ASPECTS OF MULTIDRUG RESISTANCE

IN BREAST CANCER

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DEDICATION

This thesis is dedicated to my wife, Julie, and my two children, Elizabeth and Jennifer, for their endless support and encouragement during the writing of this work.

DECLARATION

I declare that the work described in this thesis was carried out solely by me with the following exceptions. All the people mentioned were in the Department of Medical Oncology, University of Glasgow unless otherwise stated.

The assessment of P-glycoprotein staining and tumour histology, described in Chapter 2, was carried out by Dr JJ Going, Department of Pathology, Glasgow Royal Infirmary. The establishment of the 2780AD xenograft in nude mice (Chapter 4) and the *in vivo* modulation of this xenograft (Chapter 5) were carried out in collaboration with Dr Jane Plumb.

The immunoassay, used for the detection of plasma quinidine in non tumour-bearing mice (Chapter 4), was carried out in the Department of Biochemistry, Western Infirmary, Glasgow. The quantification of epirubicin by High Pressure Liquid Chromatography in mouse plasma, tissues and tumour and in human breast cancers (Chapter 5) was carried out by Gilmour Morrison. In addition all intravenous injections to nude mice (Chapter 5) were given by Tom Hamilton, Animal House Technician.

Finally the toxicity data from the clinical trial described in Chapter 7, coordinated by myself on behalf of the West of Scotland Clinical Trials Group, was analysed by Jim Paul, Statistician, Beatson Oncology Center, Western Infirmary, Glasgow.

ABSTRACT

The successful use of chemotherapy in the treatment of breast cancer is confounded by the development of clinical drug resistance in a large number of cases. One mechanism of resistance, multidrug resistance (MDR), is associated with expression of a 170kDa transmembrane protein called P-glycoprotein (P-gp) which acts as an energy-dependent drug efflux pump. Although P-gp can be encoded by several MDR genes it is only expression of the mdr-1 gene which is associated with MDR in humans. P-gp has been identified in several human tissues and tumours but previous studies have failed to provide clear evidence of its presence in human breast cancer.

Detection of P-gp, by immunohistochemistry using specific monoclonal antibodies, allowed confirmation of P-gp expression at low levels in the majority of 29 untreated, primary breast cancers suggesting a population of resistant cells was present even before exposure to cytoxic drugs. Furthermore this expression was detected in stromal cells, as well as malignant cells, and this stromal cell immunostaining has been confirmed as mdr-1 expression by competitive inhibition using a specific blocking peptide. Examination of paired samples of normal breast and tumour from the same patient revealed P-gp expression only in ductal epithelial cells, but not stromal cells, of normal breast. Finally those patients whose tumours had the highest P-gp expression in malignant cells appeared to have a worse prognosis in terms of disease-free survival.

Following confirmation that the drug-efflux pump could be reversed *in vitro* by several

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modulating agents one of these compounds, quinidine, was selected for further study. It was established that tumour quinidine concentrations, adequate for MDR modulation *in vitro*, could be achieved both in an animal model of MDR and in human breast cancers. This led to attempts to use quinidine as a potential *in vivo* MDR modulator in a human MDR xenograft in the mouse. As well as producing a significant growth delay quinidine also appeared to redistribute intracellular epirubicin in this model with increased nuclear autofluorescence compared to controls.

This demonstration of the activity of a resistance modulator *in vivo*, together with confirmation of MDR expression in breast cancer, provides a sound basis for attempts to modulate MDR in patients. The combination of epirubicin and quinidine can be given to patients with breast cancer without any increase in systemic toxicity. Quinidine, however, did not increase total tumour epirubicin levels and had no apparent effect on intracellular distribution. A Phase II placebo-controlled double blind trial in the West of Scotland to examine the effects of quinidine on response to chemotherapy with epirubicin in patients with locally advanced or metastatic breast cancer is underway and the results of this trial are awaited with interest.

CHAPTER 1

GENERAL INTRODUCTION

Breast cancer is the commonest cancer in women in the West of Scotland, with an incidence of 91.4 per 100,000 women, which was responsible for 693 deaths per annum during 1984-1988. It is now known that even thirty years after presentation with breast cancer the risk of death from recurrent disease is significantly increased when compared with a normal population of the same age distribution (Brinkley & Haybittle, 1984). There is therefore good evidence to support the theory that breast cancer is a systemic disease, with micrometastases already present at the time of initial referral. Thus it is essential to improve methods of systemic treatment in order to influence the clinical outcome of these patients. Chemotherapy is now widely used both as an adjuvant treatment in high risk groups to prevent disease recurrence and in the treatment of advanced breast cancer. When used as an adjuvant therapy the combination of cyclophosphamide, methotrexate and 5-fluorouracil (CMF) does significantly reduce the risk of relapse, at least in women < 50 years (Early Breast Cancer Trialists' Collaborative Group, 1988)

DRUG RESISTANCE

The use of cytotoxic agents in the treatment of breast cancer is confounded by the development of clinical drug resistance in a large number of cases. Unlike intrinsic drug resistance, where tumours infrequently respond to initial cytotoxic

therapy eg. colonic carcinoma, most breast cancers do initially respond to chemotherapy only to relapse at a later stage as a result of acquired drug resistance. The most active single agents in the treatment of advanced breast cancer are anthracyclines with a response rate of 40-50% in previously untreated patients (Young et al, 1981). Although most anthracyclines are cardiotoxic after a certain cumulative dose their use is limited not by this but by the development of clinical drug resistance. As the various cellular mechanisms which underly this development of a population of resistant cells are identified it is hoped that strategies can be planned to circumvent them. The best characterised mechanism, known to be induced by anthracyclines, is called multidrug resistance (MDR). In view of the common use of anthracyclines in the treatment of breast cancer this mechanism may well be relevant to this tumour type.

THE MULTIDRUG RESISTANCE PHENOTYPE

The most striking feature of the MDR phenotype is that cells selected for resistance to a specific cytotoxic drug, by stepwise incubation in increasing amounts of the drug, can be cross-resistant to other structurally unrelated cytotoxic agents. This was first described in Chinese hamster lung cells selected for resistance *in vitro* to Actinomycin D but which displayed cross-resistance to vinblastine, vincristine and daunomycin (Biedler & Riehm, 1970). The drugs associated with the MDR phenotype are anthracyclines, vinca alkaloids and etoposide (Seeber et al, 1982). Although these drugs are not structurally similar, and have different intracellular targets, they are all hydrophobic compounds derived from natural products and they appear to share a common mechanism

of cellular drug resistance. Further studies with both animal (Skovsgaard et al, 1978; Inaba et al, 1978) and human (Fojo et al, 1985) continuous cell lines have shown that this cross-resistance is related to decreased intracellular drug accumulation which is correlated to the presence of a specific 170,000 dalton transmembrane protein (Riordan et al, 1985). This protein, first described in Chinese hamster ovary cells selected for resistance to colchicine, was termed **P**-glycoprotein (P-gp) as the cells were thought to display altered membrane **P**ermeability (Juliano & Ling, 1976). This glycoprotein is present in many MDR cell types, but not in the corresponding drug-sensitive wild-types, and is thought to act as an energy-dependent drug-efflux pump (Chen et al, 1986).

P-GLYCOPROTEIN

The entire structure and amino acid sequences of both murine and human P-gp have been derived from the specific genes which encode them (Gros et al, 1986; Chen et al, 1986). Recent studies have shown that P-gp is encoded by a family of three genes in rodents and two genes in humans (Ng et al, 1989). Although this glycoprotein is highly conserved throughout several mammalian species, and comparison of the various P-gp isoforms indicates a similar overall structure, only certain P-gp isoforms actually confer the MDR phenotype. The link between the human mdr-1 gene and the development of the MDR phenotype has been confirmed by transfer of genomic DNA from human KB carcinoma cells into drug-sensitive mouse NIH 3T3 cells, thereby inducing the same pattern of resistance in the recipient cells as their donor cells (Shen et al, 1986). Both mdr-1 and mdr-2 are located on chromosome 7q (Gottesman & Pastan, 1988) but

only expression of mdr-1 is associated with MDR. Identification of the amino acid sequences of human P-gp has revealed several characteristics which support its role as a transport protein. The 1280 amino acid molecule encoded by mdr-1 consists of a tandem duplication, suggesting that the two halves were generated by a gene duplication event. Each half consists of six putative transmembrane regions each containing 21 amino acids and is homologous to the HlyB protein, an Escherichia coli membrane protein required for the export of alpha-haemolysin (Gerlach et al, 1986). In addition each half contains nucleotide binding sequences homologous to regions found in transport proteins found in Escherichia coli (Gilson et al, 1982) and Salmonella typhimurium (Higgins et al, 1986).

From the above evidence it is possible to suggest a model for P-gp whereby energy derived from ATP hydrolysis is coupled to drug transport out of the cell via the transmembranous part of the molecule. This would suggest that P-gp can bind reversibly to drugs to allow release at the cell surface and more than one binding site may be present to bind structurally unrelated drugs. Specific vinblastine-binding sites have been confirmed, by photoaffinity labelling with vinblastine analogues in a MDR human KB carcinoma cell line, KB-C4, which were not present in the parental cell line, KB-31 (Cornwell et al, 1986). Furthermore this binding could be inhibited by vincristine and daunomycin but not by colchicine and Actinomycin D providing supportive evidence for more than one binding site. The vinblastine-binding protein has been confirmed as P-gp by immunoprecipitation with the monoclonal antibody to P-gp, C219 (Cornwell et al, 1987).

The model of P-gp as an energy-dependent drug efflux pump fits well with the increased chemoresistance and decreased intracellular drug accumulation found in MDR cell lines. However not all P-gp isoforms are associated with the MDR phenotype and the different MDR genes may code for slight alterations in the structure of P-gp which may modulate its function. Post-translational modifications eg. phosphorylation have been found *in vitro* and it is interesting to note that two agents which can reverse the drug efflux pump, verapamil and trifluoroperazine, can also increase phosphorylation (Hamada et al, 1987).

"ATYPICAL" MULTIDRUG RESISTANCE

Although the most consistent biochemical change in MDR cells is the expression of P-gp recent studies have found cell lines which display the MDR phenotype but which do not express P-gp. This form of MDR, termed "atypical" MDR (Beck et al, 1987), has been documented in several cell lines eg. the large cell lung carcinoma line COR-L23 (Reeve et al, 1990) and the human leukaemia HL60 cells (McGrath et al, 1987) and clearly suggests the existence of alternative biochemical pathways that lead to the development of the MDR phenotype. Two enzymes which have been implicated in "atypical" MDR to date are DNA topoisomerase (I & II) and glutathione-S-transferase.

Topoisomerases are nuclear enzymes which control conformational changes in DNA, by cleaving DNA strands, and are thus essential for vital cellular processes during translation, transcription and mitosis (Zijlstra et al, 1990). These enzymes are specific targets for certain anti-tumour drugs which act by stabilising a

"cleavable complex", an intermediate in the normal enzyme pathway, and this stable complex appears to be cytotoxic (Zhang et al, 1990). The drugs involved are anthracyclines, epipodophyllotoxins and amsacrine (Topoisomerase II) and camptothecin (Topoisomerase I)(Smith et al, 1990). It is thought that cells develop resistance to these drugs by down-regulation of the amount of Topoisomerase or production of mutated forms of these enzymes (Tan et al, 1989). Since anthracyclines eg. epirubicin are used extensively in the treatment of breast cancer this mechanism of resistance may well be relevant in the development of clinical drug resistance.

Glutathione-S-transferase (GST), an enzyme involved in intracellular detoxification, can neutralise potential toxins eg. cytotoxic drugs by conjugation with glutathione. Elevated levels of this enzyme have been found in drug resistant cells including the doxorubicin-resistant breast carcinoma cell line MCF7[ADR] (Batist et al, 1986). Furthermore elevation of cellular glutathione itself may be linked to doxorubicin resistance since reduction of glutathione by buthionine sulfoximine (BSO) has been shown to restore sensitivity to doxorubicin-resistant cells *in vitro* (Hamilton et al, 1985).

It remains to be seen whether alterations to either Topoisomerase I or II and glutathione-S-transferase contribute to the development of clinical drug resistance in human tumours but the evidence implicating their role in "atypical" MDR *in vitro* does seem convincing.

MDR-1 / P-GLYCOPROTEIN EXPRESSION IN HUMAN TISSUES AND TUMOURS

In humans the MDR phenotype is associated with expression of only one of the two MDR genes, designated mdr-1. Human mdr-1 expression has been studied in a variety of normal tissues by measurement of mdr-1 messenger RNA (mRNA) and high levels were found in the kidney, adrenal, liver and gastrointestinal tract (Fojo et al, 1987). The use of immunocytochemistry, with a monoclonal antibody to P-gp (MRK16), has allowed more specific localisation of P-gp in the cells of these organs (Thiebaut et al, 1987). In liver P-gp was found on the biliary canalicular surface of hepatocytes and on the apical surface of epithelial cells in pancreatic ducts, proximal kidney tubules and the gastrointestinal tract. These findings led to the suggestion that P-gp may be involved in the normal transport of metabolites or toxins in these cells and that P-gp expression in human tumours may in fact be an enhanced form of a normal cellular transport or protective mechanism. This is supported by recent evidence of mdr-1 mRNA, detected by *in situ* hybridisation, in the zona glomerulosa and zona reticularis of human adrenal cortical tumours (Harvie, personal communication). These two zones are thought to be the main sites of production and secretion of aldosterone and glucocorticoids.

MDR expression in tumours may be assessed either by measurement of mdr-1 mRNA or its protein product, P-gp. High levels of human mdr-1 mRNA have been found in a variety of tumours including those arising from organs known to express P-gp ie. adrenal, colon and kidney (Fojo et al, 1987). In addition this

technique has shown significant expression in a series of untreated primary breast cancers (Keith et al, 1990).

As mentioned previously P-gp itself may be detected by specific antibodies which can be visualised by immuncytochemical techniques. C219, which reacts with an internal epitope of P-gp, has revealed expression in acute non-lymphoblastic leukaemia (Ma et al, 1987) as well as lung and ovarian carcinomas (Volm et al, 1989). In addition MRK16, which reacts with an external epitope, has detected P-gp expression in lung cancer (Sugawara et al, 1988).

Previous studies of MDR expression in breast cancer specimens, either by measurement of mdr-1 mRNA or expression of the protein product P-glycoprotein, have failed to provide clear evidence that this particular mechanism of resistance is relevant in the development of clinical drug resistance in human breast cancer. In two studies which measured mdr-1 mRNA one found no expression in 248 breast cancers, representing untreated primary and refractory relapse specimens (Merkel et al, 1989), while low levels were found in 15% of tumours in the other series (Goldstein et al, 1989). In contrast another study has shown detectable levels of mdr-1 mRNA in the majority of 49 primary breast cancers (Keith et al, 1990) with a wide variation in expression between different tumours. These different results may be explained by heterogeneity of expression of P-gp or alternatively by variable sensitivity of techniques depending on which probe is used to detect mdr-1 mRNA. It was therefore essential to carry out a detailed study of P-gp expression in human breast cancer in the first instance, by using specific monoclonal antibodies to it, to try to resolve the conflicting

conclusions reached in previously published studies.

MULTIDRUG RESISTANCE MODULATION

As mentioned at the beginning of this introduction it is hoped that the identification of the various biochemical alterations in MDR cells may lead to specific strategies to circumvent them. Specific agents are now known which are capable of restoring sensitivity to a range of cytotoxic drugs in MDR-positive cells. These agents, called resistance modifiers or modulators, are not themselves cytotoxic and often share a common molecular structure (Rothenberg & Ling, 1989).

During the last decade there has been much interest in such non-chemotherapeutic agents which may enhance the cytotoxicity of conventional chemotherapy drugs such as doxorubicin. A number of calcium channel blocking drugs eg. verapamil (Plumb et al, 1990), calmodulin inhibitors (Ganapathi et al, 1983) and quinidine (Tsuruo et al, 1984) have been shown to be effective in enhancing the cytotoxicity of natural product anti-cancer drugs ie. vinca alkaloids and anthracyclines.

VERAPAMIL

One of the earliest resistance modulators to be recognised was verapamil which was found to enhance the chemosensitivity of MDR-positive cells to vincristine or doxorubicin by increasing the intracellular accumulation of these drugs (Tsuruo

et al, 1981). Although subsequent studies by the same group noted a minor increase in intracellular drug in the drug-sensitive P388 leukaemia cells the accumulation was much more marked in the drug-resistant counterpart (Tsuruo et al, 1982). The use of verapamil as a resistance modifier has subsequently been confirmed by other groups (Beck et al, 1986; Plumb et al, 1990).

Although the resistance modifying effect of verapamil has been established the exact mechanism or mechanisms by which it exerts this effect remains unclear. The recognition that MDR-positive cells do not have inhibitable calcium-dependent calcium channels (Ramu et al, 1984) has led to the suggestion that its calcium-blocking effect is separate from the modulating effect. Future studies therefore looked for other mechanisms which could account for the modifying effect of verapamil.

It is thought that verapamil, and other calcium-channel blockers, may bring about this effect by inhibition of voltage-dependent potassium channels (De Coursey et al, 1985) and certain calmodulin-dependent activities (Epstein et al, 1982). In addition verapamil has been shown to compete with a variety of cytotoxic drugs, associated with the MDR phenotype, for binding sites on P-glycoprotein (Cornwell et al, 1987). Furthermore it has also been recognised that verapamil can alter the phosphorylation state of P-glycoprotein (Hamada et al, 1987), which may well modulate its function, and may even act as an alternative substrate for the drug-efflux pump (Yusa & Tsuruo, 1989).

Although there are many studies confirming verapamil as an extremely effective

MDR modulator *in vitro* (Plumb et al, 1990) its use in patients has been limited by cardiotoxicity manifest as severe hypotension and cardiac arrhythmias. This has resulted in plasma concentrations 10-12 times lower than the most effective for MDR modulation *in vivo* (Kerr et al, 1986) and has led to the search for other effective MDR modulators which do not have such overt toxicity *in vivo*.

CALCIUM ANTAGONISTS

Other calcium antagonists apart fron verapamil have been shown to be effective modulators of MDR *in vitro* including nifedipine and diltiazem (Tsuruo et al, 1983). More recently the tiapamil analogue N-(3,4-dimethoxtphenyl)-N-methyl-2-(2-napthyl-m-dithane-2-propylamine) (DMDP) was shown to have a greater resistance modifying effect, in doxorubicin-resistant P388 leukaemia cells, at half the dosage level (Radel et al, 1988). Furthermore this study also demonstrated that a 24 hour exposure to DMDP with a short exposure to doxorubicin resulted in the same cytotoxicity as a 24 hour exposure to both agents suggesting that the dosing schedule of potential modulators is extremely important.

CALMODULIN INHIBITORS

Calmodulin inhibitors such as trifluoperazine have been shown to potentiate the cytotoxicity of doxorubicin in doxorubicin-resistant P388 leukaemia cells *in vitro* (Ganapathi & Grabowski, 1983). The modulating effect appeared to be related to increased intracellular accumulation of doxorubicin in resistant cells following

exposure to trifluoperazine and furthermore this enhanced cytotoxicity did not occur in the drug-sensitive parental line. More recently a range of calmodulin antagonists were shown to be effective MDR modifiers in a colchicine-resistant human KB carcinoma cell line with thioridazine having the greatest sensitizing effect (Akiyama et al, 1986). However this study also demonstrated that not all calmodulin inhibitors reversed MDR and suggested that the modulating effect was not related to calmodulin inhibition. One possible explanation is that calmodulin antagonists may interfere with lysosomal function since thioridazine has been shown to delay the degradation of certain ligands within them (Kuratami et al, 1986)

CYCLOSPORINS

The immunosuppressive drug cyclosporin A has been shown to modulate resistance to daunorubicin and vincristine in acute lymphocytic leukaemia cells *in vitro* (Slater et al, 1986a) and in Ehrlich ascites carcinoma cells *in vivo* (Slater et al, 1986b). An important observation however has been that plasma levels of cyclosporin A achievable in the clinic (1-2 μg/ml) are considerably lower than the levels required for significant modulation *in vitro* (5 μg/ml) (Kahan et al, 1983). This problem has been overcome to some extent by the use of non-immunosuppressive cyclosporin analogues which are highly effective resistance modulators at lower concentrations than cyclosporin A (Twentyman, 1988).

QUINIDINE

Quinidine has been shown to be an effective modulator of MDR *in vitro*. In a series of experiments quinidine increased vincristine and doxorubicin cytotoxicity in the vincristine- and doxorubicin-resistant sublines of P388 leukaemia cells (Tsuruo et al, 1984). Furthermore this enhanced cytotoxicity was associated with increased intracellular accumulation of the respective drug. Once again the exact nature of the mechanism which brings about MDR modulation is not known although it may be due to a disturbance of the organisation of membrane lipids (Surewicz & Jozwiak, 1983). One other possible factor which may contribute to MDR modulation is a decrease in free intracellular calcium (Harrow & Dhalla, 1976). Although quinidine often has less modifying effect *in vitro* than the calcium-channel blockers (Tsuruo et al, 1984) it has attracted much attention because the levels required for modulation *in vitro* can be achieved in patients plasma without adverse toxicity (Jones et al, 1990). It is therefore an ideal candidate for further investigation as a potential modulator of MDR-positive cells in human cancers.

AIMS OF THESIS

In the light of contradictory data on MDR expression in breast cancer previously described the first aim of this project was to confirm that the MDR product, P-gp, could be detected in human breast cancers. The use of immunohistochemistry, with specific monoclonal antibodies to P-gp, is a useful tool not only to confirm P-gp expression but to examine the distribution of this expression throughout the various cell populations present in breast cancers. By deciding to look at expression in historical biopsy specimens it was hoped that some conclusions could be reached regarding the relationship between P-gp expression and certain pathological parameters as well as patient survival.

Having confirmed the presence of MDR expression in a proportion of human breast cancers the next step was to see whether MDR could be circumvented in drug-resistant breast cancer cells *in vitro* using a variety of modulators in combination with anthracyclines. Potential modulators were assessed by their effect either on chemosensitivity or intracellular cytotoxic drug concentration.

The second major aim was to establish an *in vivo* model of MDR so that known *in vitro* modulators could be assessed to see whether P-gp was a relevant, functional target in solid tumours. If this was the case then it would strengthen the case for attempting to modulate MDR in human tumours known to express P-gp ie. breast cancers. A natural progression from this work was to examine the effect of one particular modulator, quinidine, on the concentration of epirubicin in human breast cancers and its subcellular localisation. Finally the last chapter

examines the hypothesis that the use of MDR modulators, in combination with chemotherapeutic agents in patients, may lead to increased systemic toxicity.

In summary the work described in this thesis was designed to ascertain whether one particular mechanism of cellular drug resistance, MDR, has any relevance in the development of clinical drug resistance in human breast carcinoma and to look at potential therapeutic manoeuvres to circumvent this problem.

LAYOUT OF THESIS

Each chapter (2-7) is self-contained with an introduction, methods, results and discussion section. Chapter 2 describes the expression of P-gp in human, primary breast cancers and its distribution. Of particular interest was the finding of this protein in stromal cells as well as malignant cells in these tumours. This stromal cell immunostaining was confirmed as genuine expression of P-gp in Chapter 3 which also included an investigation of P-gp expression in normal breast tissue.

Chapter 4 concentrates on a comparison of the quinidine levels required for modulation *in vitro* with tumour levels achieved in a human tumour xenograft. Chapter 5 describes the establishment of a solid tumour model of MDR with which to test the modulating capacity of several agents known to reverse MDR cells *in vitro*. This provided encouraging results with two modulators and one of these, quinidine, was used in an attempt to modulate MDR cells in human breast cancers in Chapter 6.

Chapter 7 presents the toxicity data from a current phase III clinical trial which was set up to examine the effect of oral quinidine on the response to chemotherapy with epirubicin in patients with locally advanced or metastatic breast cancer. The final conclusions of this work are presented in Chapter 8.

CHAPTER 2

IMMUNOHISTOCHEMICAL DETECTION OF P-GLYCOPROTEIN EXPRESSION IN PRIMARY BREAST CANCER AND CLINICAL/PATHOLOGICAL CORRELATION.

INTRODUCTION

As mentioned in the general introduction previous studies of MDR expression in breast cancer specimens have provided conflicting evidence about the presence of P-gp in these tumours. Since the lack of agreement seems more apparent at the mdr-1 mRNA level, with one study describing no expression (Merkel et al, 1989) and another reporting expression in the majority of tumours (Keith et al, 1990), it was hoped that measurement of P-gp itself would provide a conclusive answer.

IMMUNOCYTOCHEMISTRY

Immunocytochemistry (ICC) allows the detection of tumour-associated antigens by specific monoclonal antibodies. In its earliest form monoclonal antibodies, conjugated to fluorochrome markers eg. fluoroscein, were detected using a fluorescence microscope (Coons & Kaplan, 1950). Although a relatively simple technique, which detects antigens at low concentrations, it makes the counterstaining and orientation of tissue constituents difficult and furthermore the final preparations are not permanent.

The use of immunofluorescence has now largely been superceded by immunohistochemistry where enzymes eg horseradish peroxidase (Nakane & Pierce, 1966) or alkaline phosphatase are used to produce intensely coloured, permanent precipitates which can be viewed with a conventional light microscope. Although the increase from a two-stage process (immunofluorescence) to a three-stage process (immunohistochemistry) theoretically increases the risk of non-specific binding this is not normally a problem in practice if optimal dilutions of antibodies are used. Several monoclonal antibodies to P-gp are now available allowing detection by ICC.

One such antibody C219, which reacts with an epitope on the cytoplasmic side of the P-gp molecule (Kartner et al, 1985), has been used to demonstrate P-gp expression in both untreated and treated breast cancers (Salmon et al, 1989; Schneider et al, 1989). In addition MRK16, which reacts with an external epitope on P-gp, has been used to detect P-gp expression by ICC in untreated breast cancer (Sugawara et al, 1988).

