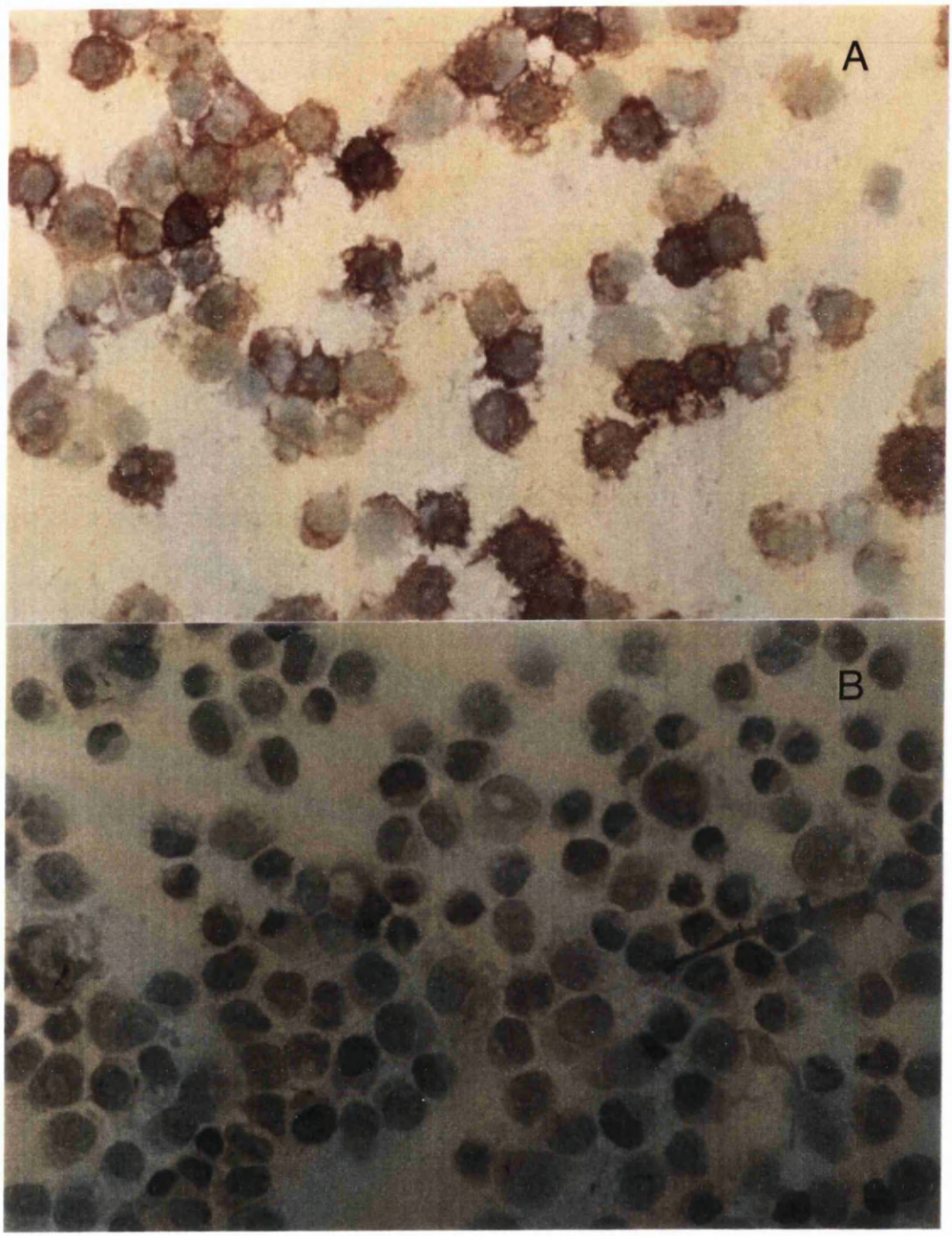


Plate 3 Immunocytochemical detection of P-glycoprotein with the monoclonal antibody C219. Cytospin preparations were fixed in acetone and stained by an indirect alkaline phosphatase method (A). In (B) cells were incubated with C219 antibody in the presence of a 15 amino acid blocking peptide at a molar excess of 1000 fold.

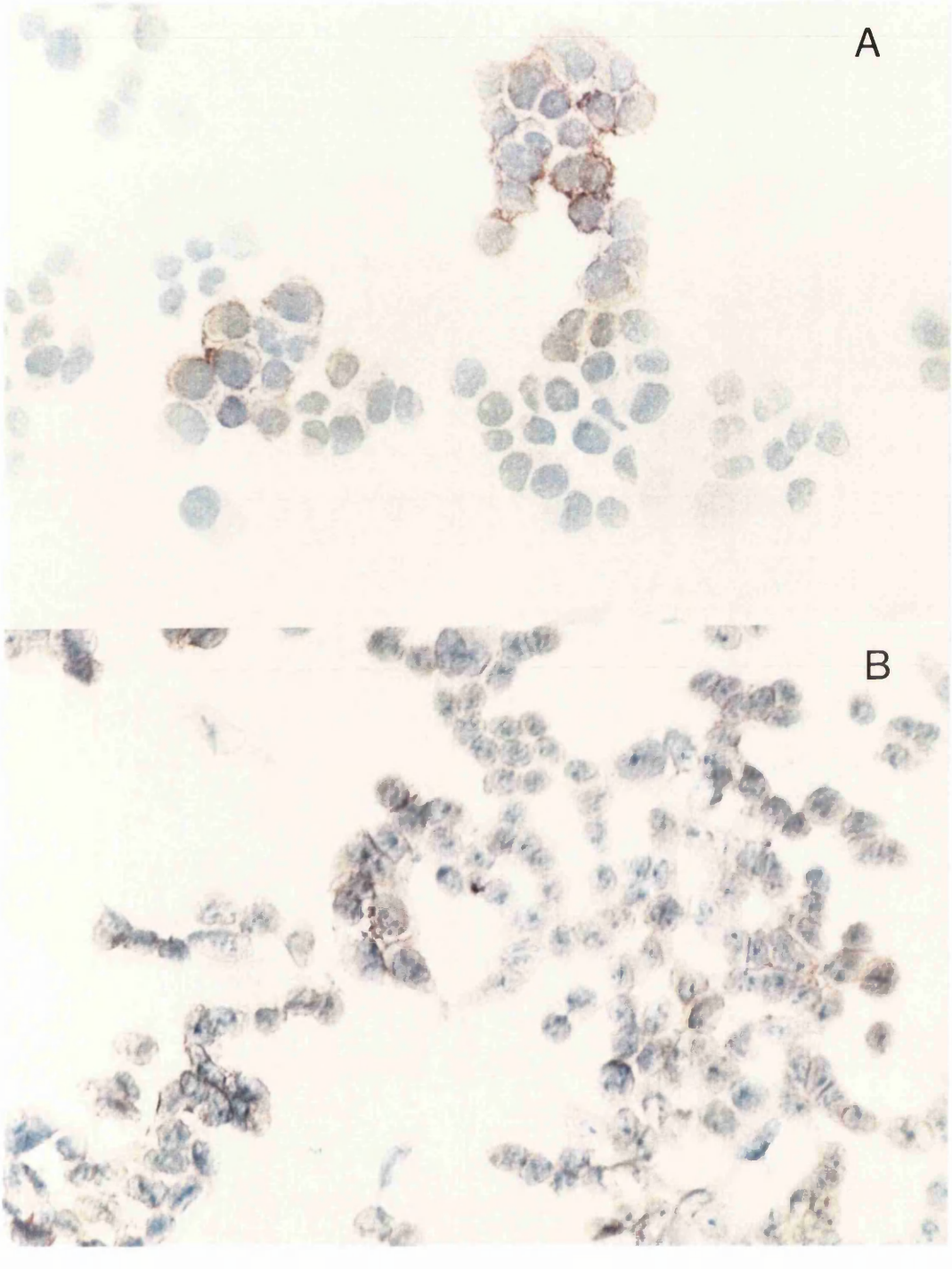


Plate 4 Immunocytochemical detection of P-glycoprotein with monoclonal antibody C219 of clonal cell line AD-10. Cells were grown in the absence of drug for 12 weeks and then stained before (A) and after (B) exposure to doxorubicin (2 μM) for 24 hours.

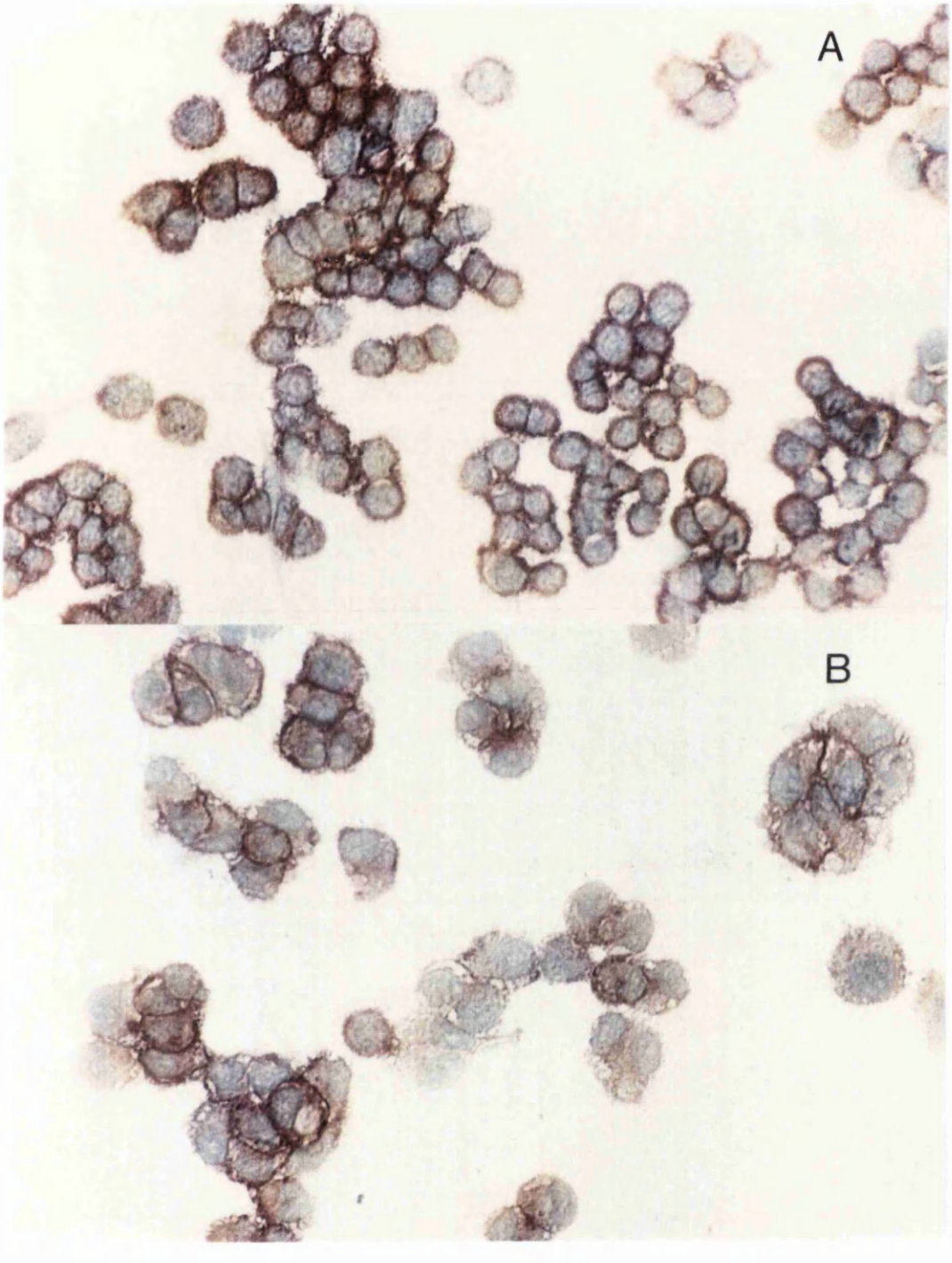


Plate 5 Immunocytochemical staining of P-glycoprotein with monoclonal antibody C219 of clonal cell lines AD-3 (A) and AD-16 (B). Clones had been grown in drug free medium for 12 weeks prior to staining.

Cisplatin sensitivity of the cloned cell lines

Cell line 2780AD is about 10 fold resistant to cisplatin when compared with the parental cell line A2780 (Chapter 5, Table I, page 135). All six clones isolated from cell line 2780AD maintained their resistance to cisplatin over the first 8 weeks of growth in the absence of doxorubicin (Fig. 11). A small decrease (about 2 fold) in sensitivity to cisplatin was observed by week 20. Only a 2 fold range in sensitivity to cisplatin was observed between to different clones.