The use of ICC appears to have several advantages when compared with other techniques. The detection of mdr-1 mRNA in a homogenised tumour sample gives no information as to which cells express the gene or how these cells are distributed within a particular tumour. In addition it may not be sensitive enough to detect a small population of MDR-positive cells. Immunocytochemical techniques can circumvent these problems by allowing detection in single, or small numbers of cells, while allowing distribution throughout the tumour to be

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examined.

PATIENT SELECTION

In certain tumour types eg leukaemia it is feasible to obtain repeat tissue samples for comparison, either before and after treatment or during the course of treatment, in order to ascertain whether P-gp expression is related to any clinical parameters. Since it is known that P-gp can be induced *in vitro* by exposure of cells to a cytotoxic drug it is possible that P-gp is induced during treatment with MDR-related drugs. This would result in failure to respond to a second course of treatment with the same drug. Unfortunately sequential sampling is not usually possible in breast cancer due to either complete excision of the original tumour or the inaccessability of metastatic sites other than skin. Moreover the long natural history of breast cancer means that relapse material for comparison with the primary tumour may not be available for many years following the initial presentation (Brinkley & Haybittle, 1984).

One way to overcome this problem and to address the question of the clinical significance of P-gp in breast cancer is to examine historical biopsy material collected several years previously and stored in tissue banks at -70°C. In this way P-gp expression can be related to the clinical outcome of these patients. Of course, any attempt to correlate P-gp expression with survival of the patient makes the assumption that this particular mechanism of resistance in some way contributes substantially to the overall resistance of the tumour, either to conventional treatment or to anti-tumour host defence mechanisms.

The study described in this chapter was therefore designed with several aims in mind. The first priority was to attempt to confirm P-gp expression in human primary breast cancer and if present to establish which cells express it and to look at their distribution within the tumour. By examining P-gp expression in historical biopsy material it was possible to look for any correlation with various pathological parameters as well as the clinical outcome of the individual patients.

MATERIALS AND METHODS

PATIENTS, HUMAN TISSUE AND CELL LINES

All twenty-nine breast cancer specimens were collected from patients undergoing breast surgery at the University Department of Surgery, Royal Infirmary, Glasgow during the years 1984-1986. The mean age of the patients, from whom biopsies were collected, was 63 (range 36-89). Of 21 informative patients 11 were oestrogen receptor positive and 10 were oestrogen receptor negative. None of these patients had pre-operative chemotherapy although 6 had adjuvant post-operative chemotherapy with either cyclophosphamide, methotrexate and 5-fluorouracil, CMF (n=3) or 5-fluorouracil, epirubicin and cyclophosphamide, FEC (n=3). All biopsies had been snap frozen in liquid nitrogen and stored at -70°C until sectioning.

As a positive control for each group of specimens cytospin preparations of the small cell carcinoma of lung cell line H69LX10, previously shown to have high expression of P-gp (Plumb et al, 1990), were used. This continuous cell line was derived from the parent cell line NCI H69 by chronic exposure to doxorubicin (to a final concentration of 1 μg ml^{-1}) and was a kind gift from Dr P Twentyman (MRC Clinical Oncology and Therapeutics Unit, Cambridge). H69LX10 was maintained in Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% foetal calf serum (FCS), sodium bicarbonate and L-glutamine (2μM). The cell line was grown in the presence of doxorubicin (1μg/ml) to maintain its multidrug resistant phenotype.

Cell suspensions of H69LX10 were centrifuged at 200g for five minutes and then resuspended in phosphate-buffered solution (PBS). Aliquots (0.5ml) of this cell suspension were then pelleted onto clean glass microscope slides in a cytospin centrifuge (Shandon). These cytospin preparations were then fixed as for tissue sections.

MONOCLONAL ANTIBODIES

For the detection of P-gp C219 (CIS UK Ltd, High Wycombe, Bucks)and MRK16 (donated by T Tsuruo, Institute of Applied Microbiology, University of Tokyo, Japan) were used, both at a final concentration of $10 \mu g \, ml^{-1}$. This concentration was found to be the optimal dilution over the range $1 \mu gml^{-1}$ - $200 \mu gml^{-1}$ by immunostaining cytospin preparations of the cell line H69 LX10.

Selected cases were also stained using CAM 5.2 (Becton Dickinson, California, USA), AE1/AE3 (ICN, High Wycombe, Bucks) to detect cytokeratins and with antibody to vimentin (Boehringer, Lewes, East Sussex). The use of these antibodies helps to differentiate epithelial cells (tumour cells) from other cells which are derived from mesenchymal tissue eg. stromal fibroblasts. In negative controls an irrelevant monoclonal antibody (Clonab LN-C, Biotest, UK) was substituted for the primary antibody.

IMMUNOCYTOCHEMISTRY

Cryostat sections 5µm thick were cut from each frozen biopsy, air dried for one hour, then fixed in acetone at room temperature. After washing in Tris saline (pH 7.6, 5 minutes) the sections were stained using an indirect immuno-alkaline phosphatase technique (Figure 1), incubating sections with specific primary mouse monoclonal antibodies for two hours. This and all subsequent incubations were carried out at room temperature in a humidified container. All antibody dilutions were carried out in Tris buffer (pH 7.6)

After washing in Tris saline (5 minutes) the second antibody was applied. This was a rabbit anti-mouse immunoglobulin conjugated to alkaline phosphatase (Dako, High Wycombe, Bucks) and used at a working concentration of 1:20 in 50% normal human serum (filtered), 100 µl per slide for 45 minutes. Following a further wash in Tris saline (5 minutes) the sections were incubated with alkaline phosphate substrate solution, 100 µl per slide, for 30 minutes, producing a red reaction in P-gp positive cells. After counter staining with Haemotoxylin (15 seconds) the sections were submerged in Scott's Tap Water Substitute (20 seconds) before mounting in a water-based mounting medium.

ALKALINE PHOSPHATE SUBSTRATE SOLUTION

2.5 mg Naphthal AS B1 phosphate (Sigma, Poole, England) was suspended in 1 drop of dimethyl formamide (Sigma), in vertical laminar flow conditions, for 20 seconds until the solution became clear (solution A). 2.5 mg Fast red TR salt was then dissolved in Veronal Acetate buffer* (pH 9.2, 5mls) in a universal container (solution B).

* Veronal Acetate Buffer:

Sodium acetate (Trihydrate)	0.9715 gms
Sodium Barbitone	1.4715 gms
Distilled water	247.5 mls
N/10 Hydrochloric acid	2.5 mls

Stored at 4°C away from light.

Solutions A and B were added together. 100 μl of Levamisole (Sigma), which blocks endogenous alkaline phosphatase activity, was then added and the final solution was filtered (Whatman no 1 filter) before use.

INDIRECT ALKALINE PHOSPHATASE METHOD

SUBSTRATE

RAM-AP

C219/MRK16

P-GP
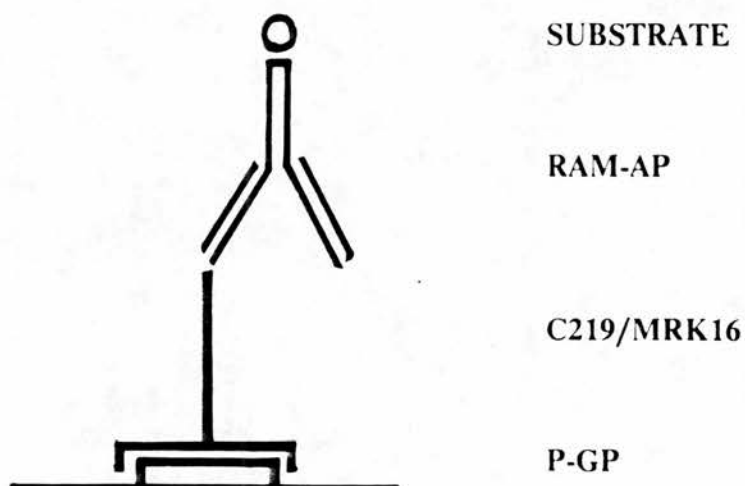
FIGURE 1
Diagrammatic representation of the immuno-alkaline phosphatase method. In this case the antigen **(P-gp)** is recognised by one of two mouse monoclonal antibodies **(C219 or MRK16)** which in turn bind to a rabbit anti-mouse second antibody conjugated to alkaline phosphatase **(RAM-AP)**. This enzyme acts on the substrate solution, producing a red precipitate in positive cells.

ASSESSMENT OF P-GP EXPRESSION AND PATHOLOGICAL PARAMETERS

All tumour sections were examined by an experienced pathologist (Dr JJ Going, Department of Pathology, Royal Infirmary, Glasgow) and an estimation of the percentage of cells showing positive staining was made. No attempt was made to quantify the intensity of the staining as this can vary slightly between experiments and cells were normally clearly positive or negative. An adjacent section of each tumour biopsy was stained with Haematoxylin and Eosin (H & E) to confirm that tumour was present in the biopsy.

The same pathologist examined these H & E sections in order to assess the overall grade (Elstron & Ellis grades I-III), the histological type (ductal, lobular or medullary) and the presence of vascular invasion (positive or negative). The extent of ipsilateral axillary node involvement $(0, 1\text{-}3, \geq 4)$ was obtained from historical pathology reports and the patient survival status was obtained from the hospital case records.

RESULTS

IMMUNOHISTOCHEMISTRY

Both C219 and MRK16 stained the positive control H69 LX10. Staining with C219 (Figure 2a) was more intense than with MRK16 (Figure 3a) in this cell line and in tumour frozen sections as well.

Twenty-nine breast cancers (all untreated) were incubated with C219 and MRK16 and the results from all patients are summarised in Table 1. C219 revealed a heterogeneous pattern of staining in epithelial cells (Figure 2b) in 21 of 29 tumours. However, in 26 of the patients C219 showed marked staining in stromal cells (Figure 2c). Furthermore, the proportion of positively stained stromal cells was notably higher than that for the epithelial cells. Although the number of cells staining with MRK16 was less than than with C219, the same pattern was observed with expression in both epithelial (16 of 29) and stromal (12 of 29) cells (Figures 3b,c). Immunostaining of epithelial cells, with both antibodies, occurred in either single cells (Figure 2d) or occasionally groups of cells (Figure 3b). In the presence of irrelevant antibody (Clonab LN-C) no areas of staining at all were visible (Figure 3d).

	NUMBER OF PATIENTS			
	Epithelial Cells		Stromal Cells	
Proportion of cells stained positive(%)	C219	MRK16	C219	MRK16
0	8	13	3	17
1-9	17	14	1	9
10-49	4	2	10	2
50-100	0	0	15	1

TABLE 1
The number of patients allocated to each group according to the percentage of positively stained cells, both epithelial and stromal, with two monoclonal antibodies C219 and MRK16.

The stromal cells which were P-gp positive, with either C219 or MRK16, were spindle-shaped cells with elongated nuclei (see Figure 2c and 3c). These cells were confirmed as non-epithelial by positive staining with a monoclonal antibody against vimentin (Figure 4a), an intermediate filament protein present in cells of mesenchymal origin, and the absence of staining when incubated with the anti-cytokeratin Cam 5.2 (Figure 4b), a monoclonal antibody which reacts with most adenocarcinomas. Furthermore, no staining of stromal cells was observed with an alternative anti-cytokeratin AE1/3 (Figure 4c).

a
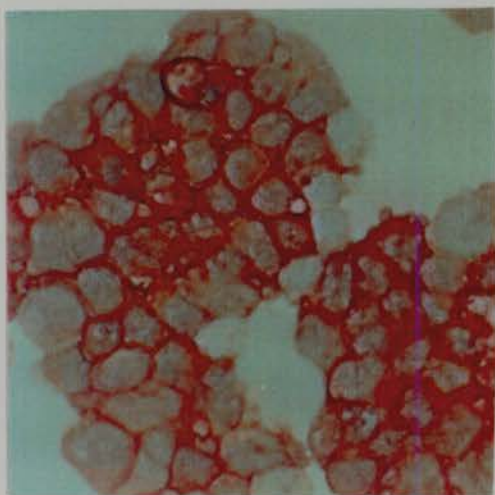
b
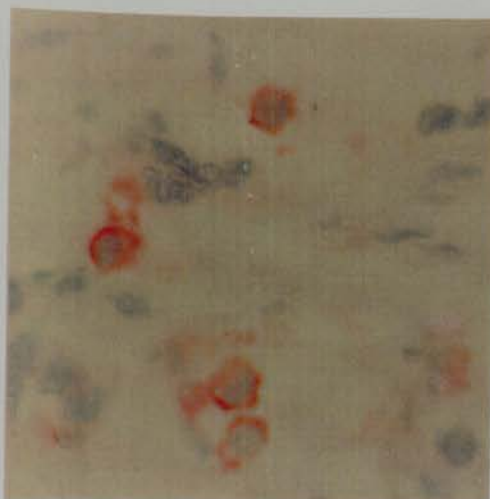
c
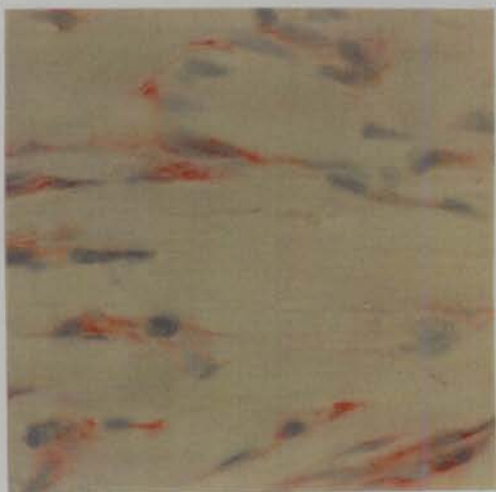
d
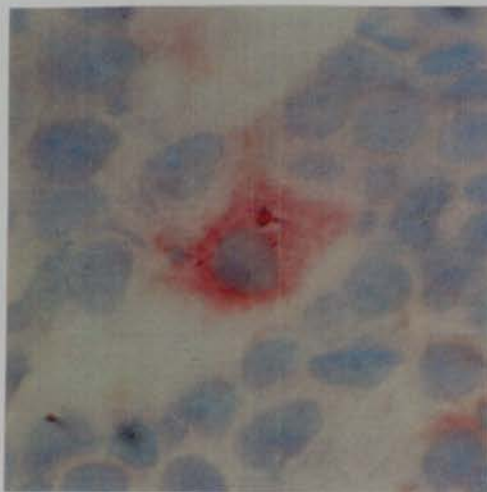
FIGURE 2
Cytospin preparation of the MDR cell line H69LX10 stained with the monoclonal antibody to P-glycoprotein, C219 (**a**). Plates **b** and **c** show heterogeneous staining, again with C219, in both tumour (**b**) and stromal (**c**) cells in a section of a primary breast cancer. Staining with C219 in an isolated tumour cell is also shown (**d**).
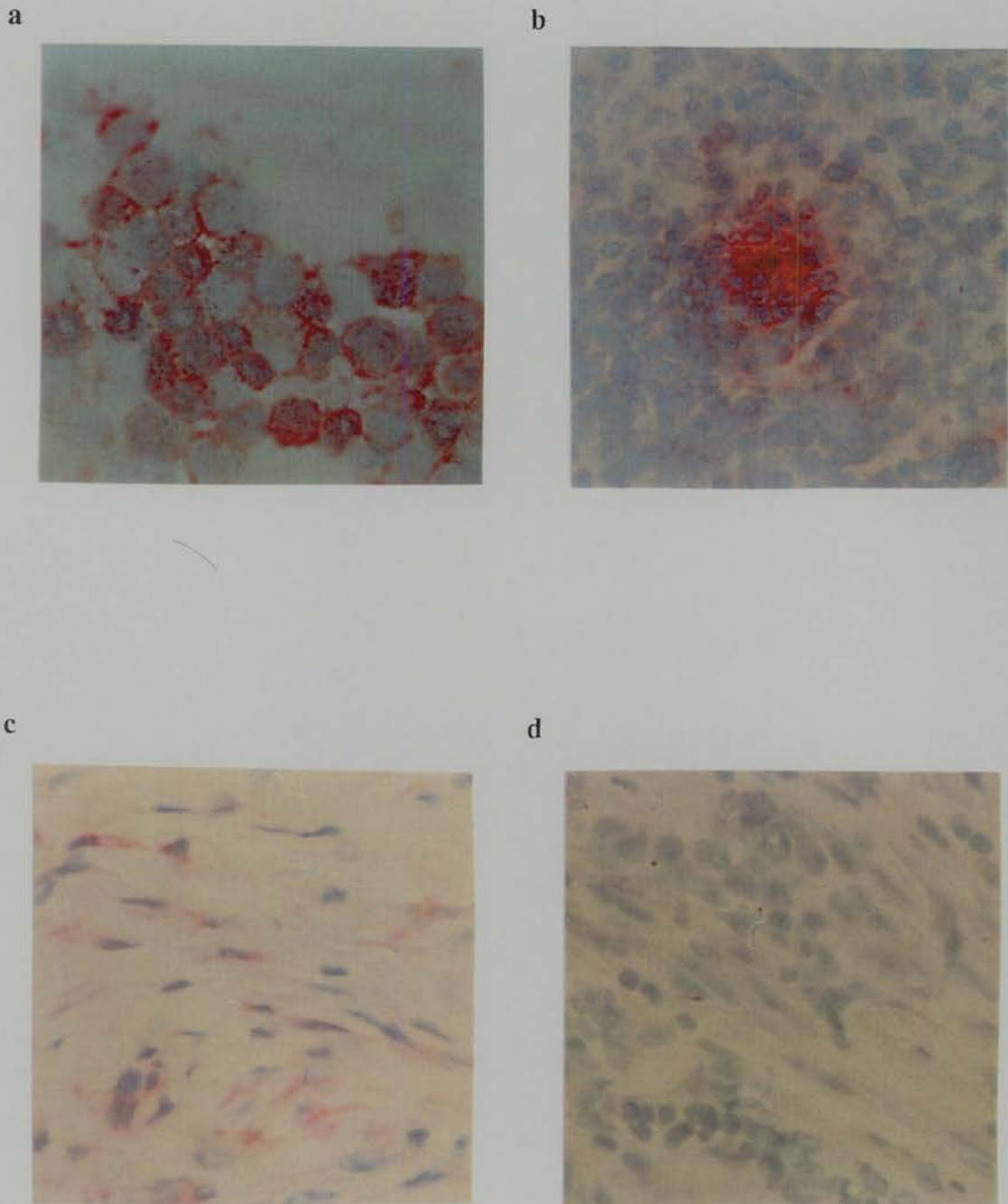
FIGURE 3
Cytospin preparation of the MDR cell line H69LX10 stained with the monoclonal antibody to P-glycoprotein, MRK16 (**a**). In sections of primary breast cancers this antibody stained both tumour (**b**) and stromal (**c**) cells. Substitution of MRK16 by an irrelevant antibody produced no areas of staining in sections of a primary breast cancer (**d**).

a
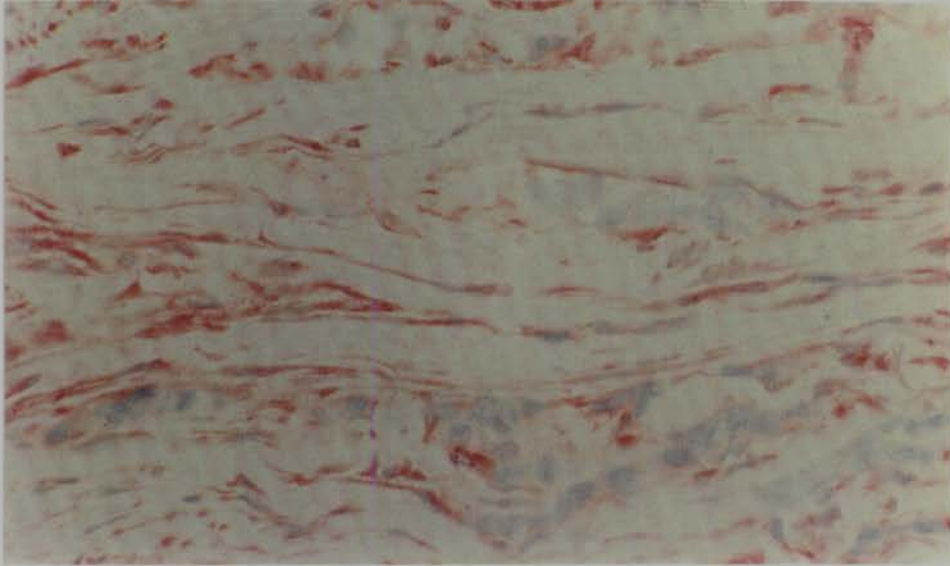
b
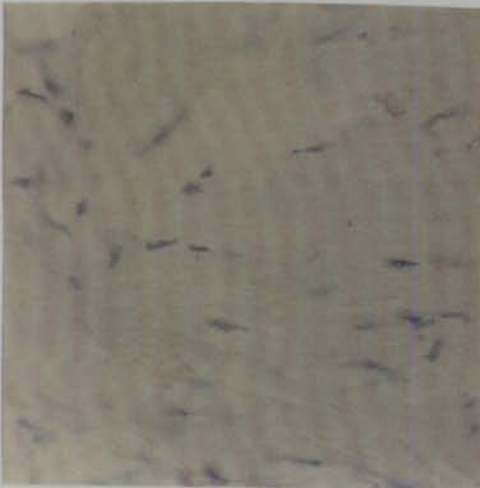
c
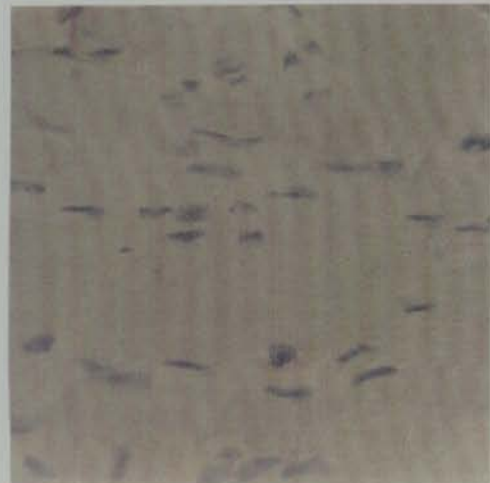
FIGURE 4
A section of primary breast cancer stained with antibody to vimentin (a) showing staining of stromal cells with this antibody. The other two figures shows the same section stained with the anti-cytokeratins Cam 5.2 (b) and AE1/3 (c) highlighting the absence of staining in the same stromal cells.

CORRELATION OF P-GP EXPRESSION WITH PATHOLOGICAL
PARAMETERS AND DISEASE-FREE SURVIVAL

There was a total of twenty-seven informative patients from twenty-nine tumour
biopsy specimens. As would be expected ipsilateral axillary nodal status predicted
prognosis in terms of patient survival (20 patients, Table 2)

NODES	ALIVE	DEAD	% SURVIVAL
0	6	2	75
1-3	5	3	63
≥4	2	2	50

TABLE 2
The relationship between ipsilateral axillary nodal status and survival with a
median follow up of 34 months.

As results of immunostaining with both antibodies were comparable, and the
range of stromal staining was much greater with C219 (0-90%) than MRK16 (0-
50%), it was decided to use the C219 results to assess any possible association
between expression and the various pathological parameters and disease-free
survival.

Immunostaining with C219, of epithelial or stromal cells, showed no correlation with the histological type of tumour, tumour grade or the number of involved ipsilateral axillary lymph nodes. In addition there was no correlation of P-gp positivity with either oestrogen receptor status or the presence of vascular invasion (Kruskal Wallace ranking test, Table 3).

Disease-free survival was assessed (median follow-up 34 months) using a Log Rank test with tumours allocated to one of three groups according to the percentage of P-gp positive epithelial cells (Gp1:0%, Gp2:$\leq$5%, Gp3:>5%). The results, from twenty-seven patients, suggest that patients in Group 3 ($\geq$5% of epithelial cells) have a worse disease-free survival than the other two groups (p=0.09; Figure 5). In contrast P-gp positivity in stromal cells showed no association with survival status (Figure 6).

NUMBER OF PATIENTS

TUMOUR HISTOLOGY	C219 POS	C219 NEG
DUCTAL	16	7
MEDULLARY	1	0
LOBULAR	2	1

TUMOUR GRADE		
1	4	1
2	6	5
3	9	2

AXILLARY NODES		
0	6	2
1-3	6	2
≥ 4	1	3

OESTROGEN RECEPTORS		
POS	8	3
NEG	7	3

VASCULAR INVASION		
YES	3	4
NO	16	4

TABLE 3

The relationship between P-glycoprotein expression, detected by immunohistochemistry with C219, and the following parameters: tumour histology, tumour grade, ipsilateral axillary lymph nodes, oestrogen receptor status or vascular invasion.
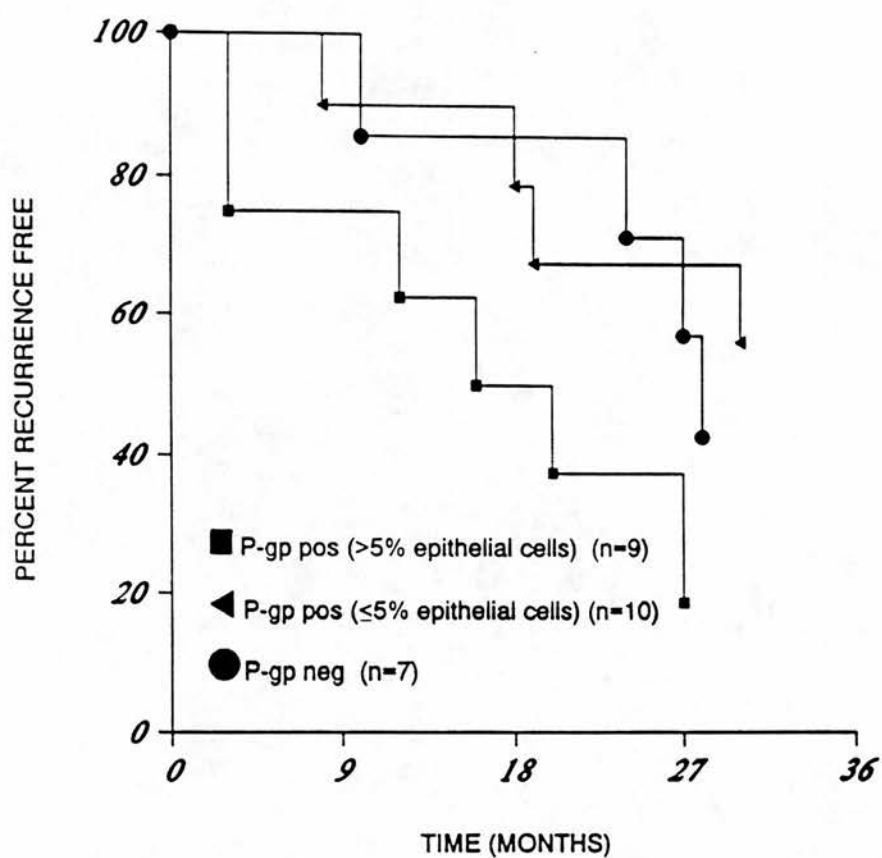
FIGURE 5

The disease-free survival according to P-glycoprotein expression in epithelial (tumour) cells, detected by immunohistochemistry with C219, in 26 patients with primary breast cancer.
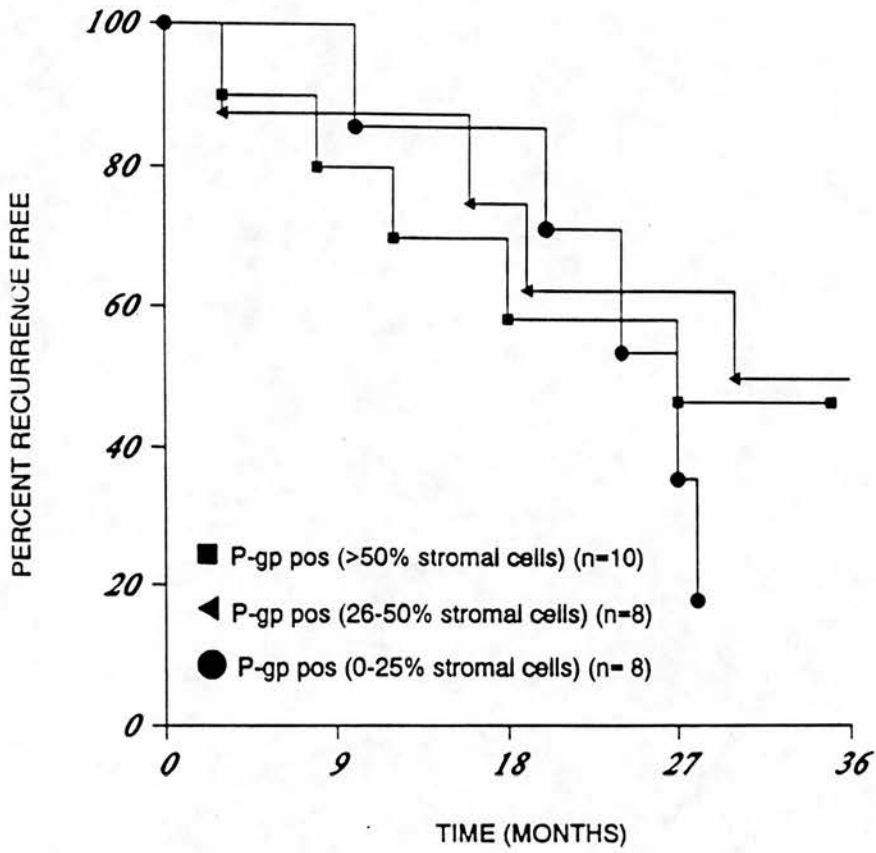
FIGURE 6
This disease-free survival according to P-glycoprotein expression in stromal cells, detected with C219, in 26 patients with primary breast cancer.

DISCUSSION

P-GLYCOPROTEIN EXPRESSION

This study has examined P-gp expression by immunohistochemistry in twenty-nine primary breast cancers (all untreated) using two monoclonal antibodies (C219 and MRK16) and an indirect alkaline phosphatase method. The results clearly show that P-gp immunoreactivity, detected by C219 and MRK16, can be demonstrated in a proportion of untreated primary breast cancers. Immunostaining was heterogeneous and interestingly appeared in epithelial and non-epithelial cells (Figure 2 & 3). It was confirmed that these non-epithelial cells were of mesenchymal origin, by immunostaining with antibody to vimentin (Figure 4a), and they are thought to be myofibroblasts.

Positive staining in epithelial cells with both C219 (21 of 29) and MRK16 (16 of 29) was detected in 1-9% of tumour cells in all but one tumour. This confirms two previous studies which found P-gp expression in one of nine (Sugawara et al, 1988) and two of twelve (Schneider et al, 1989) untreated breast cancers. The latter study considered staining in isolated cells to be negative, but it may be these cells which are selected following drug exposure, and are responsible for the development of clinical drug resistance. The staining in epithelial cells was mostly membrane bound, although cytoplasmic staining was seen, and these cells were confirmed as tumour cells by haemotoxylin and eosin staining of a separate section from each tumour.

A surprising finding was the demonstration of P-gp immunoreactivity in stromal cells with both C219 (26 of 29)and MRK16 (12 of 29). The fact that stromal staining has been detected by two monoclonal antibodies (Figures 2c & 3c), which recognise different epitopes of P-gp, suggests that it is genuine expression of the protein. This study provided the first published evidence of P-gp expression in breast cancer stromal cells (Wishart et al, 1990a) although it is known that one other group has detected expression in breast cancer stromal cells by *in situ* hybridisation (Fojo, personal communication) and P-gp positive macrophages have been detected in both lymphoid and non-lymphoid tumours (Schlaifer et al, 1990). Since *in situ* hybridisation detects mdr-1 mRNA this evidence argues against the possibility that the detection of P-gp in stromal cells is due to a cross-reaction with some other cellular constituent. It has probably not been recognised previously as other studies have often used human breast cancer cell lines (Fairchild et al, 1987) or cytospin preparations of breast cancer cell suspensions (Salmon et al, 1989) to study P-gp expression.

In all but one tumour the percentage of stromal cells staining was always greater with C219 than with MRK16 (Table 1). Recent data from one study has suggested that C219 may partially cross-react with the heavy chain of myosin in skeletal and cardiac muscle (Thiebaut et al, 1989). Since it is well recognised that scirrhous carcinomas of the breast contain a population of stromal cell with characteristics of myofibroblasts (Tremblay et al, 1979), and that explants of these cancers give rise to outgrowths of which ≥90% are myofibroblasts (Barsky et al, 1984), it was conceivable that the higher results with C219 were due to a partial cross-reaction with myosin. A more likely explanation, however, is that C219

recognises an additional isoform of P-gp not detected by MRK16. This has become apparent from a recent study which examined the expression of the three hamster P-gp isoforms using isoform-specific antibodies in a competitive immunohistochemical assay (Bradley et al, 1990). They found that the strong staining of cardiac muscle fibres and a subset of skeletal muscle fibres was due to expression of P-gp class III in these cells, an isoform not detected by MRK16, and therefore not due to a cross-reaction with the muscle component of muscle fibres. Furthermore examination of the amino acid sequences of myosin by Bradley et al (1990) did not reveal a sequence that could be recognised by C219.

Previous studies with MRK16 have used formaldehyde fixation before immuncytochemistry, as cells fixed with acetone showed only weak localisation (Thiebaut et al, 1987). It could be argued that this is one reason why the positive staining was usually less with MRK16 in both epithelial and stromal cells. However, formaldehyde fixation with MRK16, when compared with acetone fixation with the same antibody, did not increase the sensitivity of the immunostaining in this study. This highlights the benefit of using a sensitive method of P-gp detection, the immuno-alkaline phosphatase method being particularly sensitive when compared to other immunohistochemical techniques.

The use of immunohistochemistry has not only allowed confirmation of P-gp expression in human breast cancer but has revealed a heterogeneous distribution of expression. This technique, as a result of its sensitivity, can localise immunoreactivity in small numbers of single cells, unlike measurement of mdr-1 mRNA in whole tumours (Fojo et al, 1987), which is less sensitive and can give

rise to erroneous results because of tumour cell heterogeneity or expression in adjacent normal tissue. Furthermore, without this method of detection the immunoreactivity in stromal cells, previously unreported in the literature, would not have readily come to light.

These results suggest that this particular mechanism of resistance may be relevant in the development of clinical drug resistance in breast cancer and if this is the case then P-gp may provide a potential target for modulation using specific agents known to circumvent MDR *in vitro* (see Chapter 4). The novel finding of P-gp expression in breast cancer stromal cells raises interesting questions about the role of these cells in the development of drug resistance and experiments in the following chapter will attempt to address some of these questions.

P-GP EXPRESSION, PATHOLOGY AND DISEASE-FREE SURVIVAL

Despite much evidence that P-gp plays a functional role in the multidrug resistance phenotype *in vitro* little is known about the contribution of this molecule, when present, to the overall clinical drug resistance of most tumours *in vivo*. The most striking evidence that P-gp may be clinically relevant in certain tumours is described in a study of thirty cases of childhood sarcoma (Chan et al, 1990). P-gp expression, detected by a modified immunoperoxidase method, predicted a significantly shorter relapse survival (p< 0.001) in chemotherapy-treated patients as well as a significantly smaller probality of survival (p<0.001). The results from the study, described in this chapter, of twenty-six informative patients with primary breast cancer suggest that those patients with the highest

levels of P-gp expression (>5% of tumour cells) have a worse prognosis in terms of disease-free survival. Although these results do not reach statistical significance and the numbers are small they nevertheless suggest a trend which will require further investigation. In contrast P-gp expression in stromal cells was not predictive for a worse disease-free survival. The results for epithelial expression are consistent with two recently published studies which examined P-gp expression by immunohistochemistry in locally advanced breast cancer.

In one series tumour samples from forty patients were obtained at mastectomy following three courses of chemotherapy with doxorubicin, cyclophosphamide, vincristine and prednisone. P-gp expression was observed in twenty patients (<5% - >30% of tumour cells) with expression significantly greater in tumours which showed a poor response to chemotherapy. Furthermore eight tumour specimens obtained prior to chemotherapy, which were all P-gp negative, had a complete response to chemotherapy. (Ro et al, 1990). The second study showed that seven of seventeen patients with non-metastatic breast cancer, who had intense staining in the majority of tumour cells, had a significant association between P-gp expression and both response to chemotherapy and the time to disease recurrence (Verelle et al, 1991). The latter study used a semiquantitative scoring system that considered the intensity of staining as well as the number of positive cells. The intensity of staining, however, can vary from batch to batch of tumours and can be influenced by several factors ie. the intensity of counterstaining and observer variation.

Both these studies, which have suggested that P-gp expression may be relevant in breast cancer, could be explained by a poor response to chemotherapy in P-gp positive patients. In contrast, however, none of the tumours described in this chapter had been exposed to cytotoxic drugs prior to surgery and although six patients received adjuvant post-operative chemotherapy P-gp expression did not predict a worse survival within that subset. Of these six patients there were two patients in each of the three groups, according to the percentage of p-gp expression in epithelial cells (0%, $\leq$5%, >5%), and one in each group relapsed prior to P-gp assessment.

Verrelle et al (1991) describe seven patients with >75% of tumour cells being P-gp positive. These percentages are much higher than the results described in this chapter and most published series but could be explained by the fact that all the tumours in their study were locally advanced (Stage II-III) and aggressive (Grade II & III). Indeed the poor prognosis associated with P-gp expression in the series of patients with primary breast cancer in this Chapter is apparently unrelated to clinical drug resistance. P-gp may in fact be a biological marker of tumour aggressiveness and this factor would explain both a poor response to chemotherapy and a worse survival.

This hypothesis is supported by a recent study of colonic carcinomas which suggested that P-gp-positive invasive colon cancer cells have an increased potential for dissemination (Weinstein et al, 1991). There was a significantly greater incidence of vascular invasion and lymph node metastases in tumours with P-gp-positive invasive cells when compared to the tumours with P-gp-

negative invasive cells. If P-gp expression is associated with local tumour invasion and dissemination then measurement of P-gp, together with other known prognostic factors eg. c-erbB2 (Borresen et al, 1990) may help to identify patients who may benefit from adjuvant treatment. Alternatively in locally advanced breast cancer, where there appears to be an association between P-gp expression and response to chemotherapy, measurement of this protein may help to identify tumours with significant MDR expression so that MDR drugs eg. anthracyclines can be avoided or so that modulators of MDR eg. quinidine may be incorporated into their treatment.

CHAPTER 3

CONFIRMATION OF P-GLYCOPROTEIN EXPRESSION IN BREAST CANCER STROMAL CELLS

INTRODUCTION

THE INTERACTION OF EPITHELIAL AND STROMAL CELLS

It is well recognised that there is a close relationship between the epithelial (malignant) and stromal components of human breast carcinomas. Since stromal cells have been shown to influence the growth of normal mammary epithelial cells in culture, as well as carcinogenesis in the breast epithelium (Sakakura et al, 1981), their presence in invasive breast cancer may be important in the proliferation and metastatic capacity of these cancers.

The presence of large numbers of myofibroblasts in schirrous carcinomas leads to the characteristic clinical changes caused by contraction of these cells eg. an indurated, irregular mass with retraction of the surrounding breast tissue and/or skin (Tremblay, 1979). Their presence has raised some debate as to whether they represent an enhanced host response to the tumour or whether they are an inherent part of the tumour itself. The finding of P-glycoprotein (P-gp) immunoreactivity in these stromal cells (see Chapter 2) is therefore worthy of further investigation firstly to confirm that the immunostaining represents true expression and secondly to attempt to identify how it may arise.

SECRETORY CAPACITY OF STROMAL CELLS

The majority of stromal cells in breast cancers are thought to be myofibroblasts (Barsky et al, 1984) which are known to function as secretory cells laying down collagen in the extracellular matrix. The finding of P-gp in these cells is therefore consistent with the finding of P-gp in cells which also have a secretory function eg. on the apical surface of epithelial cells of pancreatic ducts, proximal kidney tubules, gastrointestinal tract and the biliary canalicular surface of hepatocytes (Thiebaut et al, 1987). It is further supported by recent evidence of mdr-1 mRNA expression, detected by *in situ* hybridisation, in the zona glomerulosa and zona reticularis of human adrenal cortical tumours (Harvie, personal communication). These are thought to be the main sites of production and secretion of aldosterone and glucocorticoids.

One recent study supports this secretory role of breast cancer stromal cells and suggests that they may play an essential role in the propensity to induce tumour invasion (Basset et al, 1990). This study reported the secretion of Stromelysin 3, a   metalloproteinase enzyme which can degrade extracellular matrix, by breast cancer stromal cells and not by epithelial cells in the same tumour. Furthermore Stromelysin-3 gene expression was restricted to stromal cells in close proximity to the tumour cells. These findings, and the absence of expression in intraduct disease, has led to the hypothesis that a diffusible factor secreted by malignant epithelial cells may induce production and secretion of Stromelysin 3 by stromal cells. These results would support the suggestion that a group of "activated" fibroblasts may exist in invasive breast cancers (van der Hoof, 1988)

and their products may be important in tumour progression.

COMPETITIVE IMMUNOHISTOCHEMICAL STAINING

The six amino acid epitope where the monoclonal antibody C219 binds to P-gp is now known from a study of hamster P-gp (Bradley et al, 1990). By using overlapping hexapeptides, which covered the entire cytoplasmic domain of P-gp, in an ELISA test with C219 the exact locus was identified (Figure 7). The discovery of this sequence, **VQEALD**, led to the synthesis of a 15 amino-acid epitope-containing peptide which could abolish the immunostaining by C219 by preincubating the antibody with the peptide (Bradley et al, 1990).
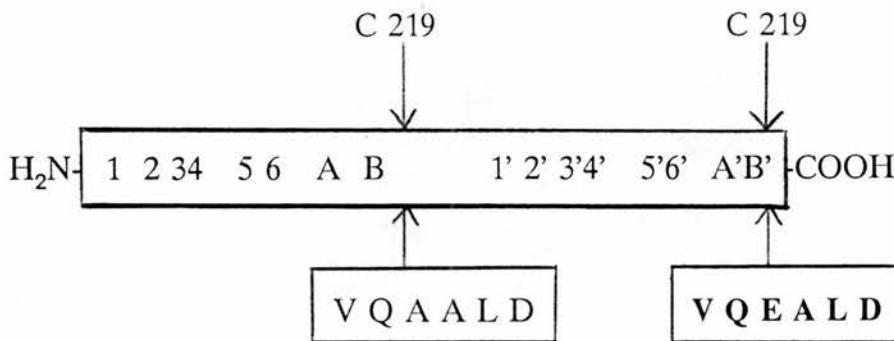
FIGURE 7
A schematic representation of the P-glycoprotein molecule showing the putatative transmembrane sites (1-6 and 1'-6'), the ATP-binding sites (A,B, and A',B') and the aminoacid sequence detected by C219.

The use of this peptide (**VVQEALDKARGGRTC**) therefore can provide a more specific negative control than either omission of the primary antibody or substitution by an irrelevant antibody. Omission of the primary antibody is used to detect staining due to non-specific binding of the second antibody and to detect the activity of endogenous enzymes which could react with the substrate. The peptide is used in the studies described in this chapter to confirm that the immunoreactivity in breast cancer stromal cells, described in Chapter 2, represents true expression of the protein and not a cross-reaction with some other antigen.

It is conceivable that breast cancer stromal cells may express P-gp either as a normal secretory process, to secrete collagen and other proteins, or as a specific part of the malignant phenotype releasing enzymes such as Stromelysin 3 into the extracellular matrix. The experiments in this chapter were therefore designed to try to address some of these questions.

AIMS OF THE STUDY

The primary aim of the study was to try to confirm genuine P-gp expression in stromal cells by the use of immunohistochemistry and competitive inhibition with a specific epitope-containing peptide.

The second aim was to compare P-gp expression, detected by immunohistochemistry with C219, in pairs of normal breast and breast cancer specimens from the same patient. If absent from the stromal cell population of normal breast tissue this would suggest that P-gp expression in breast cancer stromal cells was a transformation that arises *de novo* and not an enhanced form of a function present in normal tissue.

MATERIALS AND METHODS

TUMOUR SPECIMENS

For confirmation of P-gp expression five primary breast cancers, from the study described in Chapter 2, were selected because of the high incidence of P-gp immunoreactivity in their stromal cells (Table 4)

Tumour specimens from nine patients undergoing mastectomy at the University Department of Surgery, Western Infirmary, Glasgow were collected in theatre then snap frozen in liquid Nitrogen for storage at $-70^{\circ}C$. In the same patients a sample of "normal" breast was taken from the periphery of the mastectomy specimen and stored in a similar fashion until staining.

MONOCLONAL ANTIBODY AND BLOCKING PEPTIDE

For the detection of P-gp the monoclonal antibody C219 was used at a final concentration of 10µg/ml (see Chapter 2).

A 15 amino acid peptide was used to block the epitope on P-gp where C219 binds. This peptide (**VVQEALDKAREGRTC**) was a kind gift from Dr Elias Georges (Ontario Cancer Research Institute, Canada). The peptide was used at a final concentration of 670µM which represented a 1000 fold molar excess of peptide to antibody when incubated with C219 at a working concentration of 10µg/ml. Following incubation of peptide and antibody for one hour at room temperature

they were applied to the sections and immunohistochemistry was then performed.

IMMUNOHISTOCHEMISTRY

The indirect immuno-alkaline phosphatase method, exactly as described in Chapter 2, was used with the same positive control, the small cell carcinoma of lung line H69LX10. Immunostaining of tumour specimens was carried out on 5µm sections fixed in acetone at room temperature for ten minutes. When the peptide was used two sections from each tumour were stained, one with C219 and one with C219 and peptide.

PERCENTAGE OF P-GP POSITIVE CELLS

PATIENT 1	EPITHELIAL CELLS	STROMAL CELLS
1	<5	90
2	0	80
3	<5	80
4	<5	90
5	40	40

TABLE 4

The percentage of P-glycoprotein positive cells, either epithelial or stromal, in five tumours selected for use in the competitive immunohistochemical study.

RESULTS

<u>EFFECT OF THE PEPTIDE ON C219 REACTIVITY</u>

Incubation of the blocking peptide with C219, prior to immunostaining with an indirect alkaline phosphatase technique, completely inhibited staining in the multidrug resistant cell line H69LX10 (Figure 8a,b). When the same technique was used in the five tumour specimens it produced good amelioration of the immunostaining in the stromal cells (Figure 8c,d) but not to the same extent as in the control cell line. However, the amount by which staining was reduced in the stromal cells was the same as in the tumour cells (not shown).

<u>COMPARISON OF C219 REACTIVITY WITH NORMAL AND TUMOUR TISSUE FROM THE SAME PATIENT</u>

Immunostaining of the nine tumour sections revealed P-gp expression in both tumour (7/9) and stromal (7/9) cells with marked stromal staining (>50% of stromal cells) in 3/9 patients. In contrast however there was no staining in the stromal cells in any of the "normal" breast (Figure 9)and only some apical staining was seen in ductal epithelial cells (Figure 9c) in 2/9 patients. The two patients with expression in ductal cells of normal breast also had the tumours with the highest percentage of stromal cells which were P-gp positive.

a
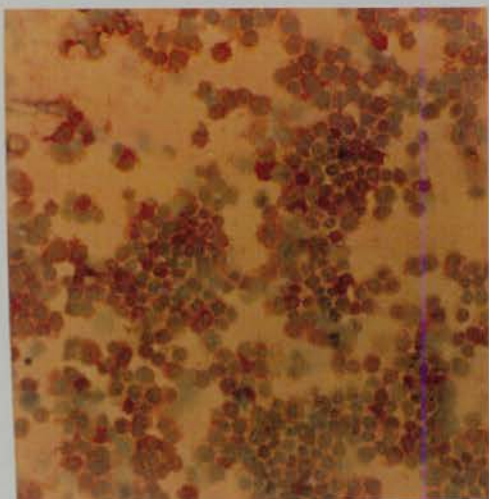
b
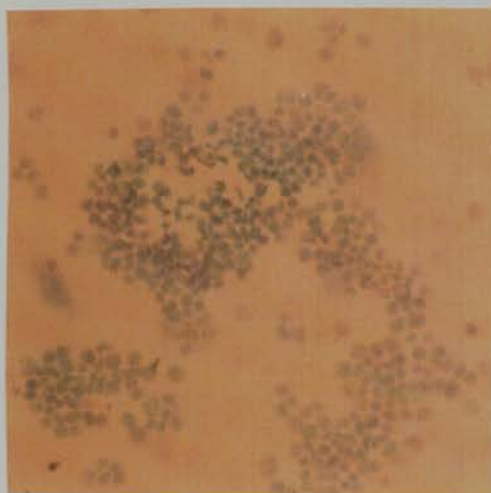
c
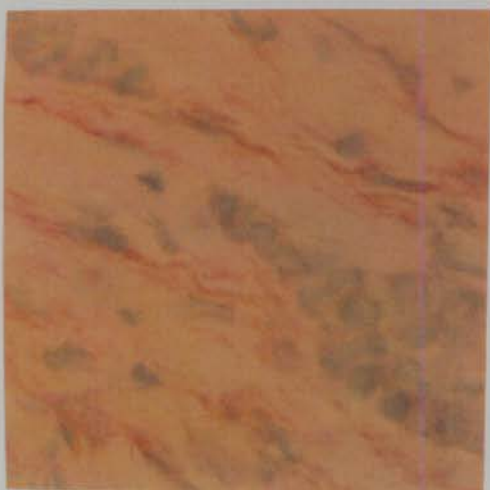
d

FIGURE 8

Immunohistochemical detection of P-glycoprotein (P-gp) in the multidrug resistant cell line H69/LX10 without **a** and with **b** blocking peptide. Immunostaining of P-gp in stromal cells of a primary breast cancer with **d** and without **c** blocking peptide.

a

b
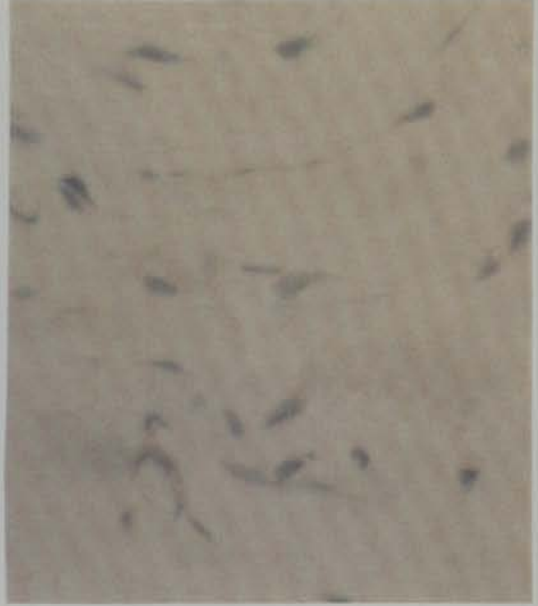
c

FIGURE 9
Immunostaining of P-gp in stromal cells of a primary breast cancer **a** and "normal"
breast specimen from the same patient **b**. P-gp expression in the apical portion
of ductal epithelial cells in a normal breast specimen **c**.

DISCUSSION

These results suggest that the immunoreactivity in breast cancer stromal cells, detected by the monoclonal antibody C219, represents genuine expression of P-gp. Although the staining was not abolished completely by the peptide blocking, as found in the positive control cell line H69LX10, this degree of amelioration would not be expected from a non-specific peptide. The use of a specific blocking peptide therefore provides a more specific negative control than omission of the primary antibody or substitution by an irrelevant antibody. In addition the expression of P-gp by MRK16 (see Chapter 2), which binds to a quite different epitope from C219, provides further confirmation of these findings.