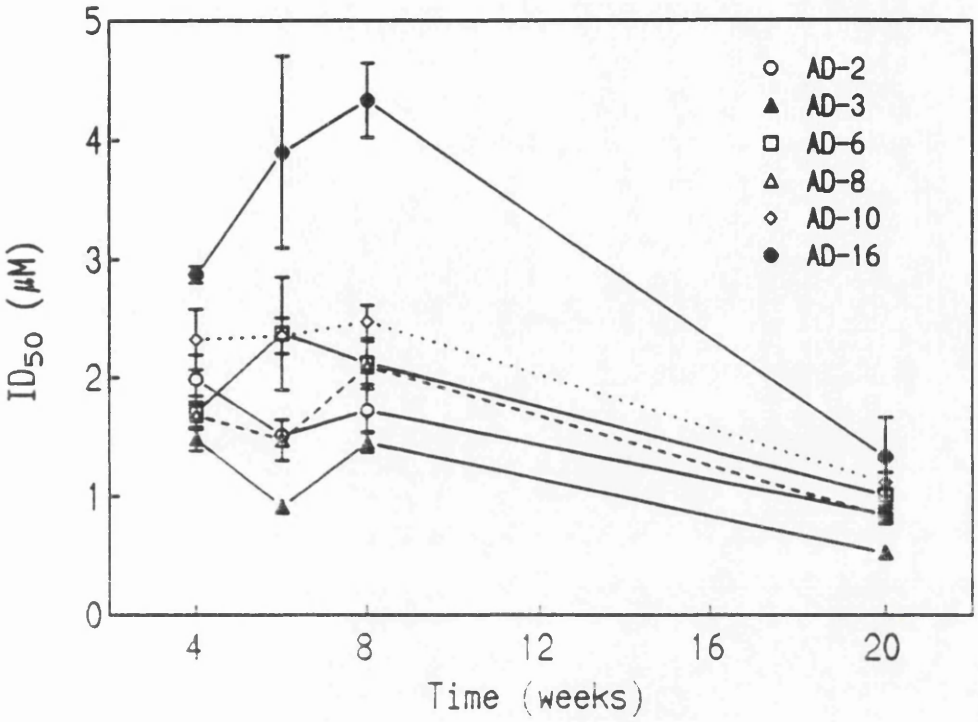


Figure 11 Sensitivity of the clones derived from cell line 2780AD to cisplatin determined at various times after growth in the absence of doxorubicin. Results are the mean ± standard error of 3 observations.

Discussion

A total of 9 clones were isolated from cell line A2780 and 14 from cell line 2780AD. Cloned cell lines showed morphological homogeneity on the whole but there were marked differences between clones. Clonal populations derived from A2780 showed similar sensitivities to daunorubicin with a range of only 1.6 fold. In contrast, clonal cell lines isolated from cell line 2780AD showed a range of sensitivities from 2 to 17.5 fold, depending on when they were last exposed to doxorubicin. Six cell lines were selected from 2780AD for detailed studies. Of these 2 maintained their resistance even after growth for 20 weeks in the absence of drug. The other 4 showed a gradual loss of resistance and this loss was associated with increased drug accumulation and decreased expression of P-glycoprotein.

Cell lines A2780 and 2780AD demonstrate marked morphological heterogeneity to an extent that is not observed amongst at least 30 human tumour cell lines in routine use in the Department of Medical Oncology. Interestingly, the cisplatin resistant cell line developed from A2780 does not show this heterogeneity. The majority of clones isolated from A2780 and 2780AD showed morphological homogeneity and the distinct morphology of each clone was maintained for more than 20 weeks in culture. This observation is consistent with that of Brown *et al* (1990) who studied clones derived from a human bladder cancer cell line.

Drug sensitivity of the clones was determined immediately after selection

and expansion and at this stage the cells had been grown in drug free medium for 4 weeks. Cell line 2780AD is normally maintained in the presence of doxorubicin (2 μM) and is only removed from drug for about a week before use in experiments. There was little difference between the drug sensitivities of clones isolated from cell line A2780 (Table I). A five fold range in sensitivity to daunorubicin was seen between clones isolated form cell line 2780AD (ID_{50}; AD-4, 1.2 μM cf AD-3, 6.2 μM). The range was small but consistent between experiments. Surprisingly, when the cells were exposed to doxorubicin (2 μM) for 24 hours the range of sensitivities decreased to only about 2 fold (Fig. 2). This suggested that in the absence of the selecting agent the drug sensitivity of some, if not all, of the clones was changing. In order to test this hypothesis 6 of the clonal cell lines derived from 2780AD were selected for detailed study. These were chosen over the range of drug sensitivities observed at initial testing and cells with reproducible growth and plating efficiencies were favoured. Only two clones were selected from cell line A2780 since these showed little difference in drug sensitivity and were required only as control cell lines for comparison.

The six clones selected from cell line 2780AD have similar doubling times and cell volumes (Table II). Similar doubling times were expected in order for the different subpopulations to be maintained in continuous culture of the original cell line 2780AD. However, the two clones studied from cell line A2780 differed by a factor of 2 in both doubling times and cell volumes. This observation may indicate differences in autocrine growth control between subpopulations in cell line A2780 such that both clone A-5 and A-10 respond to a particular growth

factor but only clone A-5 can produce and respond to the factor in an autocrine manner (Brown *et al*, 1990; Burgess, 1989; Luetteke & Lee, 1990).

Clonal cell lines derived from 2780AD were exposed to doxorubicin (2 μM) for 24 hours and drug sensitivity was determined at various times during growth in drug free medium. Two clones (AD-3 & AD-16) maintained their resistance to daunorubicin even after growth for 20 weeks in the absence of drug (Fig. 2). The other four clones selected from 2780AD showed a gradual increase in drug sensitivity over the 20 weeks of study such that the sensitivity of 3 of them (AD-2, AD-8 & AD-10) was not significantly different ($P > 0.05$) from that of clone A-5 selected from cell line A2780 (Table III). Verapamil was able to sensitise the two clones with stable resistance by 13.5 fold (AD-3) and 14 fold (AD-16). Two weeks after exposure to doxorubicin clone AD-6 was about 600 fold resistant to daunorubicin when compared with clone A-5 derived from the parental cell line A2780. After growth for 20 weeks in the absence of drug clone AD-6 was only about 150 fold resistant to daunorubicin. However, verapamil was able to increase the drug sensitivity of this clone to a greater extent (18 fold) than for clones AD-3 and AD-16 (Table III). This is an interesting observation and suggests that modulation of drug sensitivity by verapamil is limited and is not dependent on the degree of drug resistance present. Previously, it has been observed that for verapamil maximal activity is obtained *in vitro* at a concentration of 6-7 μM (Plumb *et al*, 1990). Increasing the concentration of verapamil to 10 μM had no further effect on drug sensitivity. Furthermore, the modulation factor for doxorubicin sensitivity was about 10 fold in each of 3 multidrug resistant cell

lines even though the cell lines themselves showed resistance factors ranging from 60 fold to 7400 fold. If as has been suggested verapamil exerts its effects by competition for closely related binding sites on P-glycoprotein (Yusa & Tsuruo, 1989) these observations indicate that it is not a very efficient competitor.

The changes observed in drug sensitivity of the clones isolated from 2780AD agreed with the alterations in drug accumulation. Thus, clones AD-3 and AD-16 which maintained their resistance throughout the period of study also maintained a markedly reduced drug accumulation when compared with cell line A2780 (Fig. 3). The remaining four clones from cell line 2780AD showed a gradual increase in drug accumulation with time such that by week 12 three of the clones accumulated almost as much drug per cell as did cell line A2780. Clone AD-6 accumulated about half as much drug as A2780 by week 12 and this is consistent with the observation that the drug sensitivity of this clone could be increased by verapamil at week 20. In order to allow the study of all six clones from 2780AD along with the two parental cell lines drug accumulation studies were limited to measurements of accumulation at a single time point (1 hour). It was shown in Chapter 2 that the profile of drug accumulation with time for cell lines A2780 and 2780AD differed. Thus it was possible that the changes observed at 1 hour might be explained by an alteration in the profile without any overall change in drug accumulation over a prolonged time course. It should be noted that the drug sensitivity measurements involved a 24 hour drug exposure. Drug accumulation was, therefore, determined over a five hour time course for clones that had been grown in drug free medium for 12 weeks. These results showed

clearly that differences between the clones were observed throughout the time course and that accumulation over the first hour is an accurate indication of cellular drug accumulation (Fig. 4).

Since alterations in drug sensitivity were closely related to changes in drug accumulation the results suggested that four of the clones were losing a functional drug efflux pump. This hypothesis was supported by a comparison of drug efflux rates for clones grown in drug free medium for 12 weeks (Fig. 5). Only a small amount of drug was lost from the 3 most sensitive clones (AD-2, AD-8 & AD-10) and there was no significant drug efflux from the drug sensitive cell line A2780 which does not express P-glycoprotein. Cells were loaded with drug in the absence of an energy source and in the presence of azide in order to inhibit the activity of the efflux pump. As a result cell line 2780AD and clone AD-3 accumulated as much drug as did cell line A2780. However, clone AD-3 and 2780AD lost over 80% of cellular associated drug in the first 5 minutes after transfer to drug free medium containing an energy source. Clone AD-16 was unusual in that at the end of the 1 hour loading point it had accumulated only about a third as much drug as the other cell lines studied. This suggests either that deprivation of an energy supply did not inhibit the efflux pump in this cell line or that drug accumulation is decreased by some other mechanism such as increased exocytosis (Sehested *et al*, 1987a & b). In contrast, if verapamil was added during the loading period drug accumulation was increased to that of the drug sensitive cell line A2780 (Fig. 6). This observation appears to contradict the suggestion that verapamil is maximally effective at 6-7 μM as a resistance

modulator. However it is not known what effect verapamil has on drug sensitivity when cells are exposed to drug and verapamil (50 μM) for 1 hour only. A 24 hour exposure to verapamil at 50 μM is toxic to the cells (J.A. Plumb personal communication).

As expected, P-glycoprotein was clearly detected by immunocytochemistry in cell line 2780AD and in clones AD-3 and AD-16 even after growth in the absence of drug for 12 weeks (Plate 5). In contrast, after 12 weeks only patchy staining was observed in the other four clones derived from 2780AD (Plate 4). Thus it appears that these four clones become more sensitive to daunorubicin due to a gradual loss of P-glycoprotein. Furthermore, exposure to doxorubicin (2 μM) for 24 hours induced P-glycoprotein expression and restored the resistance of the clones (Plate 4, Table IV) to greater than that observed after initial selection. The expressed P-glycoprotein was clearly functional since drug accumulation was markedly reduced (Fig. 7) and drug efflux increased (Fig. 8, Table V).

In Chapter 3 evidence was presented which indicated that there is an inconsistency between the effects of verapamil on drug sensitivity and the magnitude of the associated effects on drug accumulation. This inconsistency is further demonstrated here. When the drug sensitivity of the clonal cell lines grown for 2, 4, 6 and 8 weeks in drug free medium is plotted relative to the intracellular drug content a clear relationship exists (Fig. 9). There is an apparent discrepancy for the two clones isolated from cell line A2780 when cellular drug is expressed as pmoles/10^6 cells. However, if the cell volume is taken into account and drug content is expressed as a molar concentration the discrepancy

is accounted for (Fig. 9A cf 9B). In contrast, when the drug sensitivity of clones determined in the presence and absence of verapamil (6.6 μM) is plotted relative to intracellular drug content the relationship is lost (Fig. 10). Verapamil clearly increases drug sensitivity to a much greater extent than expected for the apparent increase in drug content, it should be noted that the drug accumulation measurements were made with a fixed extracellular drug concentration of 1 μM. Schuurhuis *et al* (1989) were able to demonstrate a relationship between drug sensitivity and drug accumulation with verapamil when drug accumulation studies were carried out at the respective ID_{50} drug concentration.

Cell line 2780AD is cross resistant to cisplatin. This resistance does not appear to be related to P-glycoprotein expression since the clones derived from 2780AD retained their resistance to cisplatin for 8 weeks and showed only a small increase in sensitivity by week 20 (Fig. 11).

It is not known why two of the clones retain P-glycoprotein expression in the absence of the selecting agent yet four clones do not. It has been suggested that in the absence of selection pressure resistant cells may "back-mutate" (Conter & Beck, 1984) due to a high reversion rate of the mutations associated with resistance (Courtenay & Robins, 1972). In the absence of a detailed cytogenetic analysis of the resistant cell line 2780AD it is not known if this is relevant to this study. However, the fact that a single exposure to doxorubicin can induce P-glycoprotein expression in the clones derived form 2780AD but not in A2780 suggests that this is unlikely. Cell line 2780AD has a modal chromosome number of 47 whereas that for cell line A2780 is 46 (I.F.D. Stephens, personal

communication). It is interesting to note that the four clones with unstable resistance have modal chromosome numbers of 46 whilst clone AD-16 has a modal chromosome number of 47 and for clone AD-3 there is range from 45 to 47. The significance of this observation is not known. It is possible that clones AD-3 and AD-16 would in time lose P-glycoprotein expression but that the time course for these two clones is much longer than for the other four clones studied. Clinical observations would suggest that it is the stable resistance reflected by these two clones that is relevant to the failure of chemotherapy. Relapse often occurs more than 3 months after chemotherapy and can occur after 2 to 3 years (Kaye, 1990). If the initial chemotherapy either induces or selects the resistant population then resistance is maintained over a period of months in the absence of the selecting agent.

In summary, cell line 2780AD contains subpopulations that differ in the stability of their multidrug resistance phenotype. The stability or otherwise of the resistance phenotype appears to be caused by differences in the stability of the induced expression of P-glycoprotein, the drug efflux pump.

Part of this work was presented at the British Association for Cancer Research meeting in Manchester in April 1991 (Luo *et al*, 1991).