These results are in keeping with recently published data which found P-gp expression by immunocytochemistry, with both C219 and MRK16, in stromal macrophages and endothelial cells in a variety of tumours (Schlaifer et al, 1990). Furthermore a recent study has identified a 150,000 dalton transmembrane protein, implicated in multidrug resistance, which is not P-gp and is also expressed in breast cancer stromal cells as well as malignant cells (Hickson et al, 1991). It remains to be established whether these transmembrane proteins in breast cancer stromal cells are involved in the MDR phenotype or whether they have a separate secretory function.

It has recently been established that P-gp is a small family of proteins with three members (Class I,II and III) in rodents and two members (Class I and III) in humans (Bradley et al, 1990). The Class III isoform, which is found in cardiac

muscle and a subset of skeletal muscle fibres, is not involved in multidrug resistance like the Class I isoform. The detection of P-gp by MRK16, which does not detect the Class III isoform in muscle (Wishart et al, 1990b), as well as C219 suggests that the P-gp detected in breast cancer stromal cells is the Class I isoform. Since the Class I isoform is present in many normal tissues including the liver and intestine (Fojo et al, 1987) it was important to ascertain whether P-gp expression was a feature of stromal cells in normal breast as well as breast cancers.

The finding of P-gp expression only in occasional ductal, epithelial cells, and not in stromal cells in "normal" breast suggests that the stromal cell expression in breast cancer is not an enhanced form of a normal mechanism. The stromal cells in "normal" breast and breast cancer would therefore appear to be quite diferent in certain respects. These results are consistent with the suggestion that a group of "activated" (Tremblay , 1979) or "abnormal" (Grey et al, 1989) fibroblasts may exist in malignant breast tumours. The latter study found that a population of fetal-like fibroblasts in breast cancers produce "migration stimulating factors", not produced by adult fibroblasts, which may contribute to the pathogenesis of breast cancer by interfering with the normal epithelial-mesenchymal interaction. Furthermore this group have demonstrated a fetal-like migratory phenotype of skin fibroblasts in approximately 90% of patients with familial breast cancer and 50% of clinically unaffected first degree relatives of these patients (Haggie et al, 1987). The latter observation implies that some genetic abnormality may lead to the development of a migratory phenotype in fibroblasts in patients with breast cancer.

It is well known that breast cancer fibroblasts can release a variety of proteolytic enzymes,which can break down extracellular matrix, including elastase, cathepsins and metalloproteinase (van der Hoof, 1988). There has been much debate as to whether the breakdown of extracellular matrix by fibroblast products is important in initiating tumours to metastasize. The finding of the Stromelysin 3 gene in invasive breast cancer stromal cells in close proximity to malignant cells (Basset et al, 1990) not only implicates these cells in the ability to metastasize but also raises the possibility of a close interaction between both stromal and malignant cells, possibly by the secretion of growth factors. The finding of P-gp expression in breast cancer stromal cells may have a secretory role in the "activated" fibroblast described by Tremblay (1979) or it may have a role in MDR not yet established. Whether this expression arises as a drug-resistant mechanism or not the presence of P-gp in breast cancer stromal cells may well interfere with current chemotherapeutic regimens. It is possible that P-gp may actually be involved in the secretion of some of these products in breast tumours. If this is the case then these "activated" stromal cells, or the external factors which stimulate them, may themselves become specific targets for anti-cancer therapy.

CHAPTER FOUR

CHARACTERISATION OF QUINIDINE AS A RESISTANCE MODULATOR WITH POTENTIAL FOR USE *IN VIVO*

INTRODUCTION

A greater understanding of the cellular mechanisms which underly the development of multidrug resistance (MDR) has raised hopes that this particular mechanism of resistance may be a suitable target for circumvention. Development of MDR is often associated with expression of P-glycoprotein (P-gp). This protein is thought to act as an energy-dependent drug efflux pump (Chen et al, 1986) such that resistant cells maintain a much lower drug concentration than their drug-sensitive counterparts (Kessel, 1968). The results presented in Chapter 2 show that P-gp is expressed in the majority of 29 previously untreated breast cancers. This protein, therefore, would seem an appropriate target for MDR modulation *in vitro* and potentially *in vivo*.

Further evidence that P-gp plays a functional role as a cytotoxic drug efflux pump is its ability to bind some of the drugs involved in the MDR phenotype eg. vinblastine (Cornwell et al, 1986). Cornwell and colleagues showed that photoaffinity labelling of two vinblastine analogues to P-gp was inhibited by vinblastine and vincristine but not actinomycin D, all drugs associated with the MDR phenotype. The fact that not all MDR drugs compete for the specific vinblastine-binding sites has led to the suggestion of the existence of more than one drug binding site on P-gp.

An important observation, however, is the ability of certain non-cytotoxic drugs eg. quinidine and verapamil, which have been shown to overcome MDR *in vitro*, to compete for these binding sites (Cornwell et al, 1987). A number of other membrane-active compounds have also been shown to partially reverse multidrug resistance *in vitro* including nifedipine (Tsuruo et al, 1983), calmodulin inhibitors eg. trifluoperazine (Ganapathi et al, 1983), cyclosporin (Twentyman et al, 1987) and also amiodarone (Chauffert et al, 1987). It is not certain how all of these modulators bring about reversal of MDR *in vitro* although it is known that verapamil causes a significant increase in phosphorylation of the P-gp molecule (Hamada et al, 1987) and may actually act as a competitive substrate for P-glycoprotein (Horton et al, 1989).

Although there are many compounds which do circumvent MDR *in vitro* their clinical potential is often limited by the inability to achieve plasma levels, adequate for MDR modulation *in vitro*, which do not cause adverse toxicity. As a result of dose-limiting hypotension and heart block in patients with ovarian cancer (Ozols et al, 1987) the plasma levels of verapamil achieved without toxicity are several fold lower than the most effective concentration *in vitro* (Kerr et al, 1986). Verapamil therefore has limited clinical use and attempts are now being made to identify compounds which are as potent MDR modulators as verapamil but which do not have the same patient toxicity.

Quinidine (6'-Methoxycinchonine), a well recognised MDR modulator *in vitro* (Tsuruo et al, 1984), has been used for a number of years as an anti-arrhythmic agent in patients (Mason et al, 1977) raising hope that it may have potential as an

in vivo MDR modifier if quinidine levels adequate for MDR modulation in vitro can be achieved without patient toxicity. Despite the recognition that quinidine (Figure 10) induces several functional changes in biological membranes the exact mechanism underlying its therapeutic effect is not known although it has been suggested that an interaction with the lipid bilayer can cause increased membrane fluidity (Surewicz & Jozwiak, 1983).
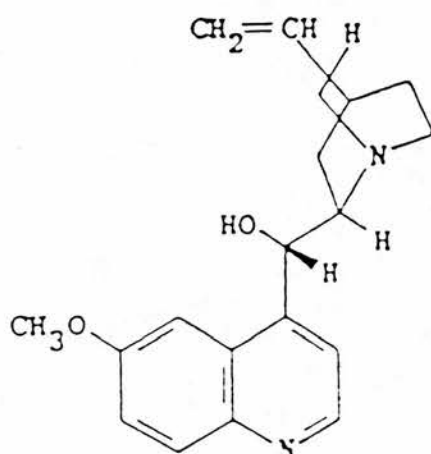
FIGURE 10
The molecular structure of quinidine (6'-methoxycinchonine).

A pilot study of quinidine, combined with epirubicin in the treatment of advanced breast cancer, has shown that a mean steady state plasma level of 5.6μM can be achieved following a four day course of oral quinidine (250mg twice daily) with minimal toxicity (Jones et al, 1990). Although symptoms of cinchonism (dizziness, tinnitus, visual disturbance and vomiting) can occur at higher doses of quinidine they are uncommon at this dose. This is encouraging since quinidine has been shown to be as effective as verapamil *in vitro* on a mole per mole basis and the maximum plasma levels of verapamil achievable are only about 1-2 μM.

One further problem encountered in attempting to modulate MDR *in vivo* is that many of the circumventing agents are highly protein bound in plasma eg. quinidine (Iven, 1977). In addition it has also been shown that the addition of exogenous alpha-1 acid glycoprotein (AAG) can reverse the modulating effect of verapamil on doxorubicin cytotoxicity in doxorubicin-resistant Chinese hamster ovary cells (Chatterjee et al, 1990). Chatterjee's study concluded that in addition to a plasma membrane site there may be an endosomal site of action of verapamil in MDR cells suggesting that resistance modifiers may not be effective in patients with high plasma levels of AAG. These results have raised some doubt that tumour levels of modifiers may not be adequate for *in vivo* MDR modulation in patients. Furthermore, with increased levels of the acute phase plasma protein alpha-1 acid glycoprotein in many cancer patients (Snyder & Ashwell, 1971), which is known to bind to other modifiers eg. verapamil (Piafsky et al, 1978), the free plasma quinidine may be decreased even further.

The aims of the study described in this chapter were firstly to characterise the MDR modulatory activity of quinidine *in vitro* to confirm whether a concentration of 5.6μM represents the optimal concentration for modulation. The second aim was to determine whether such quinidine concentrations could be achieved in tumours. Ideally it would have been more appropriate to carry out a basic pharmacokinetic study of quinidine, including plasma and tumour levels, in patients with breast cancer. Clearly the number of patients required to establish a plasma time-course of quinidine due to the wide variation in quinidine metabolism between patients make such an approach unrealistic. The study was therefore carried out in a human MDR xenograft model where multiple time-course experiments are possible and there is less variation between animals. However, in addition paired samples of plasma and tumour, for quinidine estimation, were obtained from three patients with breast cancer.

In order to carry out this pharmacokinetic study of quinidine tumour levels *in vivo* it was neccessary to have a readily usable method for the estimation of plasma and tissue quinidine concentrations in the laboratory. This was therefore established and was compared to the quinidine immunoassay used in the clinical study mentioned previously (Jones et al, 1990).

MATERIALS AND METHODS

CELL LINES

The human breast carcinoma cell line, MCF7 ^{ADR}, was used as an *in vitro* model of MDR. This cell line, which exhibits resistance to doxorubicin *in vitro,* was derived from the drug sensitive parent cell line MCF7 by chronic exposure to doxorubicin. Both cell lines were obtained from Dr K Cowan (National Cancer Institute, Bethesda, USA).

The cells were maintained in RPMI 1640 (Northumbria Biologicals, Cramlington, Northumberland, England) supplemented with glutamine 2mM (Gibco, Paisley, Scotland), 10% fetal calf serum (Globepharm, Esher, Surrey, England) and sodium bicarbonate. The resistant subline, MCF7^{ADR}, was exposed to doxorubicin (10 µM) for 24 hours every six weeks and has been shown previously to express P-gp (Fairchild et al, 1987).

HUMAN TUMOUR XENOGRAFT

The drug-resistant human ovarian carcinoma cell line 2780AD, known to express P-gp (Sugawara et al, 1988), was obtained from Dr RF Ozols (National Cancer Institute, Bethesda, USA). The cell line was maintained in RPMI 1640 culture medium supplemented with glutamine 2mM (Gibco, Paisley, Scotland), 10% fetal calf serum (Globepharm, Esher, Emgland) and insulin (0.25 units/ml). The resistance of this line was maintained by growth in the presence of doxorubicin

(2uM).

The 2780AD cell line was grown as a xenograft in female MF1 nunu athymic mice by subcutaneous innoculation of approximately 10^6 cells in an injection volume of 0.5 ml PBS. Following establishment of the xenograft three weeks later the tumour was passaged into a further fifteen mice and allowed to grow. The mice were kept at a constant temperature using a twelve hour light/dark cycle with free access to food and water.

HUMAN TUMOUR BIOPSIES

Three patients undergoing breast surgery at the University Department of Surgery, Royal Infirmary, Glasgow were treated with oral quinidine bisulphate (Astra, Kings Langley, England) 250mg twice daily for four days prior to surgery (total dose 2g) to achieve a steady-state level. This study was carried with the approval of the local Ethical Committee and with informed consent from the patients. Following excision of the tumour, 3-4 hours following the last dose of quinidine, a representative sample was snap frozen in liquid Nitrogen and stored at -70°C until assayed. In addition a blood sample was also taken during surgery and plasma stored at -20°C until assayed.

MODULATORS OF MDR

Three potential modulators of MDR were investigated: quinidine, verapamil and tamoxifen (all Sigma Chemical Company, Poole, Dorset, England). Each modulator was used at a final concentration of 6.6 μM, made up in RPMI 1640, and added to the cells for 24 hours with the doxorubicin. In addition cells were also exposed to epirubicin in combination with various concentrations of quinidine (3.3μM, 6.6μM and 9.9μM).

CYTOTOXICITY ASSAY

Drug sensitivity was determined by a tetrazolium dye based microtitration assay (Plumb et al, 1989). The assay is based on the ability of live, but not dead, cells to reduce a tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to produce a water-insoluble formazan product and the resulting colour reaction can be quantified by an enzyme-linked immunoassay (ELISA) plate reader. The system is rapid, economical and the optical quality of the formazan product lends itself to automatic handling. Moreover, the results are comparable to more time-consuming techniques eg. clonogenic assay (Plumb et al, 1989). Since the assay is carried out in 96 well microtitre plates combinations of potential modulators and drug concentrations can be used in the one experiment.

Cells were plated out in 200 μl of medium at a concentration of 1000 cells per well in nine 96 well microtiter plates (Linbro; Flow Laboratories, Irvine, Scotland). The first and last columns contained medium only. The plates were incubated for three

days at 37°C in an atmosphere of 2% CO_2 in air to allow the cells to attach and grow.

The medium was then removed from the wells and replaced with 200 μl of fresh medium containing drug and modulator. Each modulator was added to three 96 well plates. Serial dilutions of eight different concentrations of cytotoxic drug were made up in RPMI 1640 at twice the final concentration. These were then diluted with either drug-free medium or medium containing modifier at twice the final concentration prior to addition to the wells. Cells were exposed to either doxorubicin or epirubicin, both at a top concentration of $10^{-4}M$. Both drugs were supplied from Farmitalia (St. Albans, England). The plates were incubated for 24 hours at 37_o^0C with drug and modulator after which the medium was then replaced with drug-free medium. This medium was replaced on each of the next two days.

On the fourth day following drug exposure the cells were fed with 200 μl fresh medium. 3 - (4,5 - Dimethylthiazol - 2 - yl) - 2,5 - diphenyl - tetrazolium bromide (MTT; Sigma) was then added at a concentration of 5 mg/ml made up in phosphate buffered saline, 50 μl per well. The plates were wrapped in aluminium foil and incubated for 4 hours at 37 C. The medium and MTT were then removed from the wells and the insoluble MTT formazan crystals dissolved in dimethyl sulphoxide (DMSO), 200 μl per well. In addition 25 μl of Sorenson's glycine buffer (0.1M glycine, 0.1M NaCl, equilibrated to pH 10.5 with 0.1M NaOH) was added to each well.

The absorbance in each well was recorded in an ELISA plate reader (model 3550 microplate reader; Bio-rad Laboratories Ltd, Watford, England) at a wavelength of 570 nm. The first and last columns, which contained medium only, were used to blank the plate reader.

Cytotoxicity was expressed in terms of the ID_{50}, that is the concentration of drug which gave half the absorbance of the control untreated cells.

ESTIMATION OF INTRACELLULAR DRUG ACCUMULATION

The breast carcinoma cell line $MCF7^{ADR}$ was again used as an *in vitro* model of multidrug resistance. Doxorubicin uptake was measured in the presence of a range of concentrations of quinidine.

Cells were grown in six well plates (Nunclon) for two days to give a final density of approximately 10^6 cells per well. Following removal of the medium 4ml of fresh medium was added to each well and the plates allowed to equilibrate at 37°C in an atmosphere of 2% CO_2 in air. (^{14}C) Doxorubicin and doxorubicin were added in 0.5 ml of medium and quinidine sulphate (Sigma, Poole, England) in 0.5 mls of medium was added to the wells to give a final doxorubicin concentration of 1 µM and 0.01 uCi/ml. For comparison three concentrations of quinidine sulphate were used: 3.3µM, 6.6µM, and 9.9µM at the five hour timepoint.

At specified times (30, 60, 120, 180, 240, 300 minutes) the plates were placed on ice and the medium removed. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and incubated with 0.5 mls trypsin/EDTA (0.25%/1mM) for five minutes at 37°C. The contents of the wells were transferred to scintillation vials, the wells washed with 0.5ml PBS and the wash added to the vials. Scintillation fluid (10ml, Ecoscint fron National Diagnostics, Somerville, USA) was added to each vial and the radioactivity determined in a Packard liquid scintillation counter (Canberra, Packard, Pangbourne, England). Three wells were used for each timepoint and the cell counts were determined from an additional three wells.

QUINIDINE PHARMACOKINETICS

ANIMALS

Non tumour-bearing MF1 nunu athymic mice were used for study. They were kept at a constant temperature in a twelve hour light/dark cycle with free access to food and water.

DRUG ADMINISTRATION AND SAMPLING

Quinidine sulphate (Sigma, Poole, England) was dissolved in phosphate-buffered solution (PBS) at a concentration of 7.5 mg/ml then filtered using a 0.22um MILLEX-GS filter (Millipore, Molsheim, France). All mice were weighed and at time 0 received a bolus intraperitoneal injection of quinidine 150 mg/kg. Three mice were sacrificed at each time point (30 minutes, 1 hour, 2 hours, 3hours and 4 hours), blood collected by cardiac puncture and plasma stored at -20°C until assayed.

ESTIMATION OF TUMOUR QUINIDINE LEVELS

Nude mice bearing the 2780AD xenograft (tumour volume approximately 2cm^3) were used for study. All mice were weighed, then given a bolus intraperitoneal injection of quinidine sulphate 150mg/kg made up at a concentration of 3.75 mg/ml in PBS. Two hours post injection the mice were sacrificed, blood was obtained by cardiac puncture and plasma stored in 200ul aliquots at -20°C. In addition the subcutaneous tumours were excised, weighed and stored at -70°C until assayed.

ESTIMATION OF QUINIDINE

IMMUNOASSAY

Total (protein-bound and unbound) plasma quinidine concentrations, in non tumour-bearing mice, were measured using the Emit^R assay kit (Syva UK, Maidenhead, England), an enzyme-linked immunoassay based on competition between quinidine in the sample and quinidine labelled with glucose-6-phosphate dehydrogenase (G6P-DH) for antibody binding sites. Since G6P-DH activity decreases upon binding to the antibody the sample quinidine can be measured in terms of enzyme activity. The active G6P-DH converts nicotinamide adenine dinucleotide (NAD) to NAD-hydrogenase (NADH) resulting in an absorbance change over a period of time that is measured spectrophotometrically.

Aliquots (50μl) of plasma samples were thawed prior to assay. Each sample was diluted 1:6 with Emit^R buffer (buffer) solution. A 50μl aliquot of this diluted sample was again diluted 1:6 with the buffer. This was added to Emit^R quinidine reagent A (50μl/250μl buffer) and Emit^R quinidine reagent B (50μl/250μl buffer). The absorbance of this sample was then immediately measured in a Perkin Elmer spectrofluorophotometer at 340nm following calibration with known standard quinidine concentrations. The results were calculated from the change in absorbance over a thirty second time period.

EMIT^R REAGENTS

Reagent A : mouse monoclonal antibody to quinidine

glucose-6-phosphate

NAD

pH 5.2

Reagent B : quinidine labelled with G6P-DH

pH 6.2

Buffer : 0.055mol/L Tris buffer

pH 8.0

All these products also contain 0.05% sodium azide.

FLUOROMETRIC ASSAY

The quinidine concentration of plasma and tumours was measured using a spectrofluorophotometric technique which relies on the ability of quinidine to fluoresce in an acid medium (excitation and emission wavelengths 350nm and 450nm respectively). Measurement was carried out in a Shimadzu RF-540 recording spectrofluorophotometer which compares samples to one standard of known concentration.

The extraction was performed according to the original method of Cramer & Isaakson (1963) with the modification that quinidine was extracted into n-heptane instead of benzene (Iven, 1977). Plasma quinidine was extracted from a working volume of 200ul murine plasma made up to 1ml with distilled water. Sodium hydroxide (0.5ml 0.2N) and sodium chloride (0.5g) were added and the quinidine extracted into 2.5ml n-heptane containing 3% isoamylalcohol (v/v). Sulphuric acid (1ml 0.1N) was added to 2ml of the organic phase (upper phase) and following gentle shaking the fluorescence of the aqueous phase (lower phase) was measured in the spectrofluorophotometer (see above). In addition a 1ml sample of human plasma spiked with 10μg quinidine was used as a recovery control, to calculate the extraction ratio, and a normal human plasma was used as a negative control. The results are expressed in μg/ml.

All tumour samples were homogenised in sulphuric acid (5ml, 0.1N) and each batch processed included a spiked sample for estimation of recovery and a negative control. The homogenates were added to sodium hydroxide (0.2ml 1.5N) containing

0.5g sodium chloride and the extraction then proceeded as for plasma. The results were corrected for the extraction of the recovery control and expressed in μg/g tumour. In order to compare plasma and tumour levels 1g of tumour was equated to 1ml of plasma and a tumour/plasma extraction ratio calculated.

Quinidine was extracted from human plasma in exactly the same way as for murine plasma except a working volume of 1ml undiluted human plasma was used. Quinidine was extracted from human tumours exactly as for murine tumours.

RESULTS

<u>EFFECT OF QUINIDINE ON DOXORUBICIN AND EPIRUBICIN CHEMOSENSITIVITY IN MCF7^{ADR} CELLS</u>

The sensitivity of MCF7^{ADR} to doxorubicin is shown in Table 5. Also shown in this table is the sensitivity of these cells when exposed to doxorubicin in the presence of either quinidine (6.6μM), verapamil (6.6μM) or tamoxifen (6.6μM). All three modulators were similarly effective at a concentration of 6.6uM with a modulating effect of 7-10.5 fold.

The combination of epirubicin and quinidine (6.6μM) produces a much better modulating effect (22.5-fold) than doxorubicin and quinidine (Table 6). In addition quinidine , when used in combination with epirubicin, is an effective modulator over the range 3.3-9.9μM. The effect of quinidine 9.9uM on sensitivity to epirubicin is demonstrated in Figure 11.

<u>EFFECT OF QUINIDINE ON INTRACELLULAR DOXORUBICIN ACCUMULATION</u>

The uptake of doxorubicin in MCF7^{ADR} cells is much less than the drug sensitive cell line MCF7. Quinidine increases the intracellular doxorubicin concentration but not to the level of drug in MCF7 cells (Figure 12). Quinidine caused a very small increase in doxorubicin accumulation in MCF7 cells.

DRUG(S)	ID50 (µM)	MODULATION
Dox	43.0 ± 2.31	-------
Dox + Quin	6.17 ± 1.46	7-fold
Dox + Ver	4.10 ± 0.62	10.5-fold
Dox + Tamox	5.30 ± 2.36	8-fold

TABLE 5
The chemosensitivity, expressed as an ID_{50} of MCF7^{ADR} cells to doxorubicin alone and in combination with quinidine (quin), verapamil (ver) and tamoxifen (tamox), all 6.6µM.

DRUG(S)	ID50 (µM)	MODULATION
Epirubicin (E)	33.6 ± 4.16	-----
E + Q (3.3µM)	2.15 ± 1.23	15.5-fold
E + Q (6.6µM)	1.50 ± 0.17	22.5-fold
E + Q (9.9µM)	0.67 ± 0.21	50-fold

TABLE 6
The chemosensitivity of MCF7^{ADR} cells to Epirubicin (E) alone and in combination with Quinidine (Q) at three different concentrations: 3.3µM, 6.6µM and 9.9µM.