CHAPTER 5

Effect of polyunsaturated fatty acids on the drug sensitivity of cell lines resistant to cisplatin or doxorubicin

Introduction

Several studies have suggested that changes in plasma membrane structure and lipid composition are associated with the development of drug resistance (Perterson *et al*, 1983; Rintoul & Center, 1984; Wheeler *et al*, 1982). The degree of structural order of plasma membrane lipids was found to be higher in doxorubicin resistant P388 murine leukaemia cells than in sensitive parental cells (Ramu *et al*, 1983a) and a correlation between cell membrane fluidity and resistance to doxorubicin was observed in a series of Sarcoma 180 sublines (Siegfried *et al*, 1983). Furthermore, the differences in the lipid composition of the cell membrane was also noted between a human ovarian cancer cell line and a cisplatin resistant variant (Mann *et al*, 1988), and this was shown to account, in part, for decreased cellular drug accumulation.

Biological membranes are composed of a lipid bilayer in which proteins are associated to form a mosaic (Stryer, 1988). Membrane lipids are mostly in the form of phospholipids, sphingolipids and cholesterol. The non-polar fatty acyl

side chains form a hydrophobic interior core relatively impermeable to the unregulated movement of substances into and out of the cell.

Membrane fluidity is determined by the degree of unsaturation of the fatty acid residues in the phospholipids and by the cholesterol content. Cholesterol decreases membrane fluidity by interfering with the orderly packing of the lipids' fatty acid side chains. In higher plants and animals, the predominant fatty acid residues are those of the C_{16} and C_{18} species palmitic, oleic, linoleic and stearic acids. Fatty acids with less than 14 or more than 20 carbon atoms are uncommon. Over one half of the fatty acid residues of plant and animal lipids are unsaturated, *i.e.*, contain double bonds, and are often polyunsaturated, *i.e.*, contain two or more double bonds. Saturated fatty acids are highly flexible molecules because there is relatively free rotation about each of their carbon-carbon bonds. Fatty acid double bonds almost always have the *cis* configuration and this puts a rigid 30° bend in the hydrocarbon chain that interferes with their efficient packing to fill space. The melting point of fatty acids decreases with their degree of unsaturation. Similarly, lipid fluidity increases with the degree of unsaturation of the component fatty acids. Obviously, the fluidity of biological membranes is one of their important physiological properties since it determines the ability of the associated protein to move and interact, *e.g.*, receptors, ATPases and ion channels (Spector & Burns, 1987). Fluidity will also influence membrane permeability and this is particularly relevant to many cytotoxic drugs which are thought to enter the cell by passive diffusion such as doxorubicin and cisplatin (Siegfried *et al*, 1985; Andrews & Howell, 1990).

In animals a variety of unsaturated fatty acids may be synthesised by combinations of elongation and desaturation reactions. However, since palmitic acid is the shortest available fatty acid in animals and since animals lack delta 12 desaturases, the delta 12 double bond of linoleic acid cannot be produced. Linoleic acid is thus an essential fatty acid (EFA) that must be supplied in the diet mainly from plants. Linoleic acid is metabolised to gamma-linolenic acid (GLA) and arachidonic acid, the precursor for prostaglandins synthesis (Fig. 1). Eicosapentaenoic acid (EPA) and docosahexaenoic acid are also essential fatty acids and are found in abundance in fish oils (Murro, 1983). These differ from EFAs derived from linoleic acid in the position of the terminal double bond relative to the methyl end of the fatty acid. This bond is in position 6 in linoleic acid and position 3 in alpha linolenic acid, and fatty acids in the two groups cannot be interconverted.

The composition of the cell membrane is, therefore, determined to a significant extent by the composition of the diet. A diet rich in polyunsaturated fatty acids (PUFAs) can increase the proportion of PUFAs in the cell membrane (Awad & Spector, 1976). Similarly, the proportion of PUFAs in the membrane of cells grown in culture can be increased by supplementing the culture medium with specific PUFAs (Guffy *et al*, 1984). Since cytotoxic drugs must enter the cell to exert their effects it is possible that alteration of the membrane composition will alter drug sensitivity. Furthermore, alteration of membrane fluidity could influence the activity of membrane components of signal transduction pathways.

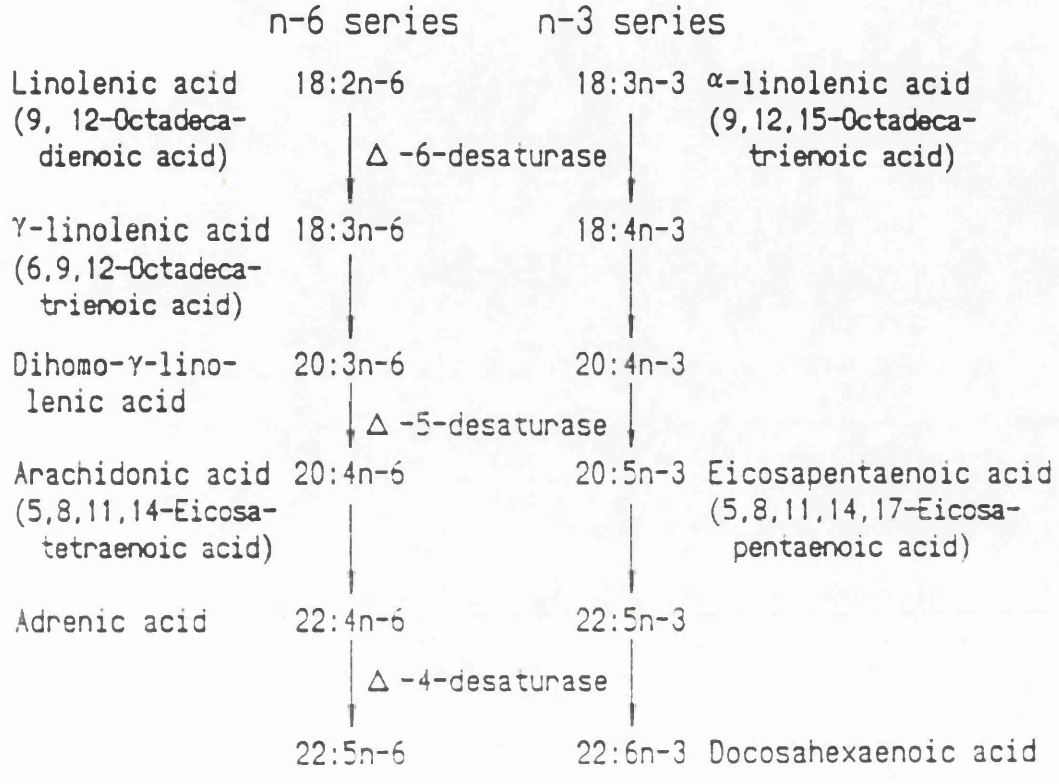


Figure 1 Relationships between two series of polyunsaturated fatty acids and between their metabolism by three desaturase enzymes.

This is particularly relevant to sensitivity to both doxorubicin and cisplatin since alterations of protein kinase C activity have been shown to alter sensitivity to both drugs (Basu *et al*, 1991; Fine *et al*, 1988; Isonishi *et al*, 1990).

Such an approach to modulation of drug sensitivity is not related to the multidrug resistance phenotype and could therefore apply to drug outwith the multidrug resistance phenotype such as cisplatin. Drug accumulation changes have been observed in cisplatin resistant cell lines (Bungo *et al*, 1990; Waud, 1987), but it is not known how important this is to the mechanisms of cisplatin resistance when compared with changes in recognition and removal of cisplatin-DNA adducts (Andrews & Howell, 1990). Verapamil does not modulate sensitivity to cisplatin but the dihydropyridine calcium channel blocker nifedipine does (Onoda *et al*, 1989). It is possible that nifedipine acts via calcium channels since cisplatin resistance has been shown to correlate with changes in calcium channels in the lipid and protein-lipid fraction of membranes (Vassilev *et al*, 1987). Changes in the lipid composition of the cell membrane could also influence these calcium channels and hence sensitise cells to cisplatin.

In the study described in this chapter both GLA and EPA have been used in an attempt to modulate sensitivity to cisplatin and doxorubicin. These two fatty acids were chosen partly through their availability and topicality (GLA is a major component of evening primrose oil), but also because they have been shown to have selective toxicities towards tumour cells in their own right (Beck *et al*, 1991).

Numerous studies have shown that certain polyunsaturated fatty acids have direct antineoplastic effects *in vitro*. A total of 30 different human malignant cell

lines derived from 19 different organs have been shown to be killed effectively by PUFAs under conditions that are not lethal to normal animal or human cells (Begin *et al*, 1985; 1986a & b; Booyens *et al*, 1984). Furthermore, tumour cells vary in their sensitivities to various PUFAs (Abou *et al*, 1988; Begin *et al*, 1986b; Begin & Ells, 1987; Karmali *et al*, 1985; Sircar *et al*, 1990). Of the PUFAs tested gamma linolenic acid, dihomogamma-linolenic acid, arachidonic acid and eicosapentaenoic acid are the most effective agents that show differential toxicity. More significantly, PUFAs have been shown to reduce the growth of human breast and melanoma and murine Ehrlich tumour xenografts in nude mice (Pritchard *et al*, 1989, Pritchard & Mansel, 1990; Zhu *et al*, 1989).

The initial study was designed to confirm that GLA and EPA are toxic to tumour cells and to determine a non-toxic concentration for use in the modulation studies. The ovarian cell line A2780 and the two drug resistant variants 2780AD and 2780CP were used for the majority of the work. In order to determine whether multidrug resistant cells were also resistant to the toxic effects of PUFAs the breast cell line MCF7 and the doxorubicin resistant subline MCF7/Adr were also used. Modulation studies were designed to determine firstly whether there is a direct interaction between PUFAs and cytotoxic drugs and secondly, whether incorporation of the PUFAs into the fatty acids of the cell alters the sensitivity of tumour cells to cytotoxic drugs. It has been shown that docosahexaenoic acid can sensitise a cisplatin resistant human small cell lung cancer cell line to cisplatin (Timmer-Bosscha *et al*, 1989). In this study cells were grown in the presence of the PUFAs for 72 hours with the PUFA replaced after 48 hours. This protocol

resulted in incorporation of the PUFA into cellular phospholipids and was therefore chosen for use in these experiments.

Since the PUFAs are cytotoxic agents it was essential in the analysis of the results to determine the nature of any interaction between the PUFAs and the cytotoxic drugs. Results were analysed by the isobologram method of Steel and Peckham (1979) which can discriminate between additive and supra-additive (synergistic) interactions. This method has been used previously in the department to demonstrate the supra-additive interaction between doxorubicin and resistance modulators such as verapamil and quinidine (Plumb *et al*, 1991).

Materials and Methods

Cell lines

The three ovarian cancer cell lines (A2780, 2780AD and 2780CP) and the two breast cancer cell lines (MCF7 and MCF7/Adr) were used. The cisplatin resistant variant (2780CP) of A2780 was obtained from Dr R. F. Ozols (Fox Chase Cancer Centre, Philadelphia, U.S.A.) and was maintained in drug free medium since resistance to cisplatin of this line was stable for up to 6 months.

The growth conditions for routine maintenance of these cell lines are described in Chapter 2.

Polyunsaturated fatty acids

The three polyunsaturated fatty acids (PUFAs), gamma-linolenic acid (GLA), eicosapentaenoic acid (EPA) and the lithium salt of gamma-linolenic acid (LiGLA), were provided by Scotia Pharmaceuticals (Scotia Pharmaceuticals Ltd., Surrey, U.K.). Both GLA and EPA were supplied as oils while LiGLA was a powder. The purity of GLA, EPA, and LiGLA was 99%, 90% and 99%, respectively.

The PUFAs were solubilised in absolute ethanol and sterilized by filtration (Millex-GS, Millipore Ltd., Watford, Herts, U.K.) at a stock concentration of 80

mg/ml. Aliquots were placed in amber-glass bottles, purged with nitrogen gas and the bottles were capped and stored at -70°C in the dark.

All the dilutions were made with complete culture medium immediately before use such that the final concentrations of ethanol did not exceed 0.2%.

Cisplatin (Sigma Chemical Company, Poole, U.K.) was prepared just before use at a concentration of 1 mM in saline and filter sterilised.

Cytotoxicity assay

Cytotoxicity was determined by the microtitration assay described in Chapter 2. The PUFAs were added either before or during drug exposure as specified. For exposure periods of up to 48 hours the PUFAs were not replaced. In experiments where cells were exposed to PUFAs for 72 hours prior to the cytotoxic drug, addition of PUFAs commenced 24 hours after plating out the cells and the PUFAs were replaced after 48 hours. Control wells were exposed to ethanol (0.2%). For all the experiments cells were allowed to recover in drug free medium for 3 days after removal of the cytotoxic drug.

Cells were exposed to the cytotoxic drug (doxorubicin or cisplatin) for 24 hours in the presence of PUFAs or following a 4 hour pre-exposure to PUFAs. For pre-exposure periods of 24 hours or more the drug exposure time was reduced to 4 hours in order to prevent cells reaching confluence in the wells during the course of the extended assay time.

Isobologram analysis

Since PUFAs are cytotoxic agents in their own right, the interaction between PUFAs and cytotoxic drugs was interpreted by isobologram analysis according to the method described by Steel and Peckham (1979).

The index of cytotoxicity used in this analysis was the ID_{50} concentration and for each drug this value was normalised to 1.0. Thus half the ID_{50} concentration is 0.5. For drugs with additive toxicity 50% cell kill can be obtained by a combination of half the ID_{50} concentration of drug A and half the ID_{50} concentration of drug B (Fig. 2A). The diagonal broken line joins the combinations of drugs A and B that will give 50% cell kill if their toxicities are additive. If cell kill is greater than 50% the points will lie to the left of this line and the interaction is classed as supra-additive. If cell kill is less than 50% the points will lie to the right of the line and the interaction is classed as antagonistic. The broken line represents additivity for ideal drugs with linear dose response curves. Where the dose response curve is non-linear a given increment of drug will have a different cell kill depending on the position on the dose response curve (Fig. 2B). As a result, an envelope of additivity is constructed that takes into account both extremes of the dose response curves of the two drugs.

Statistical analysis

All the data were subject to statistical analysis using non-paired Student's *t* test with Minitab Data Analysis Software (Release 7.1--Standard version, Minitab, Inc., 1989). Only statistically significant differences are identified.

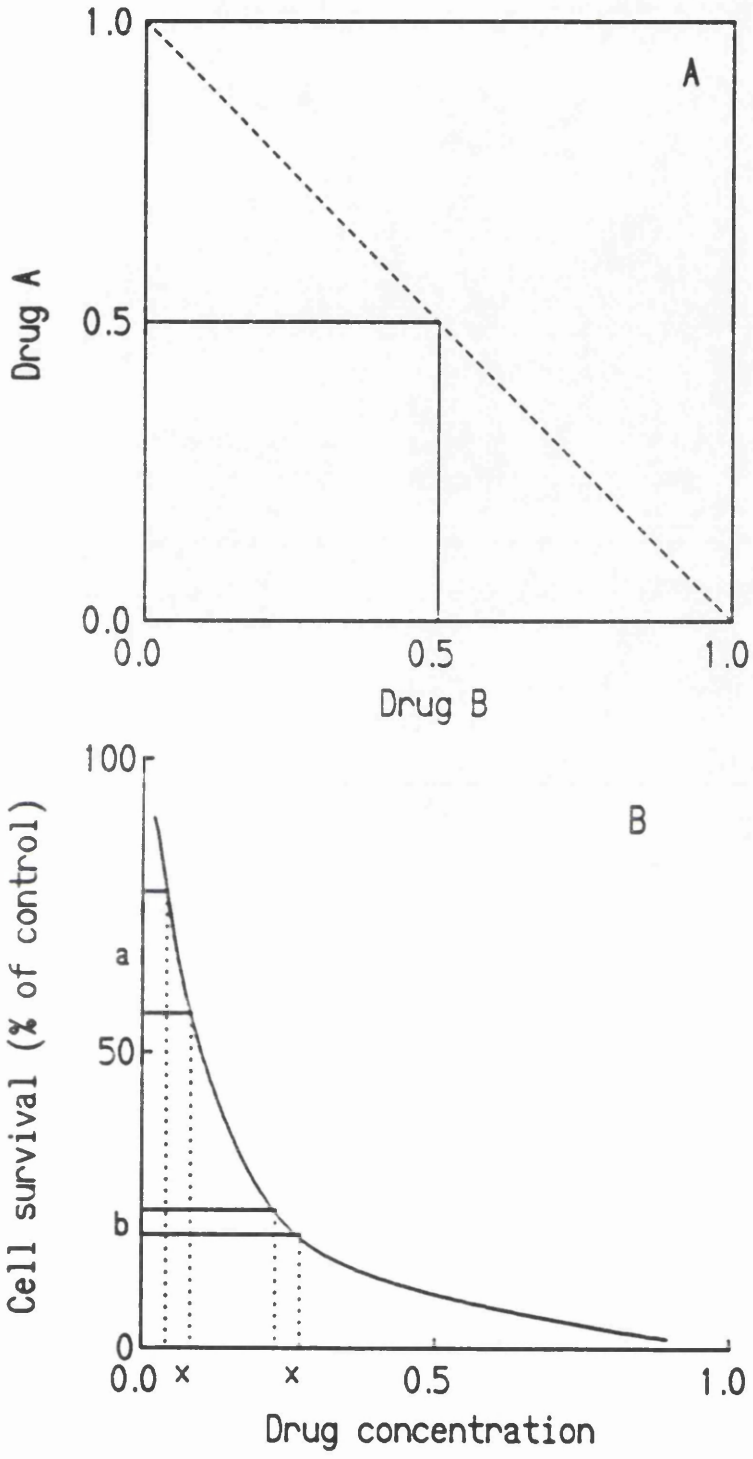


Figure 2 (A) Schematic isobologram of the interaction between two drugs A and B. The ID_{50} concentrations are normalised to 1.0. The diagonal broken line joints the combinations of drug A and drug B that will give 50% cell kill if their toxicities are additive. (B) For drugs with non-linear dose response curves the percentage of cell kill for a given increment of drug (x) will vary depending upon the position on the curve (a cf b). As a result, an envelope of additivity is constructed that takes into account both ends of the dose response curve.

Results

Cytotoxicity of polyunsaturated fatty acids

The sensitivity of three ovarian cell lines to PUFAs is shown in Table I. Also shown is the toxicity of doxorubicin and cisplatin for comparison.

The doxorubicin resistant cell line 2780AD was approximately 800 fold resistant to doxorubicin and the cisplatin resistant cell line 2780CP was about 19 fold resistant to CDDP when compared with the parental cell line A2780. Cell line 2780AD was about 11 fold cross resistant to CDDP but 2780CP was not cross resistant to doxorubicin.

Both 2780AD and 2780CP were cross resistant to all three PUFAs. However, the resistance factors were only 2.5 to 4.5 fold. The PUFAs were equally cytotoxic with a maximum difference in ID_{50} concentration of 2 fold in cell line A2780 (Table I). Steep dose response curves were obtained for all three PUFAs (Fig. 3A, 3B & 3C).

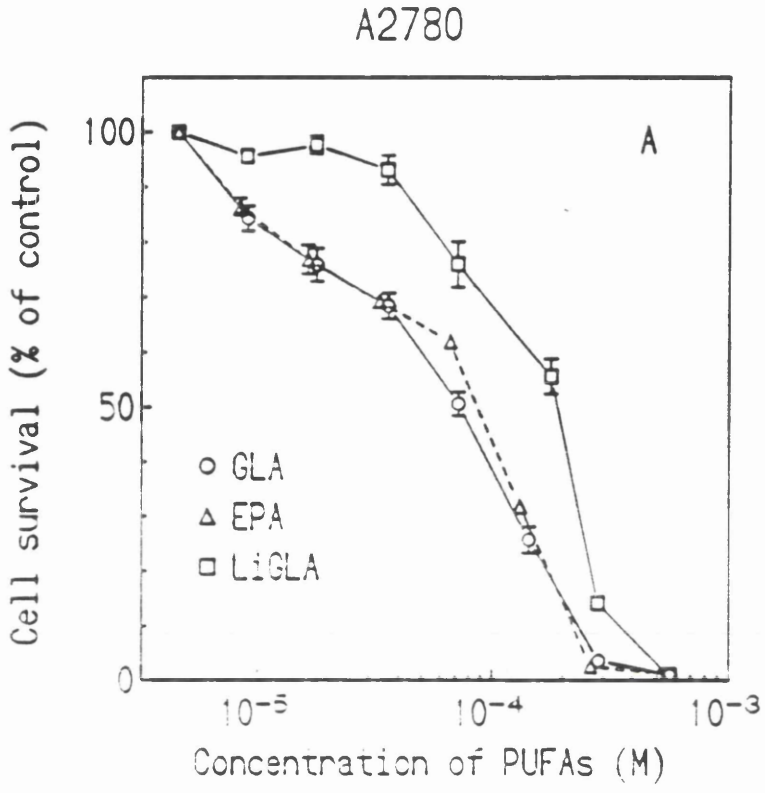
In contrast, the breast cell line MCF7/Adr was about 560 fold resistant to doxorubicin, but was more sensitive ($P < 0.05$) to all three PUFAs than the parental cell line MCF7 (Table II). Again the three PUFAs were equally cytotoxic within a cell line (Fig. 4A & 4B).

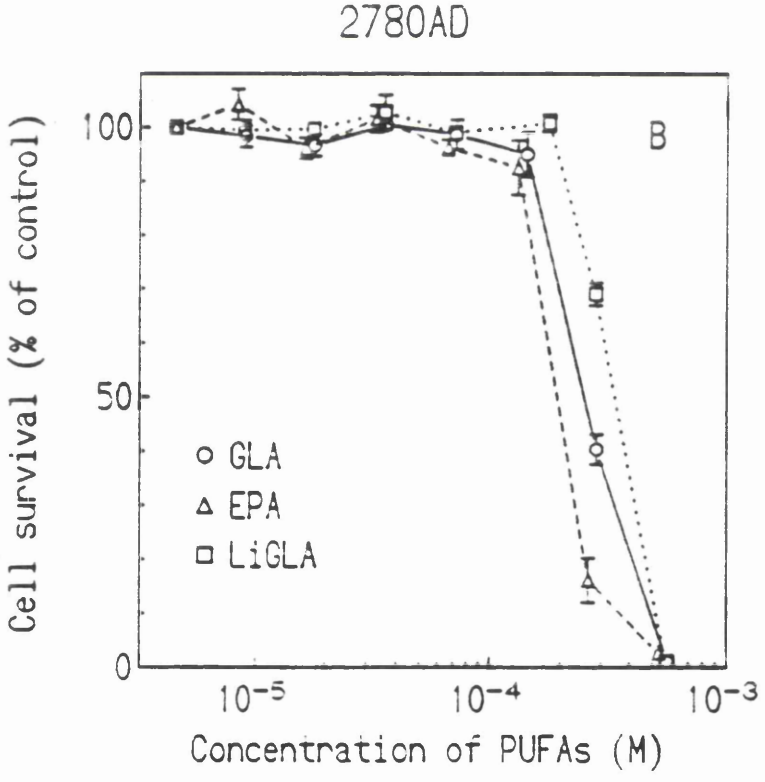
ID_{50} (μM)			
	A2780	2780AD	2780CP
GLA	72.9 ± 4.3	267.6 ± 2.9***	327.9 ± 2.5***
EPA	94.9 ± 2.0	208.3 ± 11.6**	326.3 ± 9.6**
LiGLA	147.8 ± 9.9	301.4 ± 2.1**	229.2 ± 8.2**
DOX	0.003 ± 0.0003	2.350 ± 0.100	0.003 ± 0.0003
CDDP	0.266 ± 0.017	3.080 ± 0.208	5.080 ± 0.950

Table I Sensitivities of human ovarian carcinoma cell line A2780 and its doxorubicin (2780AD) and cisplatin (2780CP) resistant variants to polyunsaturated fatty acids, doxorubicin and cisplatin determined using the MTT assay. Cells were exposed to the individual agents for 24 hours. Values are the mean ± standard error of triplicates and are representative of at least 2 determinations showing showing similar results. Asterisks represent the significant difference from control A2780 in statistics (Student's *t* test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The abbreviations used are: GLA, γ-linolenic acid; EPA, eicosapentaenoic acid; LiGLA, lithium salt of γ-linolenic acid; DOX, doxorubicin; CDDP, cisplatin.

ID_{50} (μM)		
	MCF7	MCF7/Adr
GLA	265.8 ± 30.5	156.3 ± 6.3*
EPA	275.4 ± 15.9	191.1 ± 5.3*
LiGLA	263.7 ± 12.4	200.8 ± 11.0*
DOX	0.08 ± 0.01	44.9 ± 1.1

Table II Sensitivities of human breast cancer cell line (MCF7) and its doxorubicin resistant derivative (MCF7/Adr) to polyunsaturated fatty acids and doxorubicin determined using the MTT assay. Cells were exposed to the individual agents for 24 hours. Values are the mean ± standard error of triplicates and are representative of 2 or more observations showing similar results. See Table I for statistical difference and abbreviations.