76
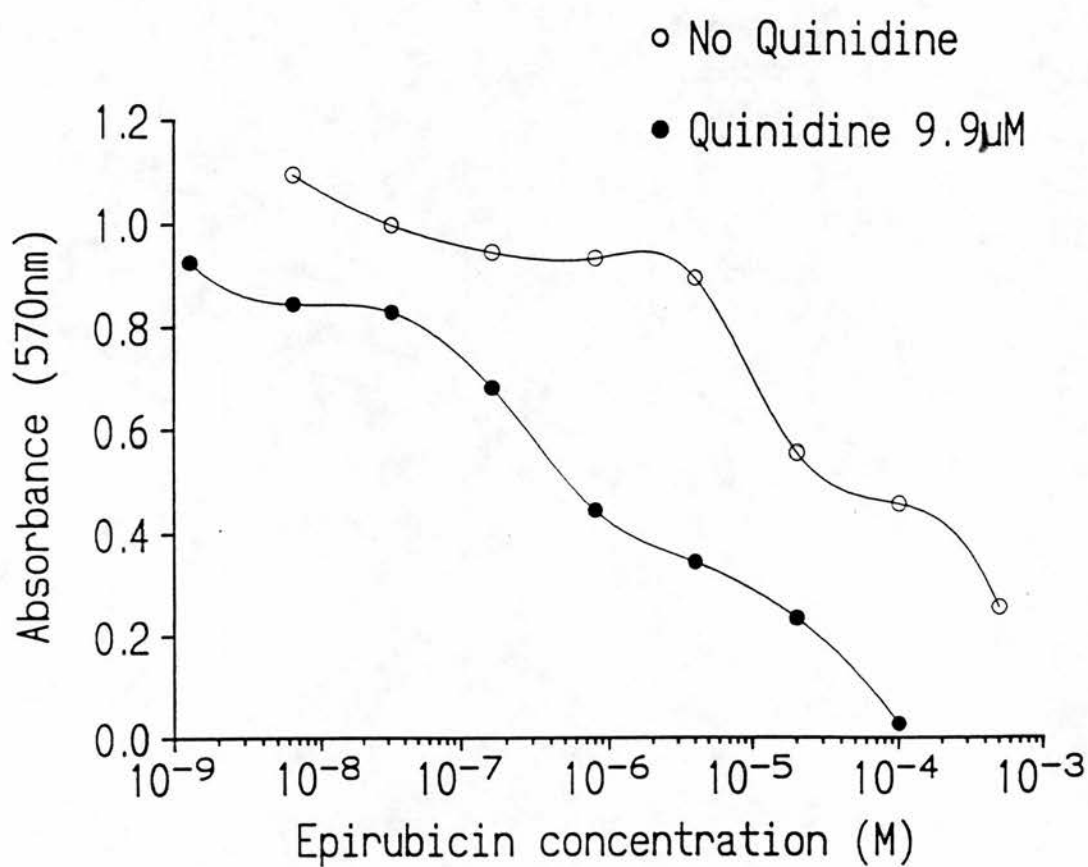
FIGURE 11
Plot of absorbance versus the concentration of epirubicin in MCF7^{ADR} cells exposed for 24 hours to epirubicin alone and in combination with quinidine 9.9μM.
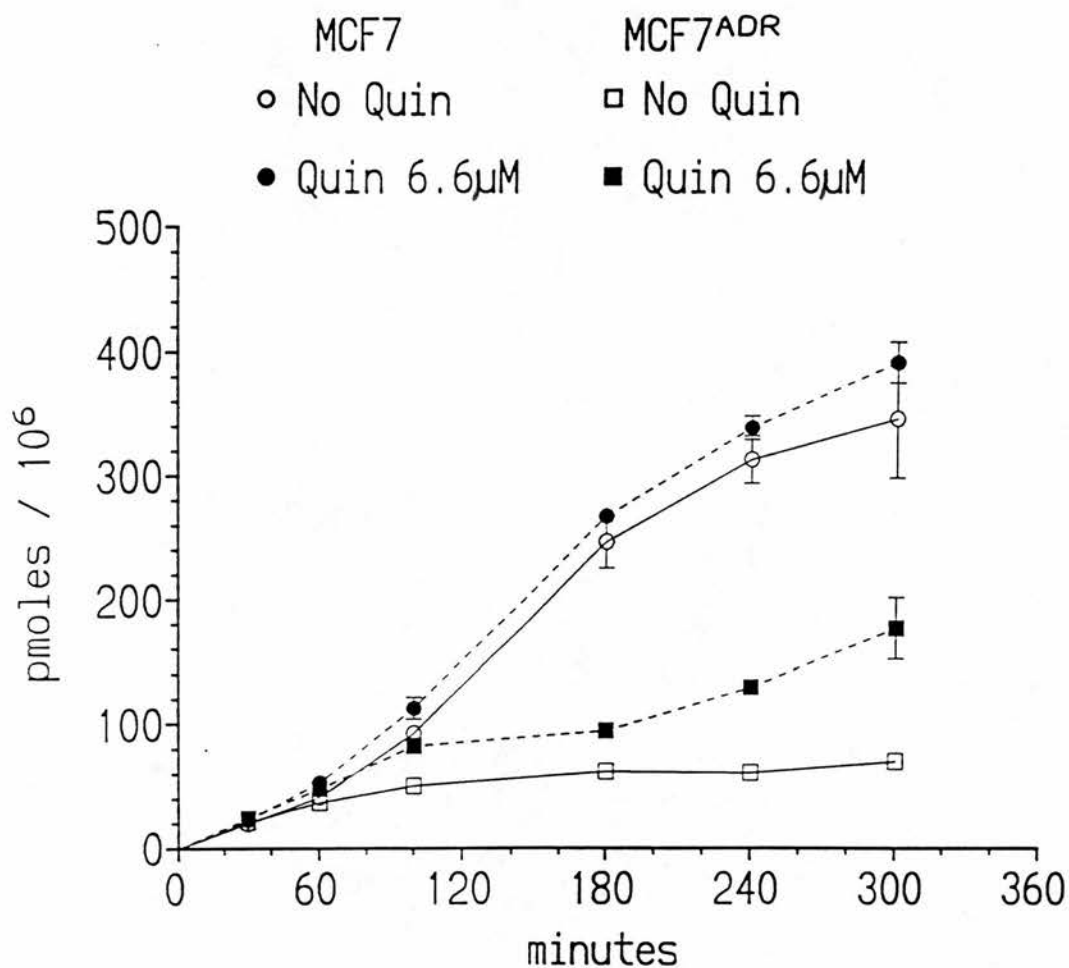
FIGURE 12

The effect of quinidine on the intracellular uptake of doxorubicin in MCF7 and MCF7^{ADR} cells exposed to doxorubicin alone and in combination with quinidine 6.6µM.

PLASMA QUINIDINE TIME COURSE IN NON TUMOUR-BEARING MICE

The results of plasma quinidine concentrations in non tumour-bearing mice following an intraperitoneal injection of quinidine sulphate are shown in Figure 13. This shows an exponential decrease of the initial high level following injection with detectable levels still present four hours post injection.
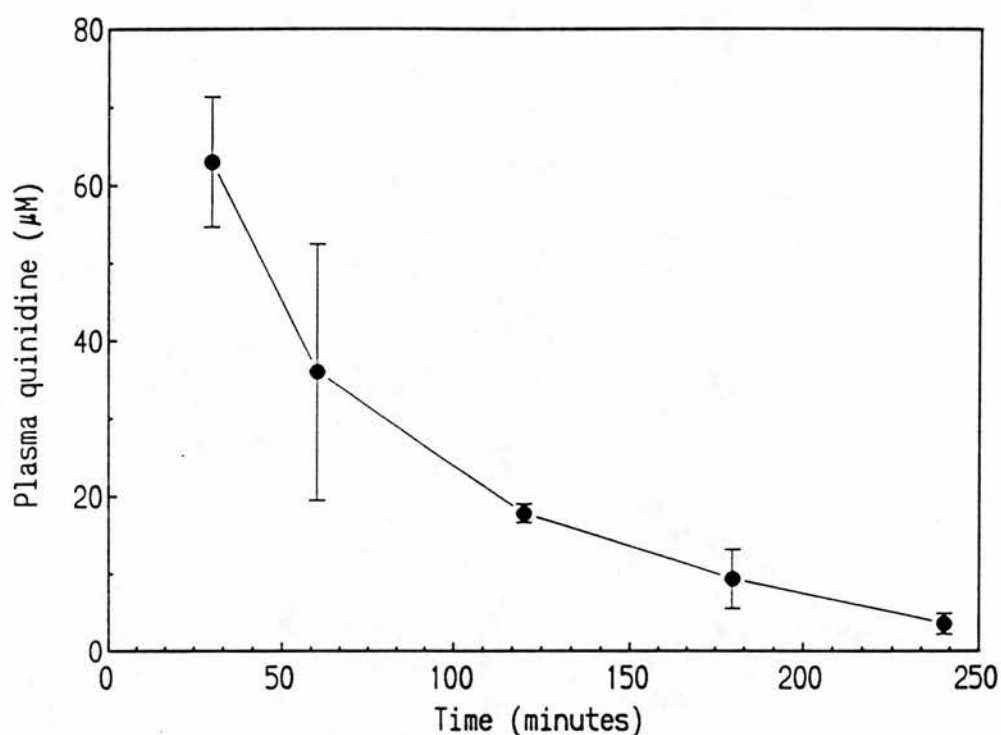
FIGURE 13
Plasma quinidine concentrations (mean ± SD) in MF1 nunu mice (non tumour bearing) following a bolus intraperitoneal injection of quinidine sulphate 150mg/kg. Each point represents the mean of three samples.

QUINIDINE ESTIMATION

The standard graphs of known quinidine concentrations versus arbitrary units produced by the spectrofluorophotometer, when samples are compared to one known quinidine concentration only, are shown in Figure 14a,b. This shows a linear relationship, both at low (10ng/ml 1000ng/ml) and high (250ng/ml-10µg/ml) concentrations confirming that quinidine concentrations can be calculated from unknown samples when the spectrofluorophotometer is calibrated with one concentration only (10µg/ml). The technique was also validated in terms of recovery, with a mean quinidine recovery of 88.2% (range 83.7-93.1%) in four spiked human plasma samples, and reproducability with the error between samples being <5%. In addition a comparison of this fluorometric assay with the immunoassay provided results that were comparable.

COMPARISON OF PLASMA AND TUMOUR QUINIDINE LEVELS

Plasma and tumour quinidine concentrations in ten tumour bearing nude mice are shown in Table 7. One plasma was lost during the extraction procedure due to a breakage. The mean plasma quinidine concentration was 1.94 µg/ml (range: 0.21-4.01). This represents a mean plasma concentration of 5.13 µM (range: 0.55-10.58). The mean tumour concentration was 6.04 µg/g (range: 2.46-12.24 µg/g). The mean tumour/plasma extraction ratio was 9.36 (range: 1.20-33.50). The recovery of the spiked plasma and tumour controls was 55% and 77% respectively.
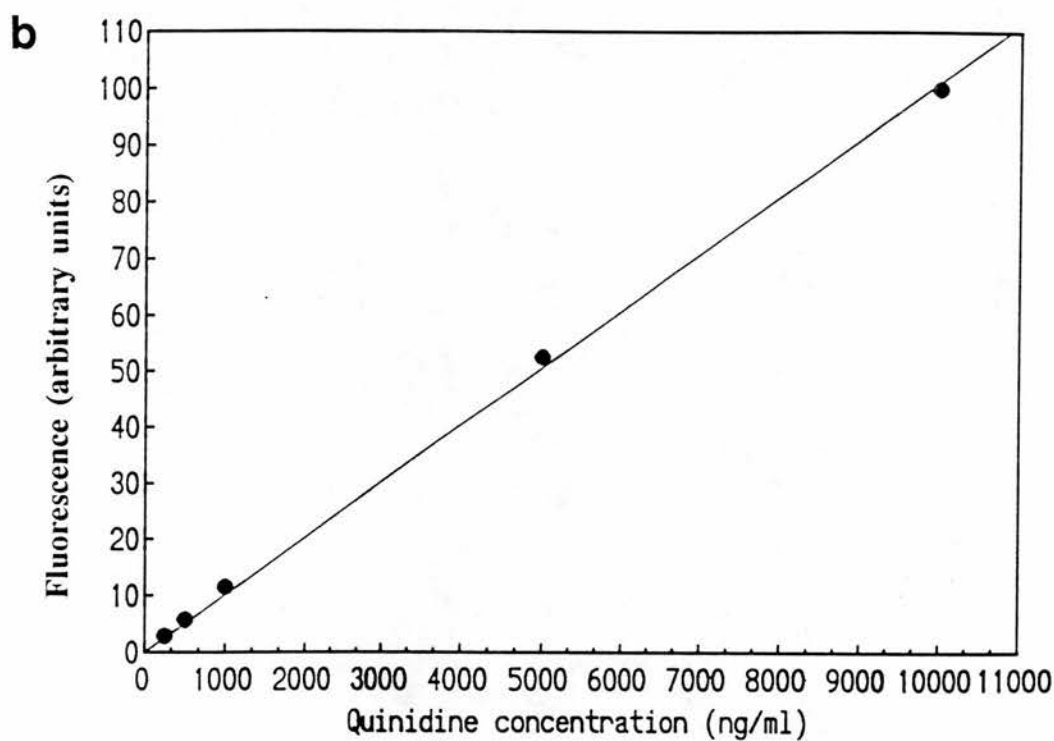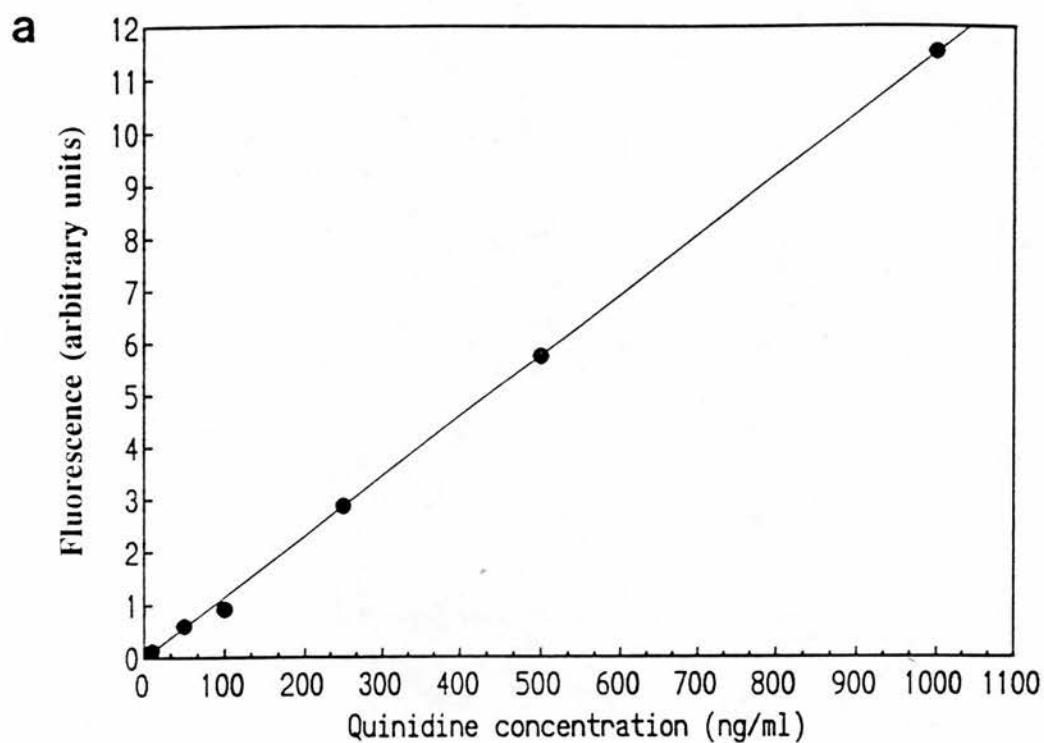
FIGURE 14
Graphs showing linear relationship between quinidine concentration and arbitrary units produced by the spectrofluorophotometer, both at low (a) and high (b) concentrations, when samples are compared to one known quinidine concentration (10μg/ml).

QUINIDINE CONCENTRATION

ANIMAL	PLASMA (μM)	PLASMA (μg/ml)	TUMOUR (μg/g)	TUMOUR/PLASMA EXTRACTION RATIO
1	8.04	3.05	4.95	1.63
2	----	----	12.24	----
3	4.20	1.59	2.46	1.55
4	4.30	1.63	8.95	5.49
5	10.58	4.01	5.87	1.46
6	9.11	3.45	5.53	1.60
7	7.93	3.01	3.61	1.20
8	0.55	0.21	7.04	33.50
9	0.62	0.24	6.21	26.40
10	0.82	0.31	3.55	11.45

TABLE 7
The results of plasma (μM & μg/ml) and tumour (μg/g) quinidine concentrations in ten tumour bearing nude mice two hours following a bolus, intraperitoneal injection of quinidine sulphate 150mg/kg. The tumour/plasma extraction ratio is also shown.

HUMAN QUINIDINE CONCENTRATIONS

Plasma and tumour quinidine concentrations in three patients undergoing surgery for breast cancer are shown in Table 8. The mean plasma concentration was 1.48 µg/ml (range: 1.05-1.84 µg/ml). This represents a mean plasma concentration of 4.54 µM (range: 3.22-5.65 µM). The mean tumour concentration was 1.39 µg/g (range: 1.25-1.46 µg/g). The mean tumour/plasma extraction ratio was 1.0 (range: 0.68-1.39). The recovery of quinidine was 99% (plasma) and 86% (tumour).

QUINIDINE CONCENTRATION

PATIENT	PLASMA (µM)	PLASMA (µg/ml)	TUMOUR (µg/g)	TUMOUR/PLASMA EXTRACTION RATIO
1	3.22	1.05	1.46	1.39
2	4.76	1.55	1.45	0.93
3	5.65	1.84	1.25	0.68

TABLE 8
The plasma (µM & µg/ml) and tumour (µg/g) quinidine concentrations in three patients with breast cancer pretreated with oral quinidine 250mg twice daily for four days prior to surgery. The tumour/plasma extraction ratio is also shown.

DISCUSSION

The results clearly show that MDR may be partly reversed in a P-gp positive human breast carcinoma cell line (MCF7^{ADR}) *in vitro* by the use of agents known to interact with P-gp. The degree of modulation achieved with verapamil (10.5-fold), tamoxifen (8-fold) and quinidine (7-fold) in combination with doxorubicin is of a similar magnitude to previously published studies (Plumb et al, 1990; Ramu et al, 1984; Tsuruo et al, 1984). Modulation with quinidine was dose-dependent over the range 3.3-9.9µM when used in combination with epirubicin. This demonstrates that quinidine can be an effective *in vitro* MDR modulator even at a concentration less than the optimal concentration (6-7µM) of verapamil required for MDR reversal *in vitro*. Furthermore it is interesting to note that quinidine, when given in combination with epirubicin, produced a much higher fold increase in sensitivity than with doxorubicin. This observation has been noted elsewhere (Plumb, personal communication) and indicates a significant difference between the interaction of epirubicin and doxorubicin and P-gp. This is surprising in view of the similarity in the structure of epirubicin and doxorubicin (Weenen et al, 1983) although it is well documented that the cardiotoxicity of epirubicin is much less than that of doxorubicin (Muggia & Green, 1991).

The results of the doxorubicin uptake study demonstrate that part of the increased chemosensitivity in MCF7^{ADR} cells, following exposure to quinidine, is due to elevated intracellular drug concentrations. Although the amount of intracellular doxorubicin could be increased approximately 2-fold by quinidine this was still much less than intracellular drug level in MCF7 cells. It is difficult to understand how

apparent 1-2 fold increases in intracellular drug concentration can effect large increases (15-50 fold) in chemosensitivity. This may however be explained by recent evidence which has suggested that modulation, as well as increasing the total amount of intracellular drug, may also redistribute this drug within the cell (Schuurhuis et al, 1989).

MOUSE QUINIDINE PHARMACOKINETICS

Following intraperitoneal quinidine sulphate (150 mg/kg) plasma quinidine concentrations of approximately $60\mu M$ were achievable 30 minutes post injection in non tumour-bearing mice. Although quinidine was still detectable 4 hours post injection there was a significant clearance of quinidine from the plasma within the first two hours. Based on the assumption that clearance from plasma is related to appearance of quinidine in tissues it was decided to use 2 hours as the time point for simultaneous measurement of tumour and plasma quinidine concentrations. Although this time was chosen empirically it was hoped that by two hours, when quinidine concentrations were in excess of the levels required for MDR modulation *in vitro*, the tumour levels would have reached a detectable level.

Two methods were used for the measurement of quinidine. The immunoassay, used to measure plasma quinidine in non tumour-bearing mice, was not readily available in the laboratory and it was therefore neccessary to establish a new assay for the detailed pharmcokinetic study. As a result the fluorometric quinidine assay was developed and this gave similar results to the immunoassay when plasma quinidine samples were measured by both methods. The fluorometric assay was standardised

over a range of concentrations of quinidine and was validated in terms of recovery and reproducability with a small error between measurements. This assay involved an organic extraction and this stage was found to be the most variable between experiments. Thus the recovery figures quoted for human plasma appear greater than that for mouse samples. However, provided recovery was assessed for each batch of samples this problem was overcome.

The results of mean plasma (1.94µg/ml) and tumour (6.04µg/g) quinidine concentrations in ten tumour-bearing mice following a bolus intaperitoneal injection of quinidine sulphate (150mg/kg) gave extremely encouraging results. Not only was the tumour quinidine detectable in all cases, the tumour/plasma extraction ratio was greater than one at a time when the mean plasma quinidine concentration was 4.5 uM. Furthermore when plasma and tumour quinidine concentrations were measured in three patients with breast cancer, who were pretreated with oral quinidine for four days prior to surgery, these results were upheld with a mean tumour/plasma extraction ratio of 1.0.

It is however notable that the extraction ratio for the mouse samples was much greater than that for humans. This is possibly explained in part by the fact that the protocol for quinidine administration differed in the two studies. However, it should be noted that the human tumour xenograft was highly vascularised and thus the measurement of tumour levels will include a significant quantity of plasma quinidine. No attempt was made to estimate the blood volume of the tumour although this would allow correction for this contamination. Since the vasculature was not confined to the periphery of the tumour but penetrated throughout the tumour the

results can be interpreted as a high concentration of quinidine in close proximity with the tumour cells. Clearly the vascular supply to human tumours varies greatly depending on the tumour type and location and no animal model will correctly reflect this pattern of vascularisation. Since it has already been shown that doxorubicin can penetrate into breast tumours (Stallard et al, 1990) the xenograft model used in this study is thought to be a fair comparison.

In conclusion the experiments described in this chapter confirm that quinidine is an effective modulator of MDR *in vitro* and furthermore that quinidine levels required for modulation *in vitro* can be achieved both in an animal model of MDR but more importantly in human tumour biopsies. It is hoped that these results will allay fears of inadequate tumour concentrations of quinidine, and other modulators, due to high plasma protein binding (Iven, 1977) and increased levels of alpha-1 acid glycoprotein in cancer patients (Snyder & Ashwell, 1971). An attempt to modulate MDR with quinidine in the human tumour xenograft, which was based on these encouraging results, is described in the next chapter.

CHAPTER 5

MODULATION OF MULTIDRUG RESISTANCE *IN VIVO*

INTRODUCTION

There is now much evidence to support the theory that MDR can be reversed *in vitro* by modulators which interact with P-gp. It is known that a wide range of human tumours express P-gp (Fojo et al, 1987) but it is not known whether this protein has a functional role in the development of clinical drug resistance. If P-gp does act as a drug efflux pump in MDR cells in human tumours then it should theoretically be possible to circumvent this function *in vivo* using modulators that have been shown to work *in vitro*. Although there is much data supporting a reversal of MDR *in vitro* there have been very few animal studies reported and this, together with the lack of suitable modulators in patients, has meant there is much less evidence suggesting that modulators have a therapeutic benefit *in vivo*.

In one of the few animal modulator studies published the calcium influx blocker, nicardipine, was shown to enhance the cytotoxicity of vincristine in the treatment of adult female mice innoculated intraperiteonally with a vincristine-resistant subline of P388 leukaemia cells, P388/VCR (Tsuruo et al, 1983). This resulted in prolonged survival in the groups treated with modulators when compared with those groups treated with vincristine alone. Calcium influx blockers also enhanced the cytotoxicity of doxorubicin in the treatment of the doxorubicin-resistant subline of the P388 leukaemia cells, P388/ADR. In contrast verapamil and a tiapamil analogue (DMDP) failed to potentiate doxorubicin activity in mice innoculated with

doxorubicin-resistant P388 leukaemia cells (Radel et al, 1988). These studies however are inconclusive with respect to the potential for MDR modulation in patients since they are not solid tumour models and the drug and tumour are being delivered to the same site. In effect the peritoneal cavity of the mice is being used as tissue culture medium for the resistant cells . In one study where verapamil was tested in a solid tumour model it was shown to partially reverse resistance to vincristine in a human epidermoid lung cancer xenograft when given with vincristine (Mattern et al, 1987). These differing results highlight the difficulty in establishing a good animal model of MDR in which to test potential modulators with the timing, route of administration, and dosage of both modulator and drug being extremely important.

To date only two published series have suggested that MDR modulation may be of benefit in MDR-positive human tumours. The first study involved six myeloma or lymphoma patients who relapsed during treatment with vincristine, adriamycin and dexamethasone (VAD). At relapse the addition of verapamil, given by continuous intravenous infusion with each pulse of chemotherapy, to the VAD regime resulted in an objective response in three of six (50%) patients with P-gp positive tumour cells but no response in P-gp negative tumours (Dalton et al, 1989). A second study of 18 lymphoma patients, who had all failed to respond to a doxorubicin containing regime, reported a 72% response rate when these patients were further treated with cyclophosphamide, VAD, as well as a five day verapamil infusion (Miller et al, 1991).

In contrast to both these studies a phase I-II study failed to demonstrate a potentiating effect for doxorubicin in eight drug resistant ovarian cancer patients

(Ozols et al, 1987). However, P-gp expression was not documented in these tumours. The dose-limiting toxicity of verapamil in both clinical studies was transient cardiotoxicity, manifest as hypotension and heart block, which was reversible on stopping the verapamil. This cardiotoxicity, which limits plasma levels to between three and twelve times lower than the most effective concentration *in vitro* (Kerr et al, 1986), is thought to be due to calcium antagonism, a function separate and not neccessary for MDR modulation.

Current studies, therefore, are attempting to identify compounds which are effective modulators of MDR, both *in vitro* and *in vivo*, but which do not cause unacceptable toxicity in patients when administered in combination with chemotherapeutic agents. Plasma levels of quinidine, which were shown to modulate MDR *in vitro* (see chapter 4), can be achieved in patients without adverse toxicity (Jones et al, 1990) suggesting a possible role for this compound as an *in vivo* modulator. Furthermore, it was also demonstrated that administration of quinidine to both mice and patients gave levels in the tumour adequate for resistance modulation. Quinidine was therefore chosen for a detailed investigation of the ability of resistance modulators to overcome drug resistance in an animal model of MDR.

AIMS OF THE STUDY

Since human breast cancers are solid tumours, like many other human tumours which acquire clinical drug resistance, it was neccessary to establish a solid tumour animal model of MDR to test the potential modulator, quinidine. As discussed the intraperitoneal models used by Tsuruo (1983) and Radel (1988) were not suitable.

Unfortunately the growth of breast cancer cell lines as xenografts in mice is extremely difficult and requires hormonal manipulation and this would interfere with any attempt to influence growth delay.