A2780
A
100
50
0
Cell survival (% of control)
GLA
EPA
LiGLA
10^{-5}
10^{-4}
10^{-3}
Concentration of PUFAs (M)


2780AD
B
100
50
0
Cell survival (% of control)
GLA
EPA
LiGLA
10^{-5}
10^{-4}
10^{-3}
Concentration of PUFAs (M)

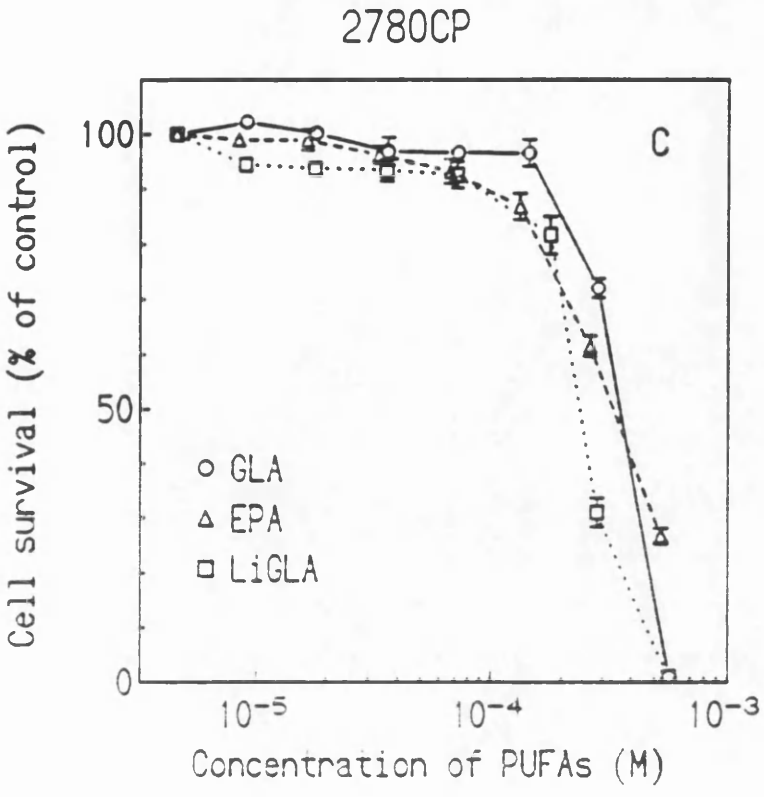


Figure 3 Dose response curves for polyunsaturated fatty acids in cell lines A2780 (A), 2780AD (B) and 2780CP (C). Cells were exposed to γ-linolenic acid (GLA), eicosapentaenoic acid (EPA) and the lithium salt of GLA (LiGLA) for 24 hours, grown in drug free medium for 3 days and cell number was estimated by MTT reduction. Cell survival (absorbance) is expressed as a percentage of the absorbance of control untreated wells. Points are the mean ± standard error of triplicate plates.

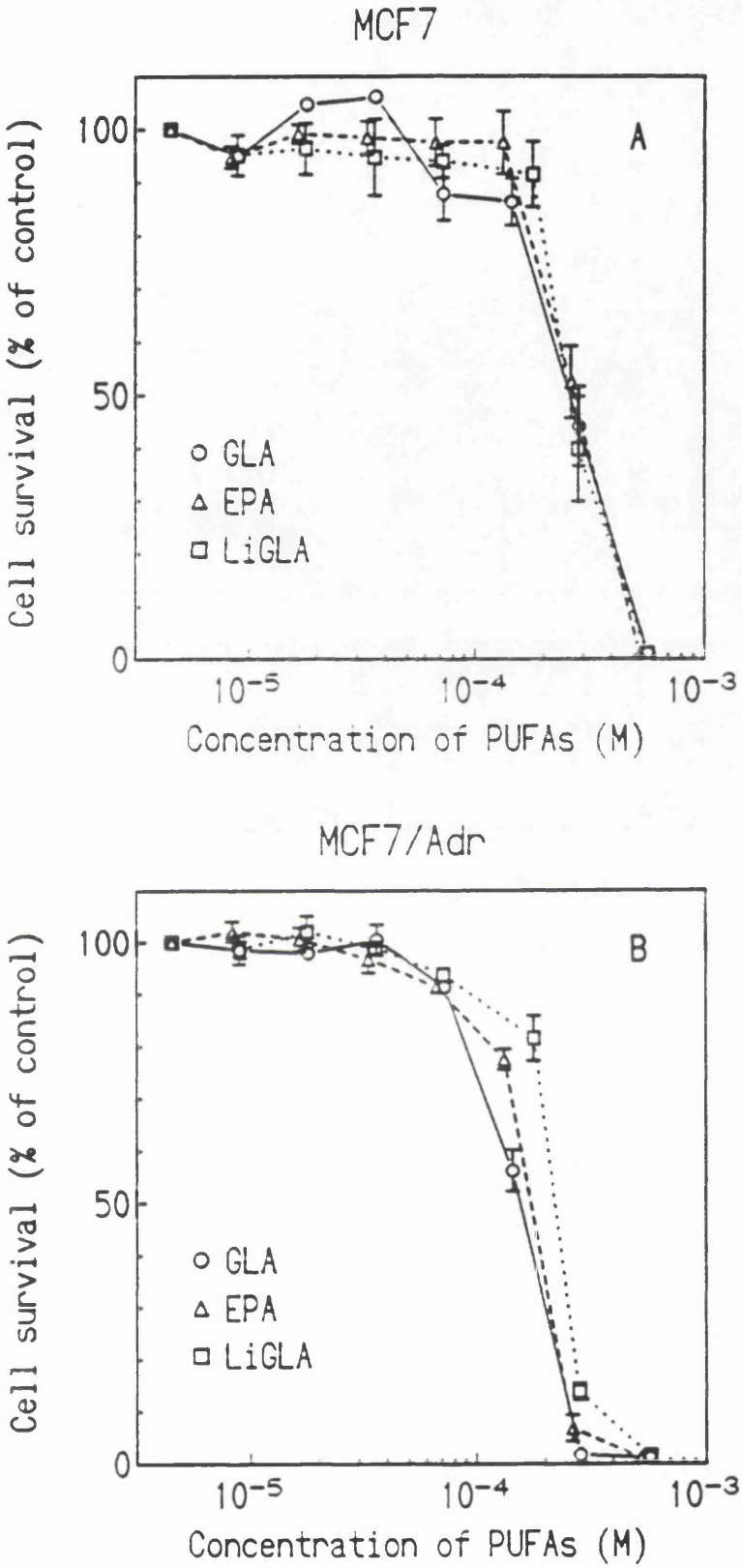


Figure 4 Dose response curves for polyunsaturated fatty acids in cell lines MCF7 (A) and MCF7/Adr (B). See Figure 3 legend for details.

Sensitivity of ovarian cell lines to cisplatin and doxorubicin when exposed in the presence of PUFAs

Since A2780 was more sensitive to PUFAs, lower concentrations were applied to this cell line. The concentrations of PUFAs used for the two resistant cell lines in all studies were within a range not exceeding 40 μg/ml (GLA, 143.7 μM; EPA, 132.2 μM; LiGLA, 142.1 μM) at which no significant inhibition of cell growth was observed after a 24 hour exposure (Fig. 3A & 3B).

The lithium salt of GLA had no effect on the sensitivity of all 3 cell lines to cisplatin (Table III). EPA had no effect on the platinum sensitivity of A2780 and 2780AD but increased slightly the sensitivity of 2780CP at all concentrations used and this increase was just significant ($P < 0.05$). The lowest concentration of GLA used (A2780, 2.5 μg/ml; 2780AD and 2780CP, 10 μg/ml) had no effect on platinum sensitivity. However, higher concentrations produced a significant decrease in the ID_{50} in A2780 ($P < 0.01$) and 2780AD (6 fold, $P < 0.001$). For 2780CP the increase in sensitivity was only just significant at the highest concentration of GLA used (40 μg/ml, $P < 0.05$). It should be noted that all ID_{50} are calculated using a control absorbance value obtained from cells that were not exposed either to cisplatin or to PUFAs.

A 24 hour co-exposure to PUFAs and doxorubicin had no effect on the sensitivity of any of the 3 cell lines to doxorubicin (Table IV).

	ID_{50} (μM)			
	A2780		2780AD	2780CP
CDDP	0.49 ± 0.08		5.03 ± 0.30	10.52 ± 0.57
+GLA(9.0)	0.36 ± 0.05	(35.9)	4.67 ± 0.28	12.20 ± 0.75
+GLA(18.0)	0.13 ± 0.04**	(71.8)	3.02 ± 0.26**	9.37 ± 0.49
+GLA(35.9)	0.06 ± 0.01**	(143.7)	0.82 ± 0.15***	6.13 ± 0.81*
+EPA(8.3)	0.46 ± 0.06	(33.0)	6.20 ± 0.50	8.33 ± 0.46*
+EPA(16.5)	0.60 ± 0.05	(66.1)	3.90 ± 0.73	8.00 ± 0.30*
+EPA(33.0)	0.40 ± 0.02	(132.2)	ND	8.10 ± 0.97*
+LiGLA(8.9)	0.60 ± 0.07	(35.5)	3.40 ± 0.48	11.53 ± 0.83
+LiGLA(17.8)	0.29 ± 0.02	(71.1)	3.62 ± 0.49	10.30 ± 0.27
+LiGLA(35.5)	0.27 ± 0.03	(142.1)	5.15 ± 0.69	11.90 ± 0.50

Table III Sensitivities of wild type (A2780), doxorubicin (2780AD) and cisplatin (2780CP) resistant human ovarian carcinoma cell lines to cisplatin measured using the MTT assay. Cells were exposed to drug for 24 hours in the presence or absence of polyunsaturated fatty acids (PUFAs). Values are the mean ± standard error of triplicates and are representative of 2 observations showing similar results. The figures in parentheses represent the concentrations of individual PUFAs (μM). Asterisks represent statistically significant difference from control, *i.e.*, cisplatin alone (see Table I). ND, not done.

	ID_{50} (μM)			
	A2780		2780AD	2780CP
DOX	0.007 ± 0.0003		4.62 ± 0.28	0.014 ± 0.0004
+GLA(9.0 μM)	0.008 ± 0.0005	(143.7 μM)	4.92 ± 0.37	0.012 ± 0.001
+EPA(8.3 μM)	0.006 ± 0.0003	(132.2 μM)	3.83 ± 0.15	0.008 ± 0.002
+LiGLA(8.9 μM)	0.007 ± 0.0008	(142.1 μM)	4.87 ± 0.22	0.011 ± 0.003

Table IV Sensitivities of wild type (A2780), doxorubicin (2780AD) and cisplatin (2780CP) resistant human ovarian cancer cell lines to doxorubicin (DOX) in the presence or absence of polyunsaturated fatty acids (PUFAs) assessed using the MTT assay. Values are the mean ± standard error of triplicates and are representative of 2 determinations showing similar results. Figures in parentheses represent the concentrations of PUFAs used. See Table III legend for details.

Effect of pre-exposure of cells to PUFAs on the cytotoxicity of cisplatin and doxorubicin

A four hour pre-exposure of the 3 cell lines to PUFAs had little effect on sensitivity to either doxorubicin or cisplatin (Table V). A small increase (1.4 fold) in the sensitivity of 2780AD to doxorubicin when exposed to EPA (132.2 μM, 40 μg/ml) was only just significant ($P < 0.05$). Similarly, all three PUFAs produced a small increase (1.2-1.3 fold) in the sensitivity of 2780CP to cisplatin and this increase was only just significant ($P < 0.05$).

The effects of a 24 hour and 48 hour pre-exposure to PUFAs on the sensitivity of the 3 cell lines to doxorubicin and cisplatin are shown in Tables VI and VII respectively. The most significant effects were seen in cell line 2780CP. A 48 hour pre-treatment with GLA (40 μg/ml, 143.7 μM), EPA (40 μg/ml, 132.2 μM), and LiGLA (40 μg/ml, 142.1 μM) sensitised 2780CP to cisplatin by 8, 10 and 11 fold respectively ($P < 0.001$, Table VII). This effect was also apparent, but less marked after exposure to PUFAs for 24 hours (Table VI). A 48 hour pre-treatment with GLA, EPA, and LiGLA sensitised 2780CP to doxorubicin by 3.5, 6 and 4 fold respectively ($P < 0.01$, Table VI).

Pre-exposure to PUFAs for up to 48 hours had little effect on the sensitivities of A2780 and 2780AD to cisplatin or on the sensitivity of A2780 to doxorubicin (Tables VI & VII). The sensitivity of 2780AD to doxorubicin was significantly increased by pre-exposure to GLA (2.5 fold, $P < 0.01$), EPA (22 fold, $P < 0.001$) and LiGLA (2.6 fold, $P < 0.01$) for 48 hours (Table VII). This trend

was also apparent after 24 hour pre-exposure but only significant for EPA (9 fold, $P < 0.01$, Table VI).

Studies of prolonged pre-treatment of the cells with PUFAswereconfined to the most significant interactions observed in the shorter pre-exposure periods. Furthermore, the concentration of the PUFA was reduced in an attempt to avoid excessive toxicity of the PUFA itself. Pre-exposure to GLA for 72 hours sensitised 2780CP to cisplatin by 5.5 fold (GLA, 35.9 μM; $P < 0.01$) and 315 fold (GLA, 71.8 μM; $P < 0.001$; Table VIII). Pre-exposure to EPA for 72 hours sensitised 2780AD to doxorubicin by 6.5 fold (EPA, 33 μM; $P < 0.01$) and 17 fold (EPA, 66.1 μM; $P < 0.001$; Table VIII).

	ID_{50} (μM)			
	A2780		2780AD	2780CP
DOX	0.003 ± 0.0003		2.350 ± 0.100	0.003 ± 0.0003
+GLA(9.0)	0.002 ± 0.0006	(143.7)	2.180 ± 0.115	0.003 ± 0.0002
+EPA(8.3)	0.003 ± 0.0004	(132.2)	1.710 ± 0.110*	0.003 ± 0.0002
+LiGLA(8.9)	0.003 ± 0.0004	(142.1)	2.320 ± 0.087	0.004 ± 0.0003
CDDP	0.187 ± 0.005		1.367 ± 0.027	5.180 ± 0.087
+GLA(9.0)	0.163 ± 0.012	(143.7)	1.390 ± 0.049	4.450 ± 0.115*
+EPA(8.3)	0.245 ± 0.013	(132.2)	1.090 ± 0.087	3.860 ± 0.104*
+LiGLA(8.9)	0.229 ± 0.010	(142.1)	1.570 ± 0.081	4.380 ± 0.162*

Table V Sensitivities of wild type (A2780), doxorubicin (2780AD) and cisplatin (2780CP) resistant human ovarian carcinoma cell lines to doxorubicin (DOX) or cisplatin (CDDP) measured using the MTT assay. Cells were exposed to PUFAs for 4 hours and then to DOX or CDDP alone for 24 hours. Values are the mean ± standard error of triplicates and are representative of 2 observations showing similar results. Figures in parentheses represent concentrations (μM) of PUFAs used for A2780 and the two derivatives, respectively. Asterisks represent statistically significant difference (see Table III legend for details).

	ID_{50} (μM)			
	A2780		2780AD	2780CP
DOX	0.050 ± 0.006		15.70 ± 0.60	0.156 ± 0.03
+GLA(9.0)	0.032 ± 0.001	(143.7)	9.77 ± 1.38	0.045 ± 0.005
+EPA(8.3)	0.038 ± 0.002	(132.2)	0.17 ± 0.01***	0.070 ± 0.011
+LiGLA(8.9)	0.029 ± 0.005	(142.1)	13.93 ± 0.97	0.154 ± 0.032
CDDP	1.73 ± 0.13		18.00 ± 1.79	50.83 ± 1.42
+GLA(9.0)	1.19 ± 0.11*	(143.7)	25.90 ± 5.87	9.13 ± 0.54**
+EPA(8.3)	1.15 ± 0.07*	(132.2)	13.70 ± 0.56	35.13 ± 2.02*
+LiGLA(8.9)	1.14 ± 0.12*	(142.1)	33.67 ± 2.59*	19.03 ± 2.66**

Table VI Sensitivities of wild type (A2780), doxorubicin (2780AD) and cisplatin (2780CP) resistant human ovarian carcinoma cell lines to doxorubicin (DOX) or cisplatin (CDDP) measured using the MTT assay. Cells were exposed to individual PUFAs for 24 hours and then to DOX or CDDP alone for 4 hours. Values are the mean ± standard error of triplicates and are representative of 2 observations showing similar results. Figures in parentheses represent the final concentrations (μM) of PUFAs used for A2780 and the two derivatives, respectively. Asterisks represent statistically significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

	ID_{50} (μM)			
	A2780		2780AD	2780CP
DOX	0.022 ± 0.001		8.87 ± 0.52	0.080 ± 0.006
+GLA(9.0)	0.016 ± 0.003	(143.7)	3.48 ± 0.16**	0.023 ± 0.004**
+EPA(8.3)	0.018 ± 0.004	(132.2)	0.39 ± 0.11***	0.013 ± 0.002**
+LiGLA(8.9)	0.020 ± 0.002	(142.1)	3.42 ± 0.78**	0.020 ± 0.003**
CDDP	0.79 ± 0.01		13.20 ± 0.46	26.77 ± 0.82
+GLA(9.0)	0.50 ± 0.06*	(143.7)	19.07 ± 1.68	3.32 ± 1.05***
+EPA(8.3)	0.65 ± 0.14	(132.2)	8.70 ± 1.72	2.68 ± 0.59***
+LiGLA(8.9)	0.41 ± 0.03**	(142.1)	14.67 ± 1.89	2.43 ± 0.34***

Table VII Sensitivities of wild type (A2780), doxorubicin (2780AD) and cisplatin (2780CP) resistant human ovarian carcinoma cell lines to DOX or CDDP measured using the MTT assay. Cells were exposed to individual PUFAs for 48 hours and then to DOX or CDDP alone for 4 hours. Values are the mean ± standard error of triplicates and are representative of 2 observations showing similar results. Figures in parentheses represent the final concentrations (μM) of PUFAs used for A2780 and the two derivatives, respectively. Asterisks represent statistically significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

ID_{50} (μM)			
2780AD		2780CP	
+EPA	Doxorubicin	+GLA	Cisplatin
0	16.40 ± 2.16	0	56.67 ± 7.69
33.0 μM	2.53 ± 0.44**	35.9 μM	10.33 ± 4.05*
66.1 μM	0.95 ± 0.15***	71.8 μM	0.18 ± 0.02***

Table VIII Cellular sensitivities of doxorubicin resistant (2780AD) and cisplatin resistant (2780CP) cell lines to doxorubicin and cisplatin following a 72 hour pre-exposure of cells to either eicosapentaenoic acid (EPA) or γ-linolenic acid (GLA). EPA or GLA was added to the cells on day 1 and day 3 after plating, at the final concentrations indicated. The cells were subsequently exposed to either doxorubicin or cisplatin for 4 hours in the absence of PUFAs. Values are the mean ± standard error of triplicates and are representative of 2 determinations using the MTT assay. Asterisks represent significant differences (Students' *t* test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Effect of PUFA pre-exposure alone on cell survival

Although non-toxic concentrations of the PUFAs were chosen from the original dose response curves (Fig. 3A, 3B & 3C) it was apparent in the pre-exposure experiments that these concentrations were toxic and that the toxicity increased with prolonged exposure time. This is shown in Figures 5, 6 and 7. In Figure 5A the dose response curves for 2780CP pre-exposed to PUFAs for 24 hours are plotted with cell survival expressed as a percentage of control where the control contained the relevant concentrations of the PUFA. Figure 5B shows the dose response curves plotted with cell survival expressed as absorbance at 570 nm. It is clear that even at very low cisplatin concentrations significant cytotoxicity is observed and this is due to the PUFA alone. This toxicity is much more marked after a 48 hour (Fig. 6A & 6B) and 72 hour (Fig. 7A & 7B) pre-exposure to the PUFAs.

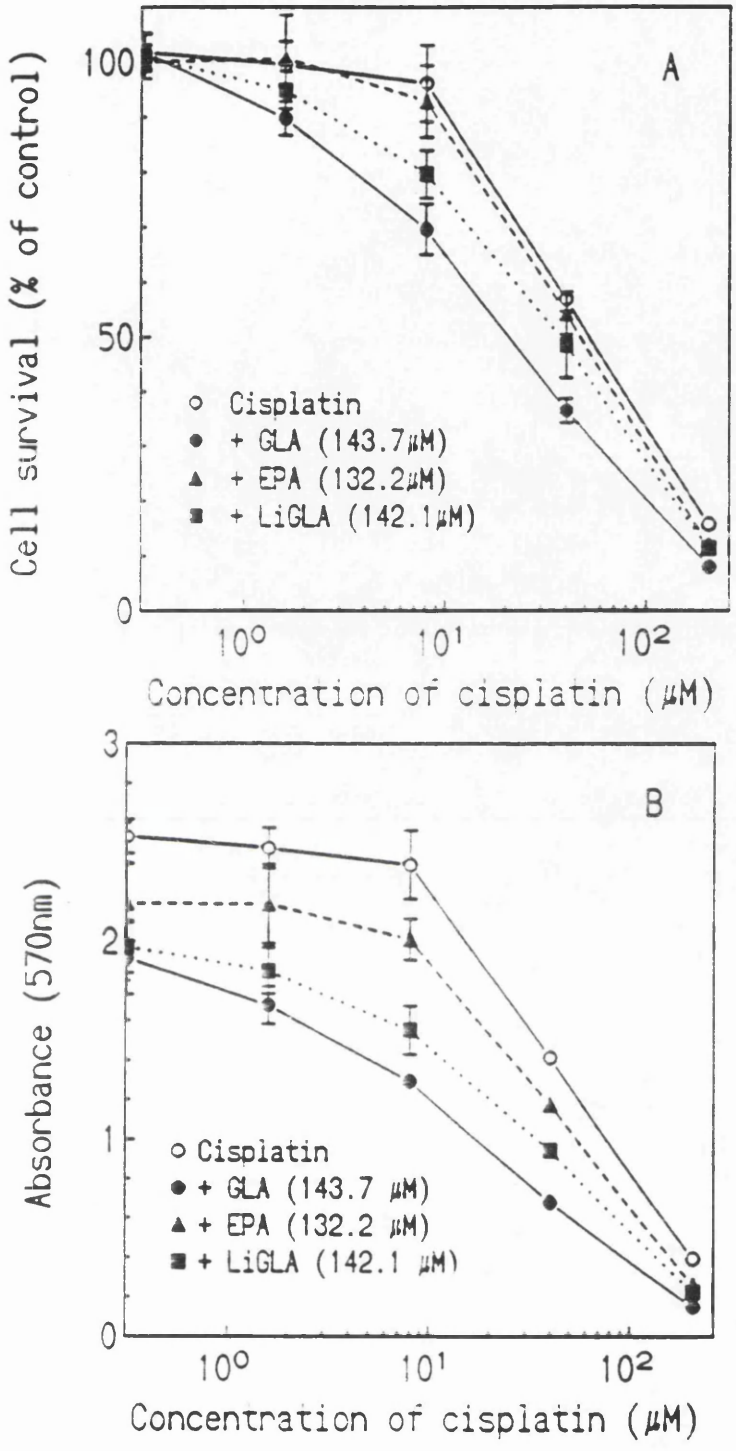


Figure 5 Dose response curves for cell line 2780CP. Cells were pre-exposed to GLA, EPA or LiGLA for 24 hours before exposure to cisplatin for 24 hours. Cell survival was measured 3 days later by MTT reduction. Points are the mean ± standard error of triplicate plates. In (A) cell survival is expressed as a percentage of the absorbance of control wells. For cisplatin alone the controls were not exposed to cytotoxic drug. For experiments where cells were pre-exposed to PUFAs the controls were exposed to the PUFAs alone. In (B) cell survival is expressed as the absorbance per well.

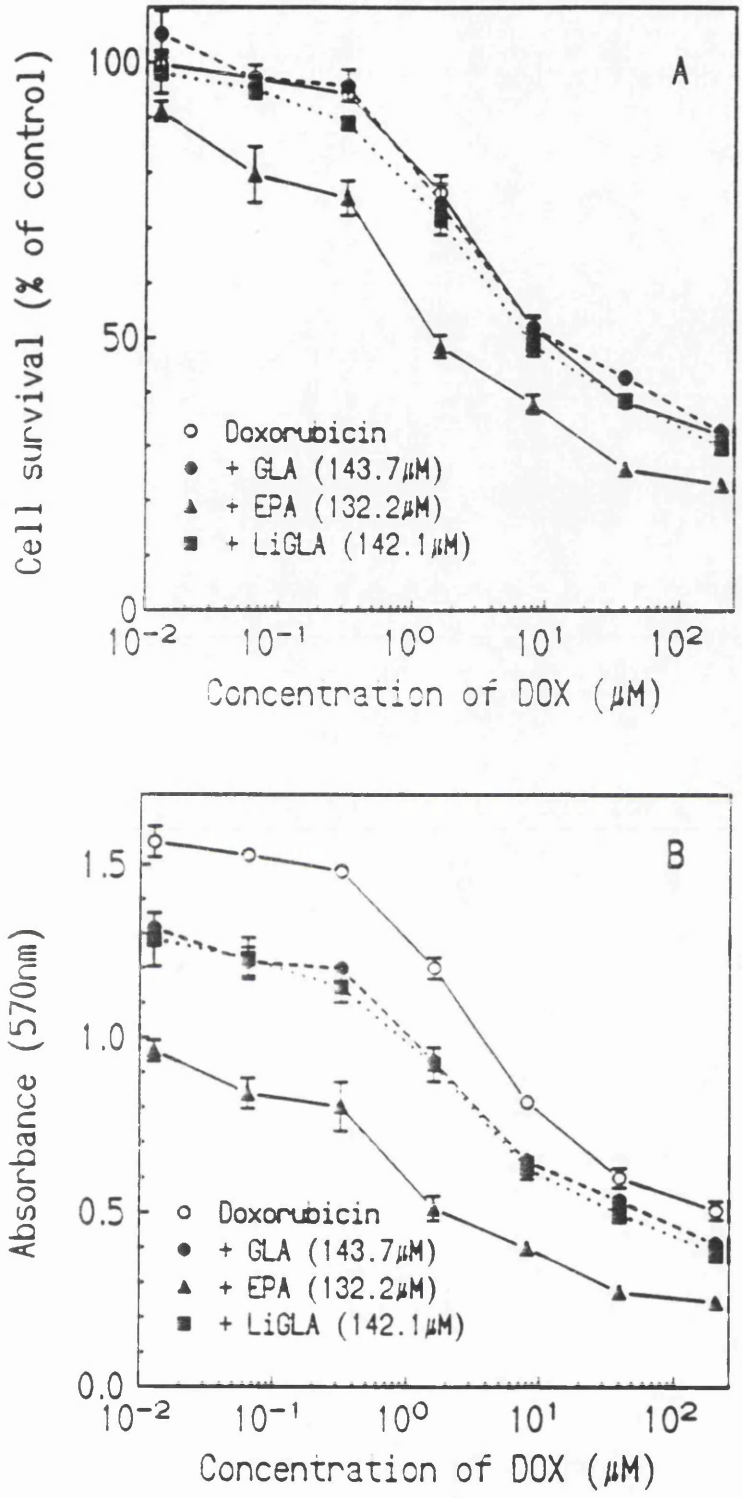


Figure 6 Dose response curves for cell line 2780AD. Cells were exposed to polyunsaturated fatty acids for 48 hours before exposure to doxorubicin for 4 hours. See Figure 5 legend for details.