The multidrug resistant ovarian carcinoma cell line (2780AD) was already established as a xenograft in the Department and this model was thus the obvious choice for these studies. The study was designed to determine whether the combination of a modulator with a cytotoxic drug could produce a significant tumour growth delay. Three modulators were included in the study. Verapamil is an effective modulator *in vitro* but it is thought that dose limiting toxicity prevents its use *in vivo*. The assumption was, therefore, that this modulator would have little or no activity. Quinidine had been identified as a modulator that was as effective as verapamil *in vitro* but had the advantage that higher plasma levels could be achieved both in animals and, more significantly, in patients. However, it was apparent that even for quinidine these plasma levels were only just adequate. It was therefore decided to include a modulator (RO11-2933) that is highly potent *in vitro* at only 2 μM, a concentration easily achieved in mice (Yin et al, 1989).

RO11-2933 is a tiapamil analogue that has little activity as a calcium antagonist. It is, therefore, not associated with the dose limiting toxicity of the calcium antagonists. It has never been used in humans and therefore does not have the immediate clinical interest associated with quinidine.

In the design of this study consideration was given to the amount of modulator used, the timing of modulator and drug administration and the route of administration. The amount of quinidine administered was aimed at the maximum tolerated dose in mice on the basis that this was most likely to show an effect. The timing of modulator administration is complicated therefore a simple regimen was used based on quinidine pharmacokinetics (see Chapter 4) and assumptions about the length of duration of activity of epirubicin ie. for several hours following administration. In terms of administration of drugs the intraperitoneal route is the simplest where large numbers of mice are involved but the cytotoxic drug was shown to be toxic via this route so it was given intravenously eventually.

It was decided to use epirubicin since greater modulation with epirubicin than doxorubicin has been demonstrated (see Chapter 4) and furthermore it is the commonest single chemotherapeutic agent used in the treatment of advanced breast cancer in the West of Scotland (Habeshaw et al, 1990). In addition a Phase III placebo-controlled, double blind trial is currently ongoing, in the West of Scotland, to assess the effect of oral quinidine on the response to chemotherapy with epirubicin in patients with advanced breast cancer.

MATERIALS AND METHODS

TUMOUR MODEL

The maintenance of the 2780AD cell line, the establishment of the subcutaneous xenograft and the maintenance conditions of the mice are described in the "Materials and Methods" section in Chapter 4.

TISSUE DISPOSITION OF EPIRUBICIN

Twenty-four tumour-bearing, female, athymic mice were weighed then randomised to receive quinidine (Sigma, Poole, England) or no quinidine prior to treatment with epirubicin (Farmitalia, St Albans, England). The quinidine treatment group were given four doses of quinidine sulphate (136.8mg/kg) intraperitoneally at -26 hours, -18 hours, -10 hours and -2 hours prior to epirubicin treatment to attempt to achieve adequate levels for MDR modulation.

At 0 hours all mice were given an intravenous, bolus injection of epirubicin, 38mg/kg, via the tail vein. The dose of both epirubicin and quinidine was converted for the mouse from human doses of 100mg/m^2 and 500mg (total daily dose of quinidine) respectively (Carter et al, 1977). At 1 and 4 hours following epirubicin administration animals were anaesthetised with ether, and blood and organs collected. A futher quinidine dose at +6 hours was administered to the 12 hour post treatment group before sacrifice.

Heart, liver, small intestine, kidney, muscle and tumour tissue was stored at -70°C prior to analysis for epirubicin levels at 1, 4 and 12 hours post epirubicin administration. Plasma was stored at -20°C until assayed.

ESTIMATION OF EPIRUBICIN IN MOUSE PLASMA AND TISSUES

All tissues were washed and weighed prior to homogenisation. A 1:10 tissue to buffer homogenate was prepared by adding a nine-fold weight/volume (w/v) excess of phosphate buffered saline. Epirubicin was extracted from 0.2ml to 1ml of homogenised tissue pre-treated with silver nitrate (33% w/v, 200ul per ml homogenate) by vortexing with chloroform-propanol (2:1). Daunorubicin was used as an internal standard for estimation of the extraction efficiency. The organic phase was evaporated to dryness and finally reconstituted in 0.1ml of methanol before analysis by High Pressure Liquid Chromotography (HPLC).

Chromatographic separation and quantification of epirubicin in tissues and plasma was performed by reverse phase liquid chromotography on a 4mm x 25cm u-bondpak C18 column. 10ul injections of standards and unknown samples were programmed to run at 15 minute intervals on a Milton Roy "Promis" autosampler. Detection was by fluorescence (excitation 480nm, emission 560nm) and integration of peak areas was performed on a JCL6000 chromatography data system.

GROWTH DELAY EXPERIMENT WITH INTRAPERITONEAL ADMINISTRATION OF EPIRUBICIN

The tumour had been passaged three times from implantation of cells into the mice prior to this experiment and tumours were approximately 1.5 cm^3 at the start of the experiment.. Epirubicin (Farmitalia, St Albans, England) was made up at a concentration of 1 mg/ml in distilled water.

TREATMENT SCHEDULE

10 tumour-bearing mice were weighed then randomly allocated to one of two groups:

Group 1   No treatment   (n = 5)

Group 2   Epirubicin alone   (n = 5)

Epirubicin was given as an IP bolus 10 mg/kg in an injection volume of 10 μl/g. Tumour volume was measured by calipers using the following formula, where d = the mean of two diameters in the horizontal plane.

VOLUME $= \dfrac{\Pi \times d^3}{6}$

Measurements were made on day 0 (just prior to treatment), day 3, day 5 and day 7. In addition the mice were weighed at the same time.

ANIMAL TOXICITY STUDIES

Mice bearing the 2780AD xenograft were used for study. A dose ranging experiment was carried out in groups of four mice for each modulator. The dose intervals used were 10mg/kg for verapamil (range 50-100mg/kg), 25mg/kg for quinidine (range 160-250mg/kg) and 20mg/kg for RO11-2933 (range 140-250mg/kg). LD50 and LD10 values were derived from probit analysis of the data.

GROWTH DELAY WITH INTRAVENOUS ADMINISTRATION OF EPIRUBICIN IN COMBINATION WITH RESISTANCE MODULATORS

Epirubicin was given by an intravenous injection into the tail vein at the same dose that was given intraperitoneally (10 mg/kg).

Tumour bearing mice were weighed then randomised to receive no treatment, epirubicin alone, epirubicin and quinidine, epirubicin and verapamil or epirubicin and RO11-2933. The modulators were given by bolus IP injection two hours prior to and four hours following epirubicin at the following doses (number of animals in parenthesis):

Quinidine 150 mg/kg   (n = 6)

Verapamil 50 mg/kg   (n = 6)

RO11-2933 30mg/kg   (n = 6)

In addition to the three groups described above there were 6 mice in each of the two

control (untreated and epirubicin alone) groups. The mice were weighed and the tumours measured daily.

STATISTICS

The tumour volume of each mouse on days 3, 5 and 7 was compared to the pre-treatment tumour volume and the difference expressed as a percentage of the initial weight to correct for any differences in size between groups. This ratio was then converted to a logarithmic function and the five study groups compared by analysis of variance. Any significant differences were further analysed by pairwise group comparison using Bonferoni P values.

ESTABLISHMENT OF THE XENOGRAFT CELL LINE IN CULTURE

A tumour was aseptically removed from a mouse. It was dissected into 1mm pieces and incubated with collagenase (200units/ml; Boehringer, Lewes, England) in RPMI 1640 medium. After several days epithelial cells were removed by incubation for five minutes at 37°C with 0.5ml trypsin/EDTA (0.25%/1mM). These cells were plated out in 25cm^3 flasks (Nunclon) and allowed to attach and grow. These cells were used both for detection of P-gp and for assessment of drug sensitivity of the cells.

Cytospin preparations were made by spinning cells suspended in PBS onto clean glass microscope slides in a cytospin centrifuge (Shandon). These were stained by an immuno-alkaline phosphatase method using a specific antibody to P-gp, C219. This method is fully described in Chapter 2.

The protocol for the cytoxicity assay was described in Chapter 4 and was carried out using the three modulators used in the study; Quinidine (6.6µM), Verapamil (6.6µM) and RO11-2933 (6.6µM).

CONFOCAL MICROSCOPY

By using a laser scan microscope in confocal mode "optical sectioning" can be carried out at levels of focus which are spaced less than 1µm apart thus eliminating out-of-focus epifluorescence. Several images can then be superimposed to produce a single, crisp image with a good depth of focus. This technique is therefore ideal for examing the subcellular localisation of fluorescent drugs in tumour sections.

The intracellular distribution of epirubicin was studied in four of the 2780AD xenografts, two from mice treated with epirubicin alone and two from mice treated with epirubicin and quinidine. Quinidine (150mg/kg) was administered as an intraperitoneal bolus two hours prior to intravenous epirubicin (10mg/kg) given as a bolus tail vein injection. The mice were sacrificed two hours post-epirubicin and the tumours stored at -70°C until sectioning.

Tumour frozen sections 20µm thick were cut from the tumours and mounted without fixation in Glycergel water-based medium (Dako, High Wycombe, England). The distribution of epirubicin was analysed on a Carl Zeiss laser scan microscope (LSM 10) by confocal microscopy with Argon laser excitation at 488nm. The sections were examined to look for any differences in subcellular localisation between the two treatment groups.

RESULTS

EFFECT OF QUINIDINE ON EPIRUBICIN PLASMA, TISSUE AND TUMOUR
CONCENTRATIONS

The epirubicin concentrations (mean $\pm$ S.D.) in plasma, tissue and tumour at
specified times following an IP bolus of epirubicin 38 mg/kg are shown in Figures
15 & 16. Also shown are the epirubicin concentrations obtained when quinidine
(136.8mg/kg) was given by IP bolus prior to epirubicin. Although the one hour
plasma epirubicin concentration was reduced by the addition of quinidine this
decrease was not maintained and was not reflected in either increased tumour or
tissue epirubicin levels over this time course.
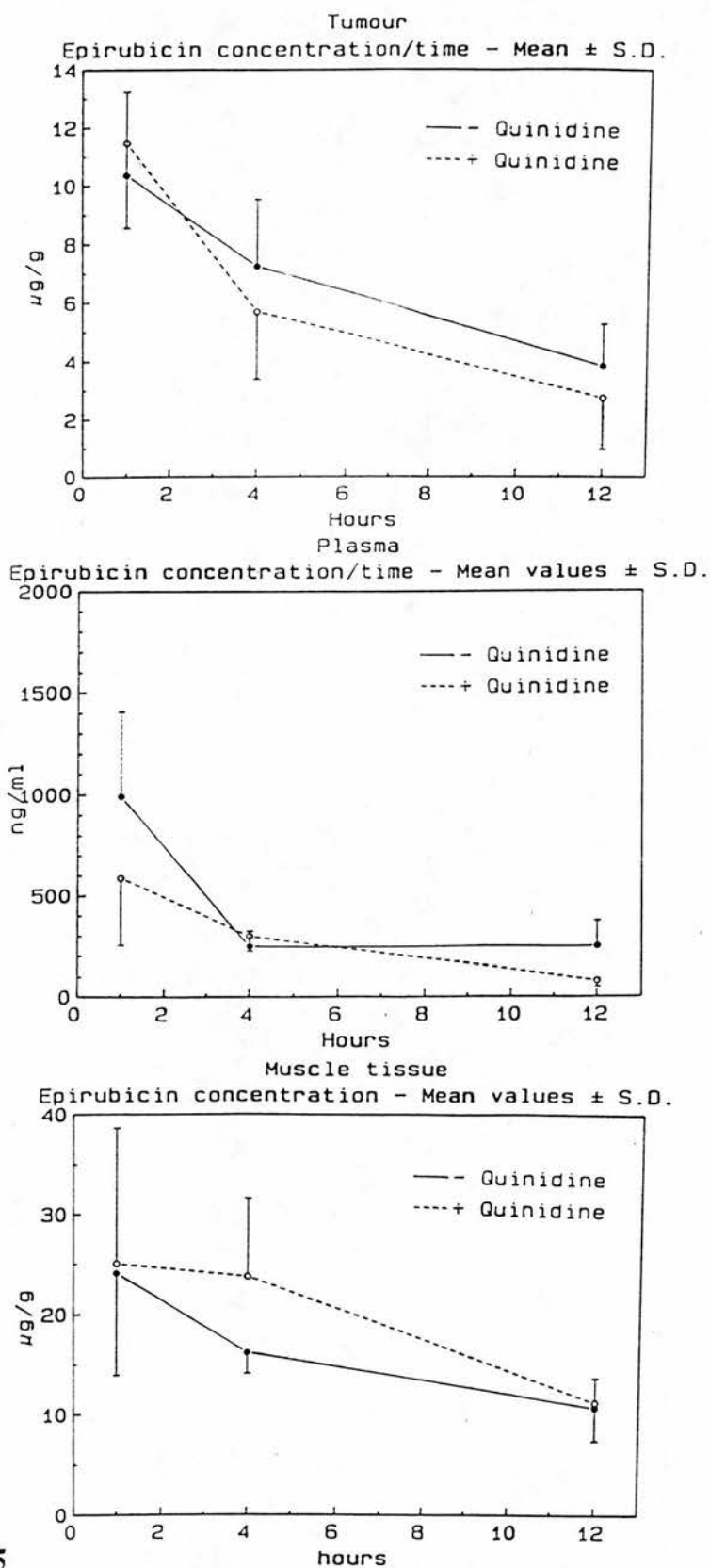
FIGURE 15

Plasma, tumour and tissue epirubicin levels at 1, 4 and 12 hours following intraperitoneal epirubicin (38mg/kg) alone and in mice pretreated with intraperitoneal quinidine sulphate. Each point represents the mean of three samples.
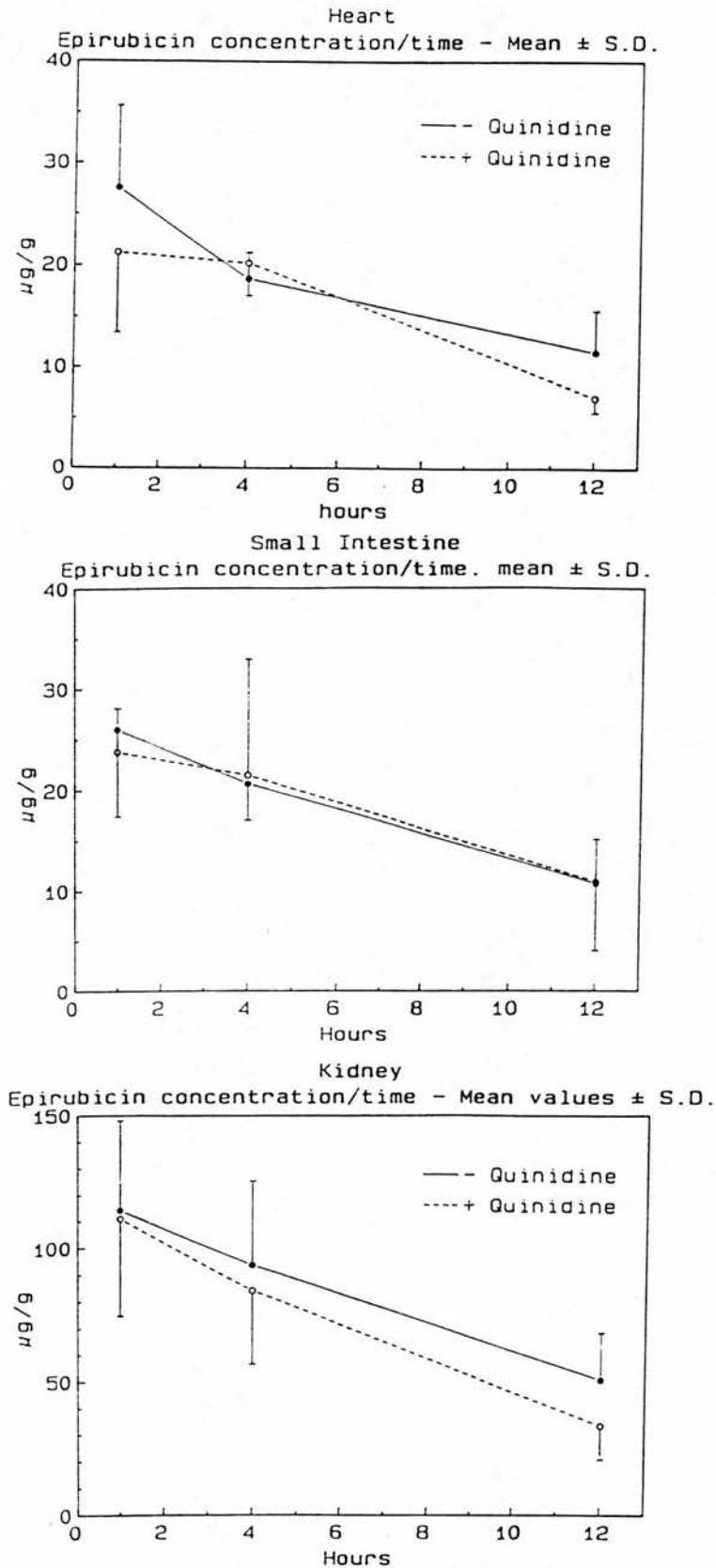
FIGURE 16
Tissue epirubicin levels at 1, 4 and 12 hours following intraperitoneal epirubicin
(38mg/kg) alone and in mice pretreated with quinidine 136.8mg/kg intraperitoneally
2 hours prior to epirubicin. Each time point represents the mean of three samples.

EFFECT OF INTRAPERITONEAL ADMINISTRATION OF EPIRUBICIN ON BODY WEIGHT

The untreated group maintained their body weight while the epirubicin-treated group lost weight rapidly and became cachectic. The mean body weight of both groups, expressed as a percentage of the initial weight, is shown in Figure 17.

TUMOUR VOLUME

Epirubicin had no effect on tumour growth. There was no significant difference between groups in the mean change in tumour volume, expressed as a percentage of the initial pretreatment tumour volume, on days 3, 5 or 7.

ANIMAL TOXIXITY STUDIES

The acute toxicity in tumour bearing mice of the three modulators is shown in Table 9. For verapamil and quinidine deaths were observed within the first hour after administration of the drug. In contrast, no immediate toxicity was observed with RO11-2933 and all deaths occured between 3 and 24 hours after drug administration.
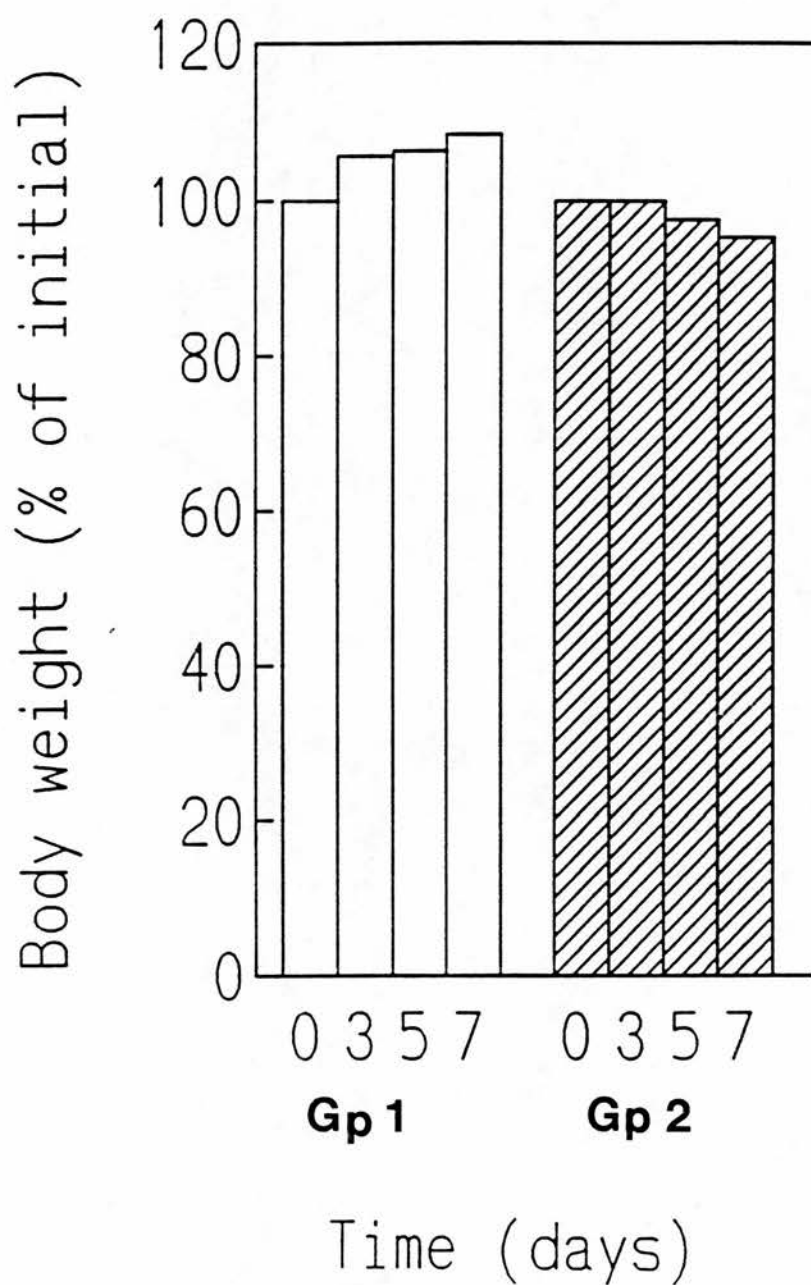
FIGURE 17
The mean body weight , expressed as a percentage of the pretreatment weight, in 2780AD tumour-bearing nude mice receiving the following treatment: Group 1, no treatment; Group 2, intraperitoneal epirubicin (10mg/kg).

MODIFIER	LD10 (mg/kg)	LD50 (mg/kg)
VERAPAMIL	51 (32-81)	75 (62-92)
QUINIDINE	185 (164-207)	204 (191-219)
RO11-2933	152 (129-180)	180 (160-203)

TABLE 9
Acute toxicity of three modulators (verapamil, quinidine and RO11-2933) in mice bearing the 2780AD xenograft. Drugs were administered intraperitoneally and 4 mice were used at each dose.

EFFECT OF INTRAVENOUS ADMINISTRATION OF EPIRUBICIN ON BODY
WEIGHT

All five groups, both treated and untreated, maintained or increased their body
weight and looked healthy during the period of study.

EFFECT OF INTRAVENOUS ADMINISTRATION OF EPIRUBICIN ON
TUMOUR GROWTH RATE ALONE AND IN COMBINATION WITH
RESISTANCE MODULATORS

The tumour doubling time was approximately two days (Figure 18). When groups
were compared according to the change in tumour volume between days 0-2, 0-5 and
0-7 significant differences were found at day 2 but not at days 5 or 7. Treatment
with epirubicin alone showed a slight, but not significant, growth delay when
compared to the untreated group. The addition of verapamil resulted in an
increased growth delay which again was not significant and resulted in two deaths
in this group, within the first 24 hours after drug treatment.

Addition of either quinidine or RO11-2933 caused a highly significant growth delay
when compared to the untreated group ($p < 0.001$) and the group treated with
epirubicin alone ($p = 0.01$). These findings are highlighted in Figure 19 which shows
the mean relative tumour volume in each group during days 1-7. Furthermore the
growth delay during days 0-2 is more apparent when the relative tumour volumes of
individual mice are plotted (Figure 20). This shows that mice treated with either
quinidine or RO-112933 show little tumour growth during days 0-2 when compared

with both control groups.
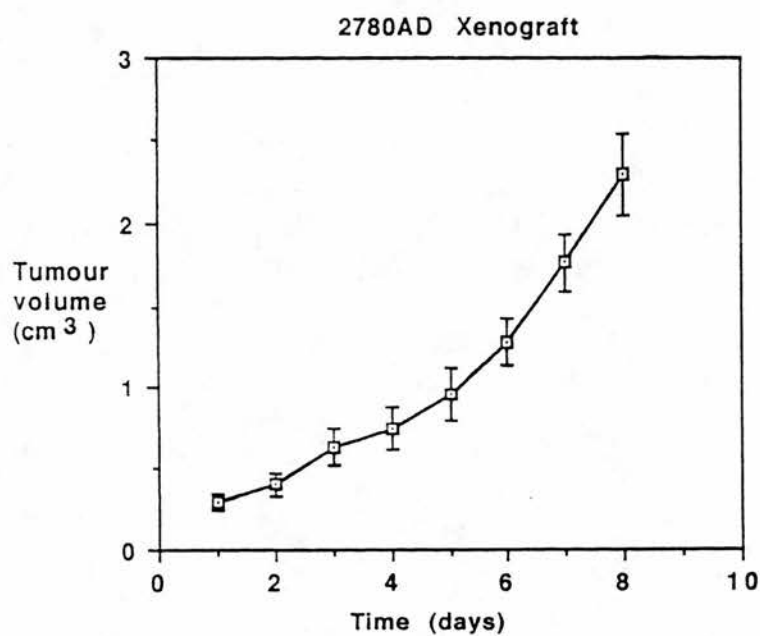
2780AD Xenograft

FIGURE 18

Tumour volume in mice bearing the 2780AD xenograft showing an approximate doubling time of 2 days.