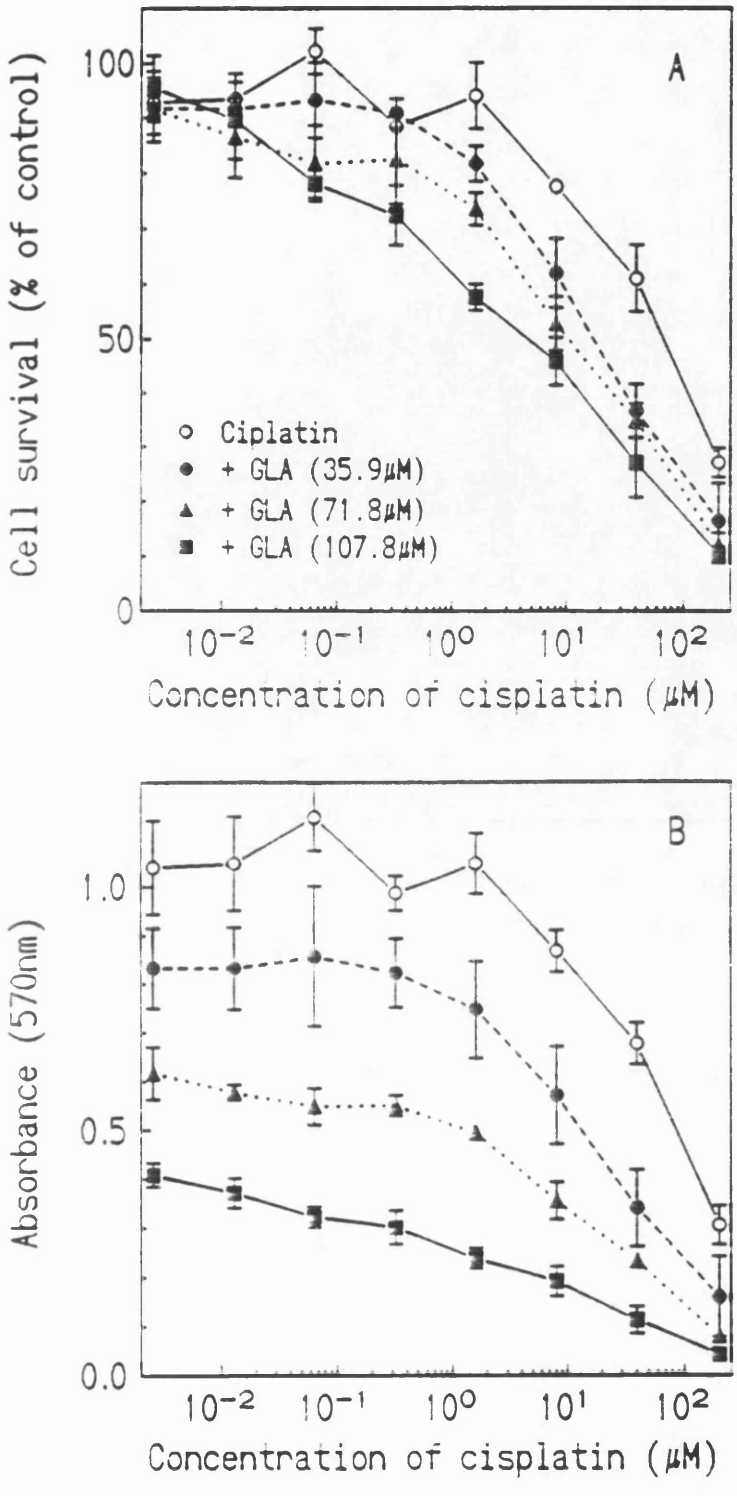


Figure 7 Dose response curves for cell line 2780CP. Cells were exposed to GLA for 72 hours before exposure to cisplatin for 4 hours. See Figure 5 legend for details.

Isobologram analysis

The interaction between PUFAs and the cytotoxic drugs was interpreted by isobologram analysis. Figure 8A shows the concentrations of cisplatin and GLA which when combined for 24 hours produce a 50% reduction in cell survival. The dotted line delineates the envelope of additivity. It is clear that the majority of points lie within the envelope indicative of an additive interaction. Figure 8B shows the isobologram for the interaction between GLA and cisplatin when cells were exposed to GLA for 24 hours before exposure to cisplatin for 24 hours alone. Again the interaction appears to be additive. Isobolograms could not be constructed for the other interactions studied since only one PUFA concentration was used.

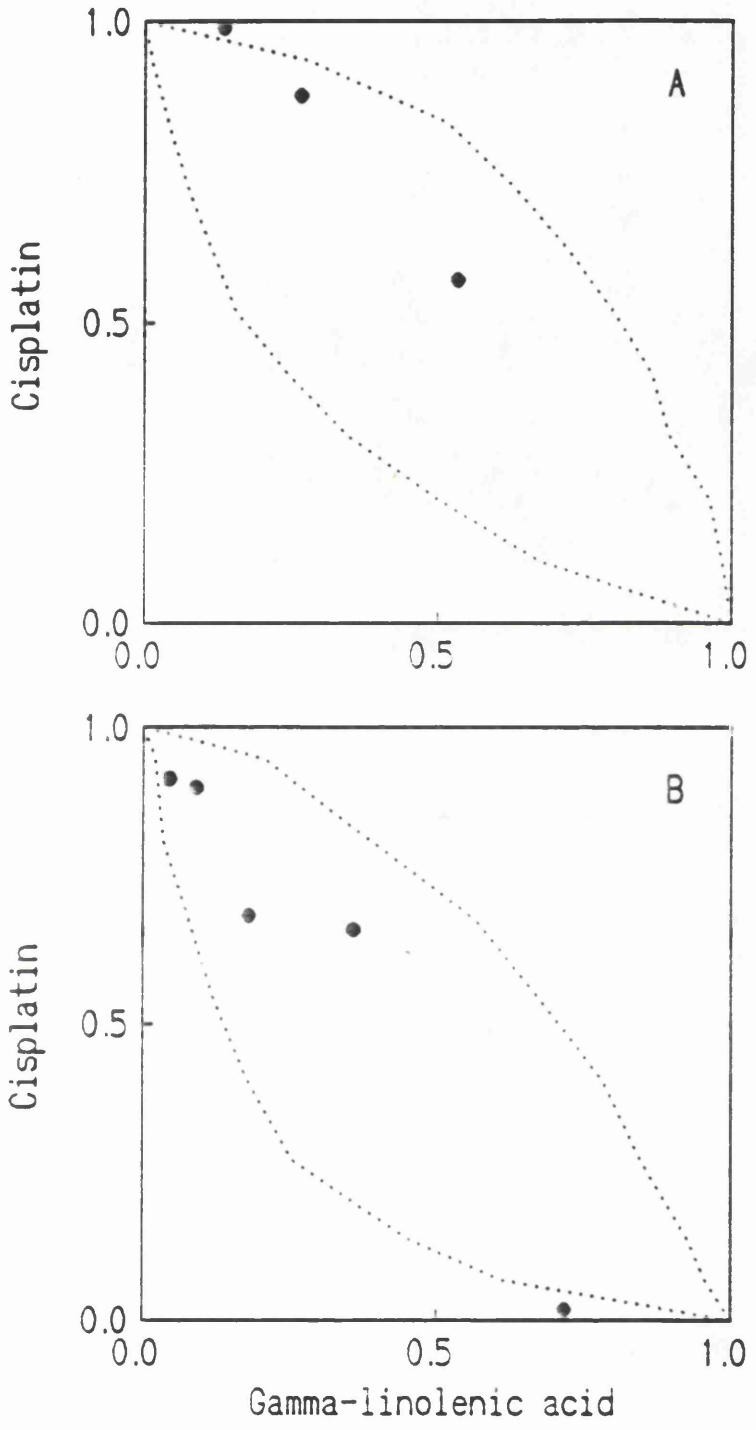


Figure 8 Isobolograms showing the interaction between cisplatin and GLA in cell line 2780CP. The dotted lines show the envelope of additivity. (A) cells were exposed to cisplatin for 24 hours in the presence of GLA. (B) cells were exposed to GLA for 24 hours before exposure to cisplatin for 24 hours.

Discussion

These results show clearly that the polyunsaturated fatty acids (PUFAs), *i.e.*, gamma-linolenic acid and eicosapentaenoic acid, are cytotoxic to tumour cell lines. Surprisingly, the doxorubicin and cisplatin resistant ovarian cell lines were cross resistant to the PUFAs although the resistance factors were much smaller than those for the selecting agent. Cross resistance to PUFAs was not a general feature of MDR cell lines since the doxorubicin resistant breast cell line showed a tendency towards greater sensitivity to PUFAs than the parental cell line. PUFAs were shown in some cases to sensitise the cells to cytotoxic drugs. However, when the drug interactions were analysed by the isobologram method the interactions were clearly additive and not supra-additive or synergistic.

The cisplatin and doxorubicin resistant sublines of the ovarian cell line A2780 showed steep dose response curves to both GLA and EPA with no apparent toxicity below 100 μM (Fig. 3B & 3C). Although A2780 was only 2.5 to 4.5 fold more sensitive to the PUFAs the dose response curve was less steep and toxicity was observed at concentrations of only 10 μM. 2780CP is 19 fold resistant to cisplatin and 2780AD is 800 fold resistant to doxorubicin (Table I). Although both resistant lines are cross resistant to the PUFAs the resistance factors are low compared to those of the cytotoxic drug against which resistance was developed. In order to confirm whether cross resistance to PUFAs is a feature common to all multidrug resistant cell lines the breast cell line MCF7 and its doxorubicin resistant subline MCF7/Adr was included in the study. The

doxorubicin resistant variant MCF7/Adr was shown to be slightly more sensitive ($P < 0.05$) than its parental cell line MCF7 to all three PUFAs (Table II). This suggests that the resistance observed for the ovarian cell lines is a specific feature of the lines and not a general phenomenon. The difference may relate to the fact that the parental cell line A2780 is highly sensitive to a number of cytotoxic drugs (J.A. Plumb, personal communication) and could thus be hypersensitive to the effect of the PUFAs.

These results are consistent with previous studies which have shown that PUFAs have direct antineoplastic activities and that tumour cell lines differ in their sensitivities to PUFAs (Abou *et al*, 1988; Karmali *et al*, 1985). The mechanisms by which PUFAs kill tumour cells is unknown but there is evidence that toxicity may be related to lipid peroxidation and production of superoxide radicals (Begin *et al*, 1986a, 1988; Das *et al*, 1987; Das, 1990). This is supported by the observation that the toxicity of GLA in human neuroblastoma cells *in vitro* is inhibited by antioxidants (Fujiwara *et al*, 1984). The most cytotoxic PUFAs appear to be those with 3, 4 or 5 double bonds and these include EPA and GLA. Docosahexaenoic acid which has 6 double bonds was much less toxic to cells (Begin *et al*, 1986a). Furthermore, EPA and GLA are more potent in terms of lipid peroxidation and superoxide radical formation than docosahexaenoic acid. Part of the selective toxicity of PUFAs to tumour cells might be explained by altered activities of enzymes that metabolise PUFAs. Both delta 6- and 5-fatty acyl-CoA desaturases have been shown to be impaired or absent in some tumour cell lines (Howards & Howard, 1974; Maeda *et al*, 1978).

Initial studies of the interaction between PUFAs and cisplatin and doxorubicin were designed to determine whether there is an acute effect of PUFAs on cellular drug sensitivity. The ovarian cell lines were used for these studies since both doxorubicin and cisplatin resistant variants were available. Co-incubation of cells with PUFAs and cisplatin had varying effects on cell survival depending on the cell line and the PUFA used. The most significant interactions were observed between GLA and cisplatin (Table III). Since A2780 was more sensitive to the PUFAs than the two resistant sublines it was necessary to use lower concentration of the PUFAs for A2780 than were used for 2780CP and 2780AD in these experiments. A range of concentrations were used that produced less than 10% cell kill alone. GLA sensitised all three cell lines to cisplatin and the effect was dose dependent (Table III). This combination was, however, least effective in the cisplatin resistant cell line 2780CP. In contrast, EPA had no effect on the sensitivity of cell lines A2780 and 2780AD to cisplatin but produced a slight decrease in the ID_{50} concentration of cisplatin in cell line 2780CP (1.3 fold, $P < 0.05$, Table III). None of the PUFAs had any effect on the doxorubicin sensitivity of the three cell lines (Table IV). The results of these experiments were not very promising and it was not possible to increase the concentration of the PUFAs without a significant increase in the toxicity of the PUFAs themselves.

In an attempt to enhance the sensitising effects of the PUFAs cells were pre-incubated with the PUFAs before exposure to the cytotoxic drugs. The rational behind this approach was based on the assumption that pre-treatment

with the fatty acids would have one of two effects. Either they would make the cells more sensitive to subsequent exposure to a second cytotoxic agent or pre-exposure would allow the fatty acids to be incorporated into cellular lipids and thus alter membrane fluidity. A four hour pre-exposure had little effect on either doxorubicin or cisplatin sensitivity in any of the three cell lines (Table V). Exposure to PUFAs for 24 hours before exposure to cisplatin was less effective for 2780AD than the co-exposure (Tables III & VI). However, it should be noted that for this experiment the exposure time to cisplatin and doxorubicin was reduced to 4 hours and this may well explain this difference. Alternatively, it could be that the PUFA and cytotoxic drug interact in some way that requires their joint presence.

A 48 hour pre-treatment period had marked effects on the sensitivity of 2780CP to both cisplatin and doxorubicin (Table VII). Both EPA and GLA were equally effective (10 and 8 fold sensitisation respectively, $P < 0.001$) and there was no difference between the two formulations of GLA. This was surprising since GLA was more effective than EPA or the lithium salt of GLA when a 24 hour pretreatment period was used (5.6 fold cf 1.4 and 2.7 fold respectively, Table VI). All three fatty acids sensitised both 2780AD and 2780CP to doxorubicin when cells were pre-treated for 48 hours with the PUFAs ($P < 0.01$, Table VI).

In order to ensure that the PUFAs would be incorporated into cellular phospholipids the protocol used by Zijlstra *et al* (1987b) and Timmer-Bosscha *et al* (1989) was followed. They used docosahexaenoic acid which is less cytotoxic than EPA or GLA. Cells were exposed to the fatty acid on days 1 and 3 after

plating such that the overall exposure time to PUFAs was 72 hours before exposure to cisplatin for 4 hours. Lipid analysis revealed significant incorporation of the docosahexaenoic acid into cellular lipids (Timmer-Bosscha *et al*, 1989; Zijlstra *et al*, 1987b). For the 72 hour pre-treatment experiments the concentration of the fatty acid was reduced but the fatty acid was replaced after 48 hours. The stability of EPA and GLA in culture medium is not known so the exact exposure time may not be 72 hours. Only the interaction between EPA and doxorubicin in 2780AD and GLA and cisplatin in 2780CP were examined and the results were consistent with the trends already observed for the shorter pre-exposure periods. However, these experiments also showed clearly that part if not all of the apparent sensitisation was due to the toxicity of the fatty acid alone (Fig. 7B). Thus at the highest concentration of GLA used for pre-treatment of 2780CP the fatty acid alone killed more than 50% of the cells and it was not possible to determine an ID_{50} concentration for cisplatin. If cell survival is expressed as a percentage of the control where the control for GLA exposure is cells exposed to GLA for 72 hours, it is possible to compare treatments. However, the parameter derived from this approach is not an ID_{50}. It is assumed that the effect of GLA alone on cell survival is constant regardless of the concentration of cisplatin used. Clearly when studying the interaction of two drugs it would be wrong to assume that GLA increases cisplatin toxicity but not *vice versa*.

Since the toxicity of the fatty acid alone was apparent in all pre-treatment experiments (Figs. 5, 6 & 7), drug interactions were subject to a more critical

analysis. The isobologram method described by Steel and Peckham (1979) was originally designed for studies of the interaction between radiation and cytotoxic drugs (Steel, 1979) and it has been used successfully to study interactions between two agents (Carter & Wampler, 1986; Church *et al*, 1988; Gessner, 1988; Plumb *et al*, 1991). Application of this method of analysis to the interaction between GLA and cisplatin showed clearly that the two agents demonstrate additive toxicities (Fig. 8) since the majority of points lie within the envelope of additivity. This is entirely consistent with previous reports that PUFAs can sensitise drug resistant cell lines to cytotoxic drugs (Timmer-Bosscha *et al*, 1989; Zijlstra *et al*, 1987b). Zijlstra *et al* (1987b) showed that the doxorubicin resistant cell line GLC_4/ADR was more sensitive to docosahexaenoic acid than GLC_4 and was sensitised to doxorubicin by 72 hour pre-treatment with the PUFA, whereas the parental cell line GLC_4 was not sensitised. Similarly, docosahexaenoic acid sensitised cisplatin resistant subline GLC_4CP to cisplatin but did not sensitise parental cell line GLC_4 to cisplatin (Timmer-Bosscha *et al*, 1989). They were unable to explain this sensitisation since cellular platinum concentration, total DNA bound platinum and the amount of the major DNA-platinum adducts increased in both cell lines. The sensitisation could well be explained if GLC_4CP like GLC_4/ADR is more sensitive to docosahexaenoic acid than GLC_4.

One of the aims of this study was to determine whether incorporation of PUFAs into the membrane could alter membrane permeability and thus alter drug permeability. However, the PUFAs themselves were more toxic than anticipated and this aim was not achieved. Several studies have suggested that

this may be a worthwhile approach (Ramu *et al*, 1983a; Siegfried *et al*, 1983). More recently, there have been a number of reports suggesting that alteration of protein kinase C activity can alter platinum sensitivity (Basu *et al*, 1991; Hofmann *et al*, 1988; Isonishi *et al*, 1990). Protein kinase C migrates to the cell membrane when activated. Alterations of the membrane composition could therefore alter protein kinase C activity and hence cisplatin sensitivity.

These results suggest that EPA and GLA do not have a role as resistance modulators in drug resistant cells. However, additive toxicities can be of value in the clinic provided that the toxicities of the drugs to normal tissues are non-additive. High concentrations of PUFAs have been given to both animals and man without apparent toxicity (Siegel *et al*, 1987; Van der Merwe, 1984; Van der Merwe *et al*, 1987; Zhu *et al*, 1989). Since it is known that tumour cells derive most of their fatty acids from the host circulation it should be possible to deliver PUFAs to tumour cells, perhaps preferentially. This aspect has already been exploited in attempts to reduce the cachexia associated with the growth of some tumours (Beck *et al*, 1991).

CHAPTER 6

General Discussion

On the whole the aims of this thesis have been achieved. These aims were:

(*1*) to characterise verapamil as a resistance modulator

(*2*) to determine whether verapamil affects intracellular drug distribution

(*3*) to find alternative means of overcoming drug resistance

The results contribute to an understanding of both the mechanisms by which tumours develop resistance to chemotherapeutic agents and the strategies to overcome such resistance.

Although verapamil has limited value as a resistance modulator in the clinic, it is one of the most potent agents identified *in vitro* and its activity was shown to include cell lines that do not overexpress P-glycoprotein (Chapter 2 Table I). This observation is significant in itself since it suggests that verapamil has more than one mechanism of action in terms of drug sensitisation. Furthermore, the two other resistance modulators studied, quinidine and bepridil, could only modulate the sensitivity of cell lines that express very high levels of P-glycoprotein. This observation was not followed up; however, subsequent studies suggested a possible explanation.

Both the human P-glycoprotein positive multidrug resistant cell lines showed a marked reduction in drug accumulation when compared with the parental drug sensitive cell lines. Sensitisation by verapamil was accompanied by an increase in drug accumulation but the degree of sensitisation was much greater

than was expected on the basis of the increased drug accumulation. This discrepancy refers to total drug accumulation per cell as measured by incubation of cells in labelled drug followed by scintillation counting. The technique of autoradiography allowed discrimination between drug located in the nuclear and cytoplasmic compartments. These studies revealed that in both drug sensitive and drug resistant cell lines the majority of the drug as revealed by grain counts was present in the nucleus. However, the ratio of nuclear to cytoplasmic grains was greater in the drug sensitive cell lines than in the drug resistant cell lines (Chapter 3 Tables I-IV). Verapamil not only increased the total grain count per cell in the drug resistant cell lines but increased the nuclear to cytoplasmic grain count ratio to equal that of the drug sensitive cell lines. These observations confirm suggestions that verapamil can alter intracellular drug distribution. It was however clear that by autoradiography only a fraction of the total accumulated drug was studied, probably due to loss of free drug during fixation of the cells. When drug distribution was observed by autofluorescence of daunorubicin there was clearly a large pool of drug in the cytoplasm yet very few grains were present in the cytoplasm of either the drug sensitive or drug resistant cell lines. The increase in grain count caused by verapamil in the human multidrug resistant ovarian cell line 2780AD (8 fold) was closer to the degree of sensitisation observed (13 fold) than that of the overall 2 fold increase in drug accumulation observed by scintillation counting of whole cells. It is therefore tempting to suggest that autoradiography detects the pool of drug that is closely linked with cellular cytotoxicity whereas other techniques detect all pools of drug.

There was a noticeable difference between the cellular handling of doxorubicin and daunorubicin. Although these are closely related molecules they have very different spectra of antitumour activities. The difference in drug accumulation between A2780 and 2780AD was 4 fold for doxorubicin but 16 fold for daunorubicin. This could be explained if daunorubicin is a better substrate for the drug efflux pump than doxorubicin. Recently, it has been proposed that P-glycoprotein is a 'flippase' molecule (Higgins & Gottesman, 1991). For this model, substrate specificity would be determined by the ability of the molecule to intercalate into the lipid bilayer of the cell membrane. Since daunorubicin is more lipophilic than doxorubicin (Arcamone, 1985; Gewirtz & Yanovich, 1987) it may intercalate into the membrane more efficiently and may thus be a better substrate for a 'flippase'.

It would be interesting to use autoradiography to study drug accumulation in the human non-small cell lung cancer cell lines investigated in this thesis. Verapamil was able to modulate the sensitivity of the four cell lines, yet only one had detectable levels of *mdr*1 mRNA. Although the effect of verapamil on drug distribution was observed in cell lines with high levels of P-glycoprotein, it is possible that this effect is not related to P-glycoprotein. To date only one study has identified P-glycoprotein in an intracellular location (Willingham *et al*, 1987) and it is not obvious how a protein in the cell membrane could directly affect drug distribution between the nucleus and cytoplasm. If verapamil caused a redistribution of drug in the non-small cell lung cancer cell lines, this would explain the effects on drug sensitivity and provide evidence that P-glycoprotein is

not involved.

One of the most interesting studies observed in this thesis arose from an observation made during the studies of drug distribution. Both of the two ovarian cell lines contained cells of markedly different morphologies. Furthermore, the amount of drug accumulated by the cells related to their morphology. For the drug sensitive human ovarian cell line A2780 differences in drug accumulation were related to cell size. Two clonal cell lines were isolated from cell line A2780 which differed in size by a factor of 2. When drug accumulation was expressed as amount per cell, clone A-10 accumulated twice as much drug as clone A-5 yet the drug sensitivity of the two clones were similar. However, if cellular drug content was expressed as a concentration there was no difference between the two clones. Thus it is important to take cell size into account when comparing drug content between cell lines.

Six clonal cell lines isolated from the multidrug resistant cell line 2780AD were studied in detail. These clones revealed a marked heterogeneity in stability of the multidrug resistance phenotype. This was an unexpected finding since it was assumed that the chronic exposure to doxorubicin would have been a sufficient selection pressure to isolate a fairly homogeneous subpopulation. Two of the clones maintained their resistance phenotype even in the absence of drug whilst the other four showed a gradual decline in resistance with increasing time in culture in drug free medium. Loss of resistance was shown to be associated with a loss of P-glycoprotein expression. However, even when drug sensitivity had reverted almost to that of the drug sensitive cell line, resistance and P-

glycoprotein expression could be reinduced by a single 24 hour exposure to doxorubicin. This observation suggests that the clonal cell lines retain the background of a resistance phenotype but that this requires constant exposure to the selecting agent to maintain a functional resistance phenotype. The relevance of these observations to clinical drug resistance is not clear. However, it is generally assumed that if P-glycoprotein is a significant factor in clinical drug resistance, relapsed tumours will express P-glycoprotein. By analogy with the clonal cell lines it is possible that the relapsed tumour is indeed a resistant subpopulation selected by the first course of chemotherapy but which does not necessarily express P-glycoprotein in the absence of exposure to the cytotoxic drug. However, the first course of chemotherapy would be sufficient to reinduce P-glycoprotein expression.

The use of polyunsaturated fatty acids to modulate drug sensitivity was a novel and rather unusual approach. It was not therefore surprising that these studies failed to demonstrate any evidence of modulation. However, a number of interesting observations did emerge. The polyunsaturated fatty acids were quite toxic in their own right and this toxicity has been shown elsewhere to be of potential use in the selective killing of tumour cells (Begin *et al*, 1985, 1986a & b; Booyens *et al*, 1984). Because of this toxicity it was difficult to determine whether the drug interactions observed were additive or synergistic and isobologram analysis proved highly effective in distinguishing between these two possibilities. The toxicities of the polyunsaturated fatty acids were clearly additive with respect to both doxorubicin and cisplatin and the polyunsaturated fatty acids

were equally effective in the drug sensitive parental cell line and in the doxorubicin resistant and cisplatin resistant cell lines. Since polyunsaturated fatty acids have been administered to patients without any undue side effects (Dodge, 1990; Van der Merwe *et al*, 1987) it is possible that they could be used in addition to standard chemotherapy in order to enhance tumour cell kill. Clearly, studies in a human tumour xenograft model would be well worthwhile pursuing.

Concluding remarks

The importance of P-glycoprotein in clinical drug resistance is not clear and it is certainly not the only mechanism involved. However, in those tumour types where P-glycoprotein is expressed to any significant degree, studies with resistance modulators are well worth attempting. Verapamil may not be suitable for use in the clinic, but the studies described in this thesis show that it has properties that are not shared by all modulators and indeed may be able to modulate in the absence of P-glycoprotein. This is an interesting property that could have significant value to the understanding of mechanisms of drug resistance. Perhaps more importantly the use of polyunsaturated fatty acids in combination with chemotherapy should be recommended since this approach appears to be effective against very different mechanisms of drug resistance.

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