Time (days)
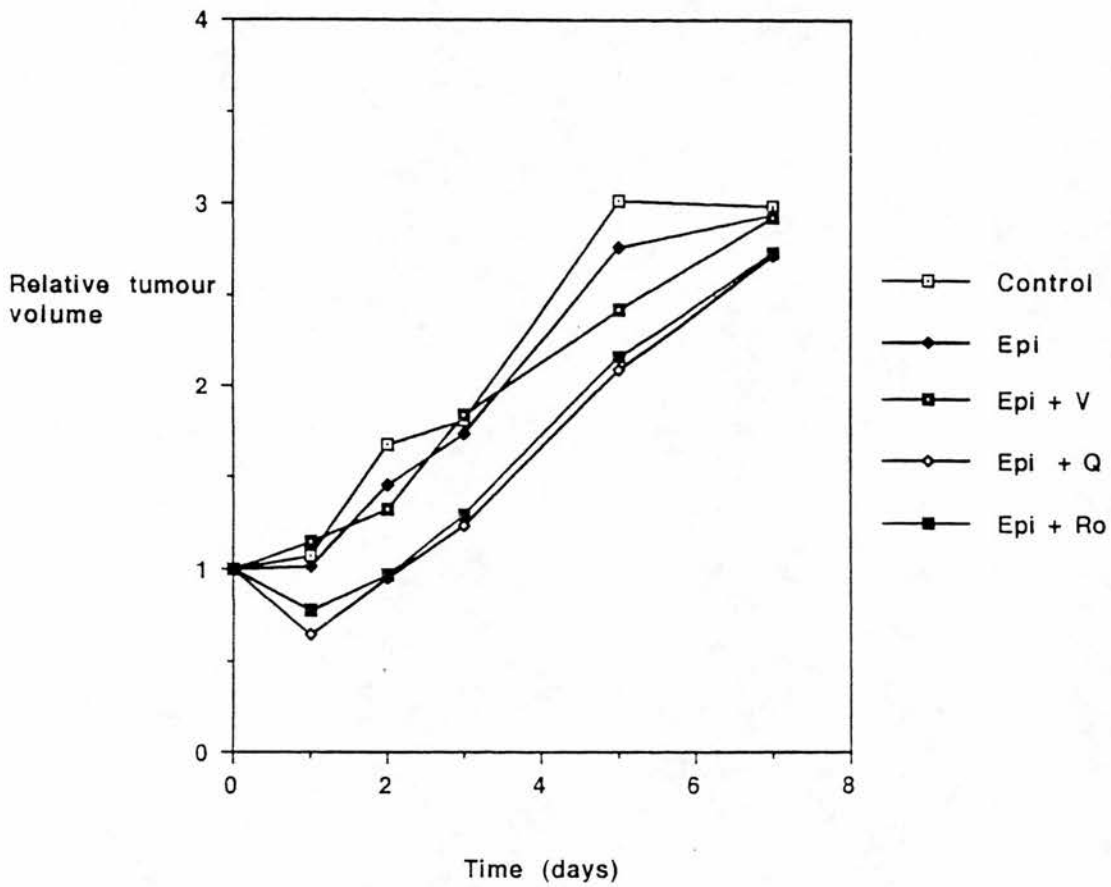
FIGURE 19

The mean relative tumour volumes in 2780AD tumour bearing nude mice during days 1-7 following treatment with the following drugs: Group1, untreated; Group2, epirubicin alone; Group3, epirubicin + verapamil; Group4, epirubicin + quinidine; Group 5, epirubicin + RO-112933. Epirubicin (10 mg/kg) was given by tail vein injection and all modulators were given as an intraperitoneal bolus 2 hours prior to epirubicin.
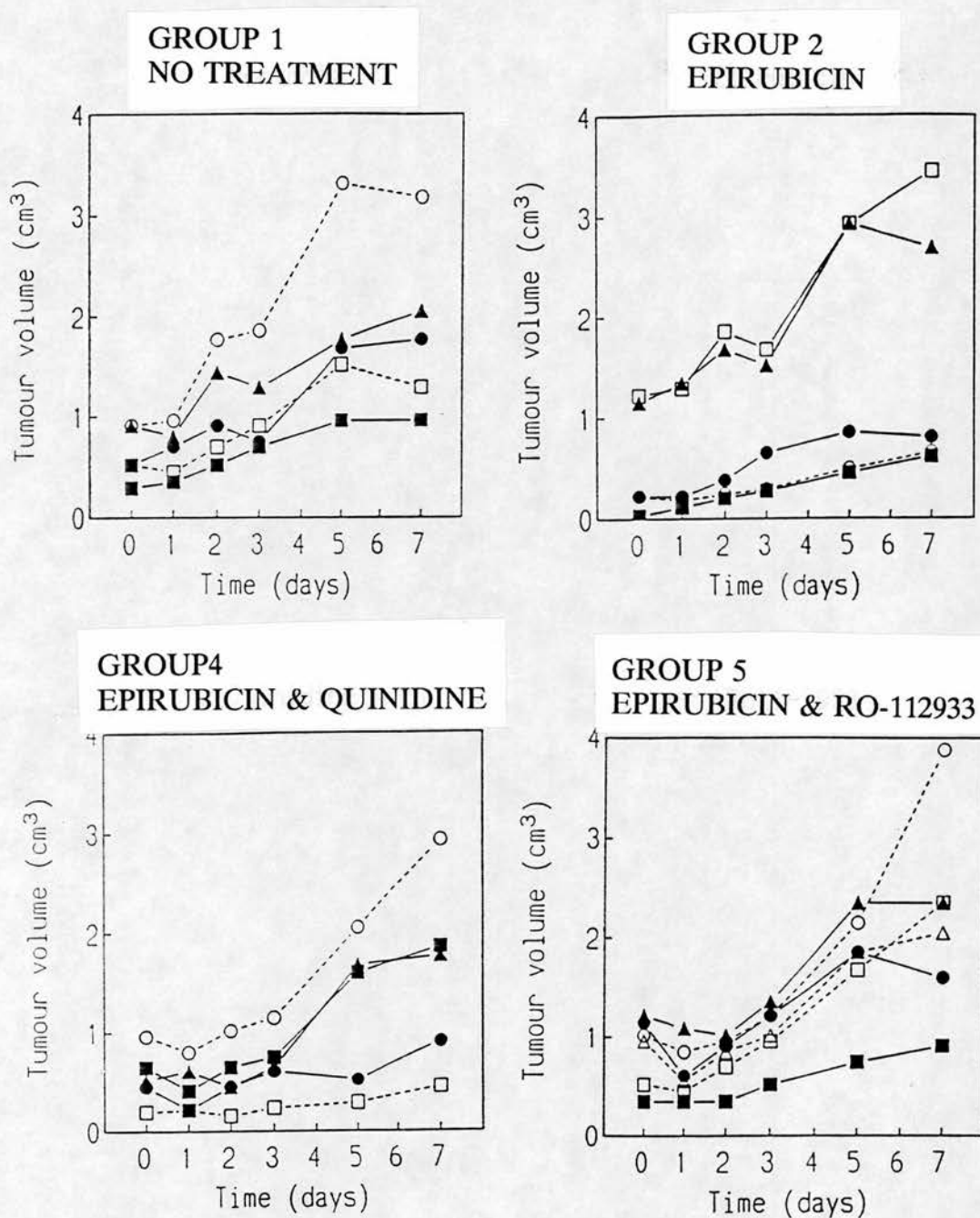
FIGURE 20

The relative tumour volumes of individual mice bearing the 2780AD xenograft following treatment following no treatment (Group1) and treatment with epirubicin alone (Group 2), epirubicin and quinidine (Group 4) and epirubicin and RO-112933 (Group 5). Epirubicin was given by tail vein injection and all three modulators were given by intraperitoneal bolus 2 hours prior to epirubicin.

EFFECT OF MODULATORS ON THE SENSITIVITY OF 2780AD
XENOGRAFT CELLS TO DOXORUBICIN

The ID50 for doxorubicin alone, and in combination with verapamil, quinidine and RO11-2933 in the 2780AD cells derived from the xenograft (2780AD/XENO) are shown in Table 10. For comparison the ID50 in 2780AD cells with doxorubicin $\pm$ verapamil is shown.

The 2780AD/XENO cells (ID50 = 295 ± 2.9) were more sensitive to doxorubicin than the 2780AD cells (ID50 = 7430 ± 920). Increased sensitivity to doxorubicin was found with verapamil (10.9 fold), quinidine (4.2 fold) and RO-112933 (19.3 fold) when they were used at a concentration of 6.6uM in combination with doxorubicin.

ID50 (nM)

CELL LINE	DOXORUBICIN	DOXORUBICIN + MODULATOR		
		VERAPAMIL	QUINIDINE	RO-112933
2780AD/ XENO	295 ± 2.9	27 ± 1.5 (10.9)	70 ± 3.5 (4.2)	15.3 ± 0.9 (19.3)
2780AD	7430 ± 920	790 ± 30.0 (9.4)		

TABLE 10
Chemosensitivity of 2780AD/XENO cells, expressed as an ID50 when exposed to doxorubicin alone and in combination with verapamil, quinidine and RO11-2933. For comparison the ID50 of 2780AD cells exposed to doxorubicin ± verapamil, again in an MTT assay, is shown.

DETECTION OF P-GLYCOPROTEIN

Immunohistochemical staining of cells derived from the 2780AD xenograft (control, untreated group) after the completion of the studies revealed P-gp expression to be present in the majority of cells (Figure 21a). The intensity of the staining in these cells was less than in the 2780AD cells prior to establishment of the xenograft (Figure 21b). Appropriate positive and negative controls were satisfactory (results not shown).

EFFECT OF QUINIDINE ON SUBCELLULAR LOCALISATION OF EPIRUBICIN

Examination of sections from the 2780AD xenograft in mice treated with epirubicin alone revealed both cytoplasmic and nuclear autofluorescence, often with intense peri-nuclear localisation (Figure 22). In contrast those tumours treated with a combination of epirubicin and quinidine had predominantly nuclear fluorescence and the intensity was greater than the tumours exposed to epirubicin alone even with the brightness of the laser reduced by approximately one fifth.
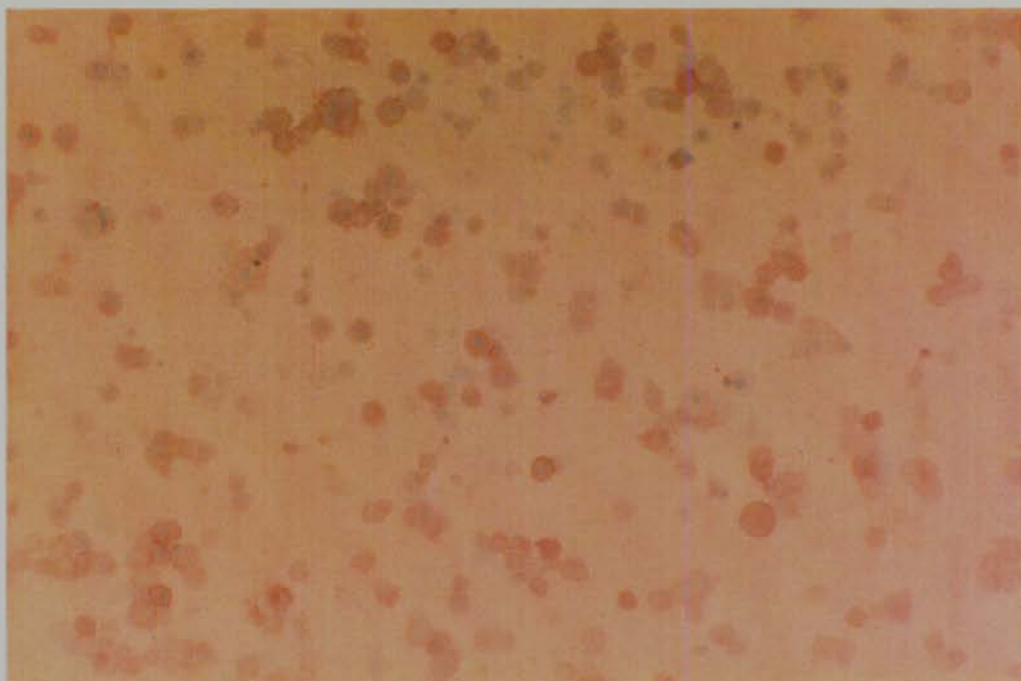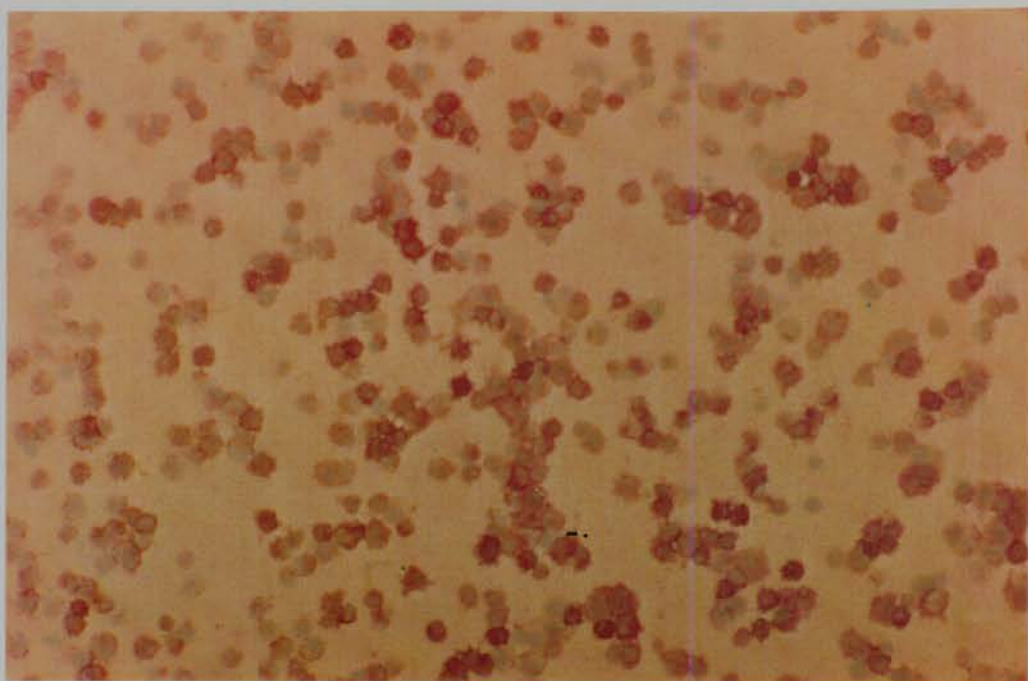
FIGURE 21
Immunohistochemical staining of P-gp, using the monoclonal antibody C219, in cells derived from the 2780AD xenograft (**a**) and in 2780AD cells prior to establishment of the xenograft (**b**).
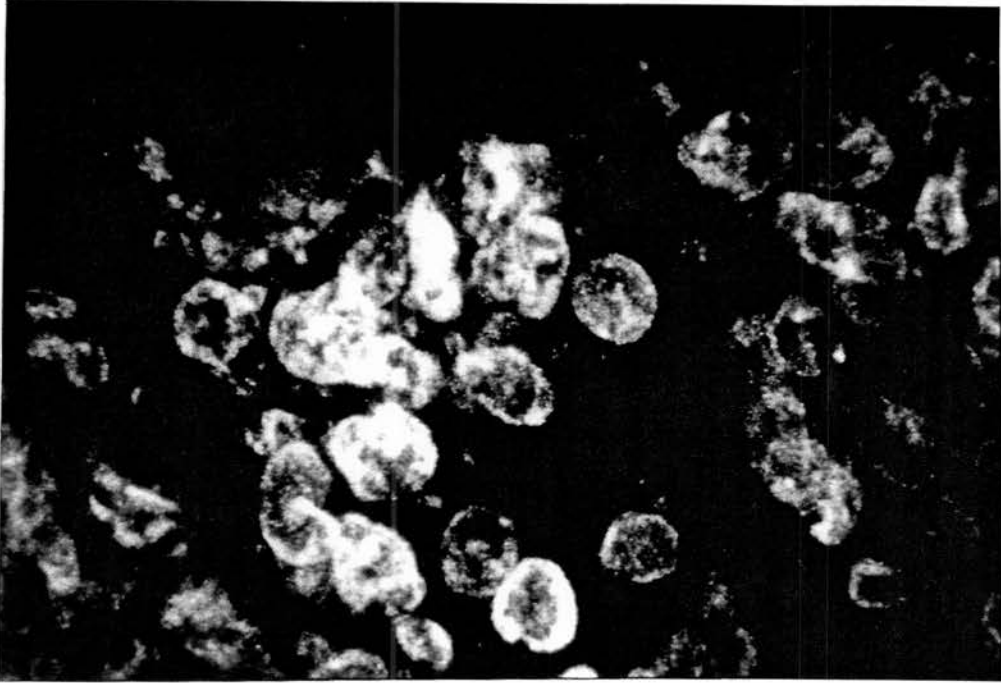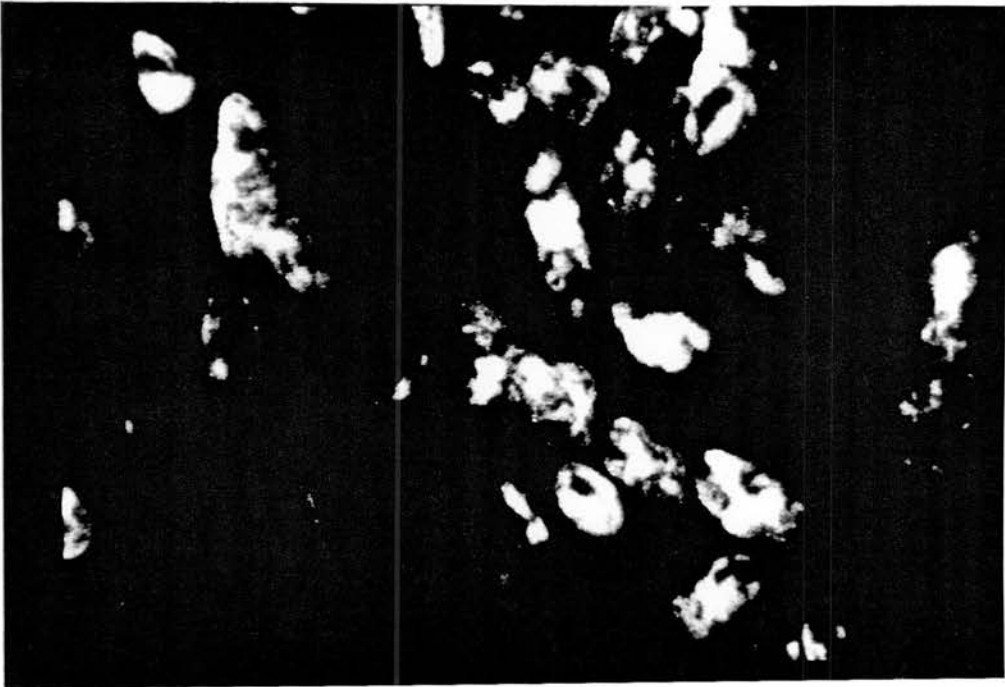
FIGURE 22
Epirubicin autofluorescence in 2780AD xenograft (a) showing both cytoplasmic and
nuclear fluorescence. When the 2780AD xenograft is exposed to epirubicin and
quinidine the fluorescence is predominantly nuclear (b) and is much more intense
even with the brightness of the laser reduced to approximately one fifth of (a).

DISCUSSION

These results demonstrate a significant growth delay by the addition of either quinidine or RO11-2933 to intravenous epirubicin in mice bearing a solid tumour model that is resistant to epirubicin. This resistance to epirubicin was confirmed *in vivo* since no growth delay in this tumour model was seen with epirubicin alone. The use of verapamil did not improve the growth delay achievable with epirubicin. Although there was no increased drug delivery to tumour cells, with the addition of quinidine, there was a change in intracellular drug distribution which may account for the improved response to epirubicin.

The resistance of the 2780AD xenograft to epirubicin was confirmed by the fact that no growth delay was seen when the mice were treated with epirubicin alone. With the addition of verapamil there was no effect even at the dose used (the LD_{10}) which was high enough to cause mice deaths from acute toxicity following injection of verapamil. When epirubicin was used in combination with quinidine, at a dose known to achieve tumour quinidine levels adequate for MDR modulation, there was a significant growth delay when compared to the untreated group ($p < 0.001$) and the group treated with epirubicin alone ($p = 0.01$). A similar result was achieved with the modifier RO11-2933 but this may have even more potential as an *in vivo* modulator of MDR since this compound was not used at the maximum tolerated dose.

There are two pices of evidence which support the theory that the significant growth delay is due to modulation of MDR *in vivo*. Firstly the tumour was shown to have maintained expression of P-gp by immunohistochemical staining with the monoclonal

antibody C219 in cells derived from one of the tumours. Secondly these same cells could be modulated *in vitro* by agents known to reverse multidrug resistance.

A potential criticism of this study would be that the significant growth delay with quinidine and RO11-2933 is not due to modulation of MDR at all. This criticism can be easily answered by repeating this study in the drug-sensitive A2780 xenograft and these studies are in progress. Another explanation for the growth delay is that the modulators in some way altered the pharmacokinetics of epirubicin to favour tumour drug delivery. Although an interaction has been described between verapamil and doxorubicin (Kerr et al, 1986) the use of quinidine had no effect on the delivery of epirubicin to tissues or tumour in this model. Finally, it is essential to exclude the possibility that the growth delay is due to the modulators themselves and these studies are also underway at present.

The choice of protocol for the administration of modulators was essentially empirical but was based on the assumption that a second dose of modulator was neccessary to maintain levels for several hours following epirubicin administration. In retrospect this was a good idea but the study could perhaps be improved by a longer administration of modulator and this will be attempted following a more detailed study of quinidine pharmacokinetics in this model. It may of course be possible to maintain the significant growth delay beyond 2-3 days by repeat dose of epirubicin. Anthracyclines, however, can cause marked tissue necrosis in patients (Muggia & Green, 1991) when extravasation occurs outwith the vein and as a result the mice tails are usually inflamed after one tail vein injection making a repeat injection often impossible. Furthermore the growth rate of this tumour (doubling time of

2 days) suggests that the doses of epirubicin may have to be very close together and the second dose may have to be reduced as a result leading to a possible loss of effect. As described also in this study an attempt to use the intraperitoneal route for epirubicin administration, for convenience and ease of repeat injections, led to unacceptable toxicity. This observation is supported by a previous study which described an increase in toxicity, manifest as early death and fibrosing peritonitis, following intraperitoneal administration of doxorubicin in a rat model (Litterst et al, 1982). Furthermore there is evidence to suggest that the anti-tumour activity of anthracyclines is decreased by this route of administration (Lenaz & Di Marco, 1972).

The drug distribution studies described in this Chapter are consistent with recent publications which have suggested that, although increased intracellular drug levels can be achieved by modulation of MDR cells *in vitro*, it appears to be a redistribution of the drug within the cell may be the most important factor (Schuurhuis et al, 1989; Coley et al, 1990a). The first of these studies showed that modifiers like verapamil and RO11-2933 act by inducing redistribution of doxorubicin from the cytoplasm to the nucleus of multidrug resistant cells *in vitro*. This helps to explain how relatively small increases in intracellular drug concentration ie.1-2 fold have apparently been associated with large increases in chemosensitivity ie.10-12 fold (Plumb et al, 1990) and agrees with the results of the tissue disposition study which suggest that quinidine has little effect on the total amount of drug in both tumour and other tissues. This failure to increase tumour drug levels concurs with a previous study which examined the effect of verapamil on vincristine concentrations in rhabdomyosarcoma xenografts in mice although in this

study verapamil enhanced retention of vincristine in liver, kidney and small intestine (Horton et al, 1989).

In summary the finding that the use of MDR modulators in combination with epirubicin can cause a significant growth delay in a solid tumour known to be resistant to epirubicin is an exciting breakthrough in the attempt to prove that P-gp is a functional target in human tumours including breast cancer. The confirmation of P-gp positive cells in the 2780AD xenograft, which could be modulated *in vitro*, together with the demonstration of a redistribution of intracellular epirubicin towards the nucleus with quinidine both support the theory that the growth delay is due to an interaction of the two modulators, quinidine and RO11-2933, with P-gp *in vivo*. If this is the case then the use of modulators of MDR may have an important role to play in the treatment of MDR-positive human tumours including carcinoma of the breast.

CHAPTER 6

THE EFFECT OF ORAL QUINIDINE ON TUMOUR EPIRUBICIN
CONCENTRATION AND INTRACELLULAR DISTRIBUTION IN HUMAN
BREAST CANCERS.

INTRODUCTION

Several factors are thought to influence the delivery of cytotoxic drugs to
malignant cells in solid human tumours including the pharmcokinetics of the drug
itself, the vascularity of the tumour and the distribution of drug within the tumour.
In patients with advanced breast cancer the best single agents are anthracyclines
with a response rate of approximately 40% in previously untreated patients (Young
et al, 1981). This figure highlights the fact that over half the patients fail to
respond to this drug from the outset possibly due to inadequate drug delivery or
due to the presence of a population of resistant cells prior to chemotherapy. Since
doxorubicin has been detected in human breast cancer biopsies following a small
dose (25mg) intravenously (Stallard et al, 1990) drug delivery is probably not a
major problem. It is therefore more likely that drug delivery to MDR-positive
cells within the tumour is a major problem.

One way of attempting to improve drug delivery to resistant cells is to increase the
total tumour drug delivery by the use of "targeting" in an attempt to deliver high
concentrations of cytotoxic drugs to the tumour whilst reducing the delivery to
normal tissues. An examples of targeting is the use of vasopressive agents in the
delivery of loco-regional chemotherapy to hepatic metastases (Anderson et al,

1991). Loco-regional therapy to the breast would be extremely difficult and at present the total dose of epirubicin is limited by cumulative cardiotoxicity and the dose intensity is restricted by myelosuppression and mucositis.(Muggia & Green, 1991). Any possible ways of increasing drug delivery to breast cancer cells would therefore be extremely important.

The finding of a proportion of P-gp positive cells in the majority of 29 primary breast cancers (see Chapter 2), together with the finding that MDR modulation can increase intracellular drug concentration in MDR cells *in vitro* (see Chapter 4), has raised the possibility that circumventing agents may increase the drug delivery to MDR positive cells in human breast cancers.

The study described in this Chapter was designed with two aims in mind. The first was to determine whether quinidine could increase the total tumour epirubicin concentration as a result of inhibition of the efflux pump in MDR positive cells. The second aim was to examine the effect of quinidine on the intracellular distribution of epirubicin in these tumours since it is now recognised that circumvention of MDR cells can alter the pattern of distribution (Schuurhuis et al, 1989) as described in the discussion in Chapter 4.

This study was carried out in patients with breast cancer rather than the mouse xenograft model for several reasons. Firstly detailed drug distribution studies in the mouse model will not answer the same question since the tumour vasculature in the xenograft is quite different from human breast cancers. Secondly the clinical trial of epirubicin and quinidine in patients with advanced breast cancer

does not assess P-gp status for each patient and therefore any benefit to P-gp positive patients may be hidden when all patients, both P-gp positive and negative, are assessed together. This therefore is a more detailed study to determine whether the principles of MDR modulation *in vivo*, as observed in the mouse model, will apply to human tumours.

The rational for selection of quinidine as the modulator in this study was described in Chapter 4. Epirubicin was chosen as the cytotoxic drug for study both to mimic the combination used in the clinical trial and since the magnitude of the resistance modulation with quinidine was much greater for epirubicin than doxorubicin.

The natural autofluorescence of epirubicin lends itself to distribution studies in tumour sections and the elimination of out of focus epifluorescence by optical sectioning, using confocal microscopy, allows the subcellular localisation of fluorescent drugs to be determined with extremely high resolution.

MATERIALS AND METHODS

PATIENT SELECTION

A total of 15 women with histologically proven breast cancer were entered into the study after informed consent was obtained. All patients were admitted for elective surgery of early breast cancer at the University Departments of Surgery at the Western Infirmary or the Royal Infirmary, Glasgow. The mean age of the patients was 60.9 (range 48-75) and 4 patients underwent mastectomy while the other 11 had conservation surgery. Patients were excluded from study if there was a history of cardiac or renal disease. In addition patients with impaired hepatic function (bilirubin > 33μmol/l or AST > 100μmol/l) were also excluded.

DRUG ADMINISTRATION

With the approval of the local ethical committee all patients in this study were given a small dose of intravenous epirubicin approximately one hour before surgery since a previous study had suggested this to be an optimal time for tissue binding of another anthracycline, doxorubicin (Cummings et al, 1986). Since doxorubicin has been detected in human breast cancers following a dose of 25mg intravenously (Stallard et al, 1990) this dose was modified to 20mg/m^2 to allow for variations in patient size. In addition patients were randomised to pretreatment with oral quinidine for four days prior to surgery or no pretreatment at all.

The dose of quinidine sulphate was 250mg/kg twice daily commencing in the evening 4 days prior to the day of elective surgery (total dose 2g). At this dose no side effects were encountered in a pilot study in the Beatson Oncology Centre, Western Infirmary, Glasgow (Jones et al, 1990) whereas higher doses do lead to increased levels of side effects.

On the day of surgery all 15 patients were given an intravenous bolus of epirubicin ($20mg/m^2$) into the side arm of a fast running drip approximately one hour before the expected time of tumour excision. Following removal of the tumour the samples were snap frozen in liquid Nitrogen and stored at -70^0C until epirubicin and DNA assays.

EPIRUBICIN ASSAY

The total epirubicin content (intracellular and extracellular) of the tumours was quantified by High Pressure Liquid Chromatography (HPLC) using the same method described in Chapter 5. Daunorubicin was again used as an internal standard to estimate the extraction efficiency of the assay and epirubicin concentrations were normalised by correcting for drug recovery. To allow for differences in cellularity between tumours, which is often quite marked in breast cancers, epirubicin concentrations were expressed as µg/mg DNA rather than µg/mg of tumour.

DNA ASSAY

A 1:10 tissue to buffer homogenate was prepared by adding a nine-fold weight/volume excess of PBS. To 0.5ml of tumour homogenate 3M NaAc (50ul) was added with an equal volume of phenol and chloroform/isoamylalcohol (24:1 chloroform:isoamylalcohol), mixed for 15 minutes, then centrifuged at top speed for ten minutes in a microfuge. The aqueous layer was removed and added to 0.5ml cool isopropanol (100%). The precipitated DNA was washed in 70% ethanol, dried and resuspended in 1ml water. The optical density (OD_{260}) was read in a spectrophotometer (Perkin Elmer, Lambda 2, Beaconsfield, UK) and the tumour DNA concentration calculated from a known standard. These tumour DNA concentrations were used to calculate epirubicin concentrations as µg/mg DNA.

INTRACELLULAR EPIRUBICIN DISTRIBUTION

The technique of confocal microscopy is described fully in the "methods" section in Chapter 5. In brief 20µm sections were cut from four of the breast cancers, two from patients given quinidine and two who did not receive quinidine. The sections were unfixed and mounted in a water-based medium (Glycergel, Dako, High Wycombe, England). The distribution of epirubicin in these sections was analysed on a Carl Zeiss Laser Scan microscope (LSM 10) by confocal microscopy with Argon laser excitation at 488nm. The sections were examined not to compare the total amount of cellular fluorescence between different tumours but to look for differences in subcellular localisation following quinidine pretreatment.

RESULTS

<u>TUMOUR EPIRUBICIN CONCENTRATIONS</u>

A total of 9 patients received epirubicin alone and 6 patients received epirubicin in addition to oral quinidine. The mean sampling time was 64 minutes (range 45-120) in the epirubicin group and 73 minutes (range 57-90) in the epirubicin and quinidine group. The results of tumour epirubicin concentrations are shown in Table 11. This shows no significant difference ($p = 0.724$) in the overall epirubicin concentration between the groups treated with quinidine (0.815 ± 0.372 µg/mg DNA) or no quinidine (0.714 ± 0.182 µg/mg DNA) (Mann Whitney U Test).

TREATMENT	EPIRUBICIN CONCENTRATION (µg/mg DNA)
EPIRUBICIN ALONE	0.815 ± 0.372
EPIRUBICIN & QUINIDINE	0.714 ± 0.182

<u>TABLE 11</u>
Tumour epirubicin concentration (mean $\pm$ SD) in patients with breast cancer treated with intravenous epirubicin alone and in patients treated with epirubicin and oral quinidine pretreatment

SUBCELLULAR LOCALISATION OF EPIRUBICIN

Fluorescence was detectable in all four breast cancer sections. The staining in tumour cells was predominantly nuclear with some cytoplasmic staining. There was no difference in intracellular distribution of drug with or without quinidine either in tumour cells or stromal cells. Examples of the staining in tumour cells is shown in Figure 23.
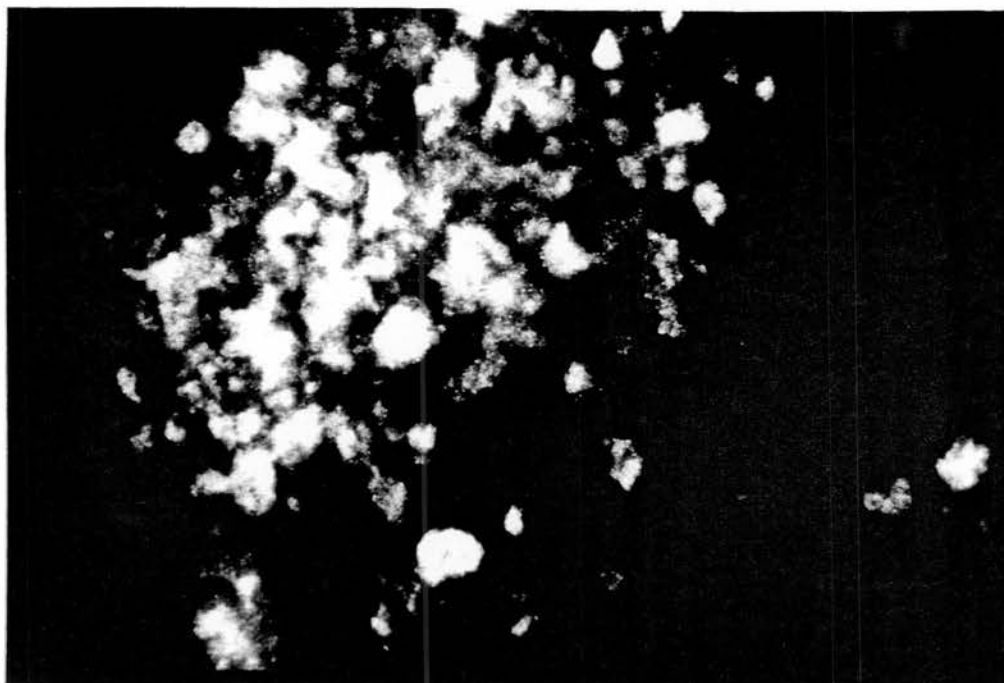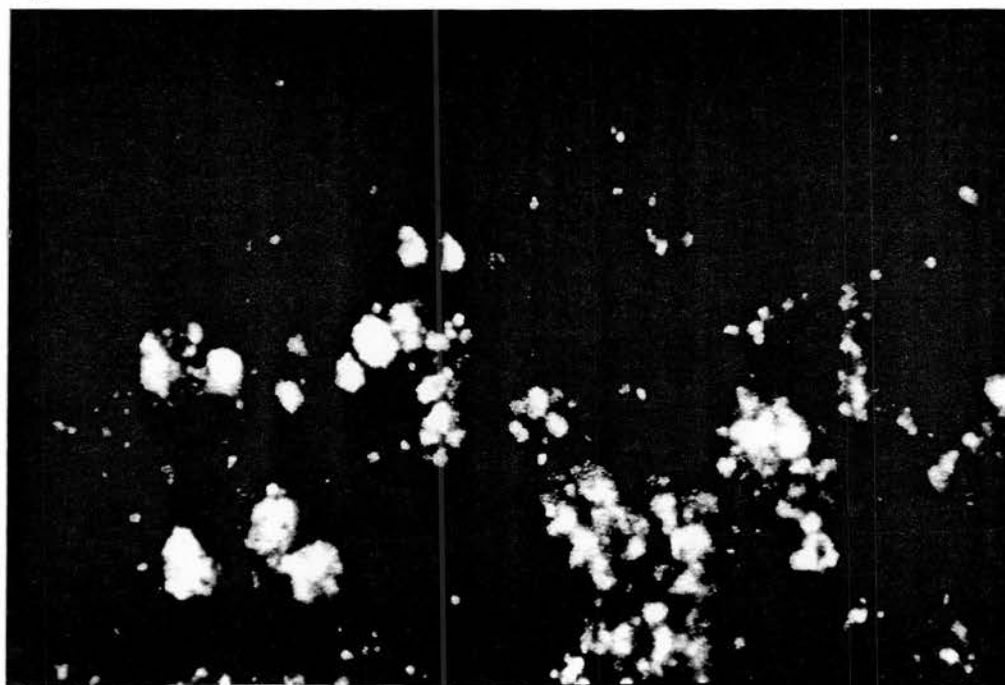
FIGURE 23
Epirubicin subcellular localisation in sections of human breast cancers treated with quinidine (**a**) and no quinidine (**b**). Both sections show a predominance of nuclear epirubicin although some cytoplasmic fluorescence is also seen.

DISCUSSION

The lack of difference in the total tumour epirubicin concentration between those tumours exposed to epirubicin and quinidine and those exposed to epirubicin alone is not altogether surprising. Given the small percentage of P-gp positive tumour cells in most breast cancers it is unlikely that successful MDR modulation in these cells, with small increases in intracellular drug concentrations, would have a profound effect on the total amount of drug in the tumour. The range of tumour epirubicin drug concentration in this study (423-1582 ng/mg DNA) shows a wide inter-patient variation and these levels are consistent with a previous study where doxorubicin (20mg) was given an hour before surgery (Stallard et al, 1990).

With the recent suggestion that MDR modulation may redistribute intracellular drug *in vitro* (Schuurhuis et al, 1989) it seemed appropriate to look for any change in the distribution of epirubicin in tumour cells *in vivo*. Although the number of tumours was small this study failed to demonstrate any alteration in subcellular localisation which could be attributed to the quinidine. It could be argued that the subcellular distribution was not specifically looked at in P-gp positive cells in these tumours. Unfortunately this was not possible since the small tumour specimens were just sufficient for estimation of total tumour epirubicin levels and for the thick sectioning (20µm) for con-focal microscopy. In addition the technique of fixation required for immunohistochemical detection of P-gp interfered with the preservation of fluorescence ruling out the possibility of looking at P-gp expression and autofluorescence in the one section.

The experiments described in this chapter confirm good penetration of epirubicin in human breast cancers with nuclear localisation at subtherapeutic doses when given approximately one hour before surgery. The use of con-focal laser microscopy does provide high resolution images of subcellular localisation but it is more likely to be of use in looking at those tumours with a higher percentage of P-gp positive cells. This was highlighted in Chapter 4 where it was extremely helpful in examining intracellular distribution of drug in a solid tumour model of MDR, the 2780AD xenograft, with and without quinidine.

The use of quinidine has failed to provide any evidence that MDR modulation in human breast cancer in any way "targets" the entire tumour and this was expected. Whether the use of this drug specifically modulates MDR in the P-gp positive cells in these tumours remains to be seen and this would hopefully be reflected by an enhanced response to chemotherapy or improved patient survival in MDR-positive tumours. It is possible however that the method used to measure tumour epirubicin is not sensitive enough to detect 1-2 fold increases in MDR-positive cells, as is observed *in vitro*, which may represent only between 1-10% of the total tumour cell population.

Finally there was no obvious difference in autofluorescence in breast cancer stromal cells with or without quinidine. It is possible that this small dose of epirubicin fails to highlight the small spindle-shaped nuclei in stromal cells but the lack of enhanced staining in these cells with quinidine suggests that the P-gp present in these cells (see Chapter 3) may not respond to modulation in vivo. The hypothesis that MDR modulation in breast cancer, with increased accumulation

in stromal cells, may actually decrease the availability of cytotoxic drug for the malignant cells in these tumours would appear to be unsubstantiated at present.

In conclusion this study once again demonstrates that cytotoxic drug delivery does not appear to be a problem in the treatment of breast cancer. The study however has failed to demonstrate an effect that could be attributed to MDR modulation *in vivo*, either in total tumour or intracellular epirubicin., although the methods used may not have been sensitive enough to detect small changes in intracellular drug in MDR-positive cells. Although the results are somewhat disappointing the rational for attempting MDR modulation in breast cancers with quinidine, based on the P-gp expression study (Chapter 2) and successful MDR modulation with quinidine in the mouse model (Chapter 5), is sound and even small, and as yet undetected, changes in cytotoxic drug delivery to MDR-positive cells may influence the growth of the tumour. The results of the clinical trial may answer these questions although future trials may have to include a comparison of MDR-positive and MDR-negative tumours when modulators are used to determine whether modulation of MDR-positive cells in human breast cancer has an effect on tumour response to cytotoxic drugs.

CHAPTER 7

THE EFFECT OF ORAL QUINIDINE ON SYSTEMIC TOXICITY WITH
EPIRUBICIN IN PATIENTS WITH METASTATIC OR LOCALLY ADVANCED
BREAST CANCER.

INTRODUCTION

Chemotherapy is a useful palliative measure in the treatment of metastatic or locally
advanced breast cancer and the most successful single agents are anthracyclines with
a total response rate of 43% in previously untreated patients (Young et al, 1981).
The use of anthracyclines is associated with conventional toxicity eg.
myelosuppression, alopecia, nausea and vomiting as well as a more specific toxicity,
cardiomyopathy after a certain cumulative dose. The use of doxorubicin
(adriamycin) has now largely been superceded in the treatment of advanced breast
cancer in this country by epirubicin (4'- epidoxorubicin) which is thought to be less
cardiotoxic at an equivalent dose (Brambilla et al, 1986). The clinical use of
anthracyclines is not limited by this toxicity however but by the development of
clinical drug resistance.

One major route by which cells develop resistance to anthracyclines *in vitro* is
multidrug resistance (Seeber et al, 1982). This particular mechanism of resistance
is associated with expression of P-gp and this protein has been detected in the
majority of 29 primary breast cancers by immunohistochemistry using two

monoclonal antibodies C219 and MRK16 (See Chapter 2). This protein, which acts as a drug-efflux pump, can be reversed by a number of compunds *in vitro* including quinidine (see Chapter 4). Since tumour quinidine levels which are adequate for *in vitro* modulation of MDR can be achieved both in a MDR-positive mouse xenograft and in human breast cancers, it would appear that *in vivo* modulation with this compound may be possible. Furthermore the concentration of quinidine achieved in the mouse model was capable of a significant increase in growth delay (see Chapter 5) of the P-gp positive xenograft. This evidence therefore makes quinidine an attractive choice as a potential MDR modulator in patients with breast cancer.

Before proceeding with quinidine as a MDR modulator in breast cancer patients it is essential to know if this compound will be tolerated by the patients and if the combination of quinidine and the cytotoxic drug is more toxic than the cytotoxic alone. This theory of increased toxicity is based on the presence of P-gp in several human tissues (Fojo et al, 1987) with possible increases in cytotoxic drug levels in these organs , following modulation, leading to increased toxicity. In practice however Jones et al (1990) have already shown that quinidine can be given, in combination with epirubicin, to breast cancer patients without any increase in acute toxicity. Furthermore this study also showed that patient compliance in taking oral quinidine was good and the side effects from the quinidine alone were minimal.

Following this pilot study a Phase III, multicentre, clinical trial is presently ongoing, coordinated by the West of Scotland Clinical Trials Group, to assess the effect of oral quinidine on response to chemotherapy with epirubicin in patients with metastatic or locally advanced breast cancer. The trial has a target figure of 260

patients, 130 in each arm , in order to have enough patients to detect any survival difference between the two arms of the trial as well as any difference in response to chemotherapy. As the trial is still ongoing response data is not yet available but the initial analysis can be used to answer some of the questions about toxicity when modulators are used in patients. The systemic toxicity data in both the quinidine and placebo arms of the trial has therefore been collated to look for any significant differences that might support this theory of enhanced systemic toxicity secondary to MDR modulation.

MATERIALS AND METHOD

<u>PATIENT TREATMENT</u>

Following randomization all patients are supplied with a six day course of capsules, either quinidine 250 mg twice daily or placebo, in a double-blind fashion. The course of capsules commences at 8pm on the fourth day prior to each course of chemotherapy and finishes at 8pm on the second day following chemotherapy (Figure 24).

DAY OF TREATMENT

	-4	-3	-2	-1	0	+1	+2
8am	-	*	*	*	*	*	*
					∞		
8pm	*	*	*	*	*	*	-

* ORAL QUINIDINE/PLACEBO

∞ INTRAVENOUS EPIRUBICIN

<u>FIGURE 24</u>
The timing of patient treatment with oral quinidine or placebo given with each course of chemotherapy in a Phase III trial of epirubicin in patients with locally advanced or metastatic breast cancer.

All patients receive epirubicin 100mg/m^2, dissolved in 25mls of water, into the side arm of a fast running drip every three weeks for a maximum of eight courses. On the morning following epirubicin a five day course of oral steroid (prednisolone 25mg twice daily) is commenced and appropriate anti-emetic treatment is given with the epirubicin at the discretion of the clinician.

Prior to each cycle of chemotherapy each patient had a 5ml sample of blood taken for quinidine estimation by immunoassay (for protocol see Method section in Chapter 5.

PATIENT SELECTION

Eligibility criteria for entry into the study included histologically proven metastatic or locally advanced breast cancer, measurable or evaluable disease and a World Health Organisation (WHO) performance status 0,1 or 2. In addition patients require to have adequate bone marrow function (haemoglobin > 10gm% ; total white cells > 4.0 x 10^9/l ; platelet count > 100 x 10^9/l) and a normal serum bilirubin. Patients are allowed to have received previous adjuvant chemotherapy (excluding anthracyclines) and hormonal therapy as long as they are stopped prior to entry into the study.

Reasons for exclusion from the study include prior chemotherapy for advanced disease, inability to give informed consent or the presence of a second malignancy (other than basal cell carcinoma of skin or carcinoma-in-situ of cervix). In addition a previous history of myaesthenia gravis or hypersensitivity to quinidine are further

reasons for exclusion as well as active peptic ulceration or another major contraindication to oral steroids.

TREATMENT MODIFICATIONS

A 20% dose reduction of epirubicin is carried out if the total white blood cells are less than 3.0 x $10^9/l$ or if platelets are less than 100 x $10^9/l$ on the day of treatment. If the full blood count has not recovered after a maximum of two weeks patients are removed from study. If grade III mucositis occurs epirubicin is deferred until recovery has taken place and subsequent doses are reduced to 80mg/m^2.

Side effects are not expected at the dose of quinidine given but can occasionally include hypotension, ventricular arrhythmias or cinchonism (dizziness, tinnitus or visual disturbance). If side effects are encountered the capsules are discontinued for subsequent doses of quinidine withour breaking the randomization code.

RESULTS

REASONS FOR STOPPING TREATMENT

Demographic data from the first 100 patients randomised shows the groups to be well matched for age, performance status and prior hormone therapy (Table 12). In the placebo (**P**) group 74.5% of patients completed three courses of chemotherapy with 66.0% of them proceeding to >3 cycles. In the quinidine (**Q**) group 66.7% completed three courses with 54.2% of these patients having further cycles of chemotherapy. The reasons for stopping treatment at or before the third cycle are summarised in Table 13. A total of 7 patients in group **Q** stopped treatment at or or before the third cycle because of "excessive toxicity" in contrast to only 4 patients in group **P**. In group **P** the main reason for this toxicity was haematological (3/4) with the one other patient stopping due to uncontrollable vomiting post chemotherapy. In group **Q** 3/7 patients stopped due to haematological toxicity and the other four patients stopped for one of the following reasons: cerebrovascular accident; breathlessness; phlebitis; prolonged vomiting.

		PLACEBO	QUINIDINE
ECOG PERFORMANCE	0	27.7%	25.0%
STATUS	1	53.2%	54.6%
	2	19.2%	20.8%
PRIOR HORMONE	YES	72.3%	81.3%
TREATMENT	NO	27.7%	18.7%
MEDIAN AGE		54	54
(RANGE)		(29-74)	(30-71)

TABLE 12
Patient characteristics in both placebo and quinidine arms of the trial showing both groups to be well matched for age, performance status and previous hormone therapy.

	PLACEBO	QUINIDINE
PROGESSIVE DISEASE	8	9
PATIENT REFUSAL	3	0
EXCESSIVE TOXICITY	4	7
DEATH (UNKNOWN/NON CANCER)	1	2
INFECTED CENTRAL LINE	0	1

TABLE 13
Reasons for stopping treatment at or before the third cycle of chemotherapy in both placebo and quinidine arms of the trial.

In the placebo arm 21.3% required epirubicin dose reductions for haematological (n=6) and other (n=4) toxicity. In contrast only 12.5% of patients in the quinidine arm had dose reductions, two for haematological toxicity and two for other toxicity. There was however a greater number of patients who had delays in epirubicin treatment in the quinidine arm (22.9%) than the placebo arm (14.9%).

WHO grading of haematological toxicity, alopecia, nausea/vomiting, diarrhoea and mucositis has shown no significant differences between placebo and quinidine limbs of the trial (Table 14).

PLASMA QUINIDINE CONCENTRATION

A total of 197 plasma samples, from patients during cycles 1-3, had detectable levels of quinidine. The mean steady-state level was 5.63µM (range 0.3-16.6 µM).

	WHO GRADE	PLACEBO	QUINIDINE
HAEMATOLOGICAL	0	36.6%	27.3%
	1	31.7%	45.5%
	2	19.5%	13.6%
	3	7.3%	6.8%
	4	4.9%	6.8%
ALOPECIA	0	0.0%	2.3%
	1	2.8%	9.3%
	2	12.5%	27.9%
	3	85.0%	60.5%
NAUSEA/VOMITING	0	17.1%	18.6%
	1	41.5%	34.9%
	2	22.0%	25.6%
	3	14.6%	18.6%
	4	4.9%	2.3%
DIARRHOEA	0	74.4%	81.4%
	1	15.4%	7.0%
	2	7.7%	9.3%
	3	2.6%	2.3%
MUCOSITIS	0	53.9%	51.2%
	1	20.5%	18.6%
	2	18.0%	23.3%
	3	7.6%	7.0%

TABLE 14
The percentage of patients, in both limbs of the trial, experiencing each systemic toxicity according to the WHO grading system.

DISCUSSION

Previous attempts to modulate multidrug resistant cells in human tumours have been associated with increased systemic toxicity which has been related to the specific modulator used. In this way the use of verapamil in combination with vincristine, doxorubicin and dexamethasone (VAD) led to increased cardiotoxicity , manifest as mild hypotension (8/8) and heart block(7/8), in eight patients with drug resistant B-cell neoplasms previously treated with VAD alone (Dalton et al, 1989). Other doxorubicin-related toxicities eg. myelosuppression and nausea/vomiting were not however significantly increased by the addition of verapamil to VAD. This data would concur with the findings described in this chapter which found no significant differences in systemic toxicities when the combination of epirubicin and quinidine is compared to epirubicin alone.

The toxicities in the various categories, graded according to WHO guidelines (see Table 13), are very similar to the toxicities encountered in the high dose arm of a study which compared high dose ($100mg,m^2$ 3 weekly) with low dose ($50mg/m^2$ three weekly) epirubicin (Habeshaw et al, 1991). There are two possible explanations why the postulated increase in systemic toxicity with the addition of quinidine to epirubicin does not appear to have occurred. Firstly, small increases in intracellular cytotoxic drug concentrations in normal cells may not be enough to significantly alter host toxicity despite the fact that these same increases in intracellular drug concentrations *in vitro* can markedly alter chemosensitivity.

A second explanation for the lack of increased toxicity with quinidine is that intracellular drug concentrations in normal cells containing P-gp may not be increased at all. This may arise as a result of a non-functional or an altered P-gp molecule in normal cells, which is not affected by conventional modulators, or as a result of inadequate tissue concentrations of quinidine. The finding of a mean plasma quinidine concentration in 197 samples of 5.63μM/l would seem to contest the latter explanation since it is known that tumour quinidine concentrations, and presumably tissue concentrations, are of the same magnitude as plasma levels (see Chapter 4). Futhermore this mean plasma concentration of 5.63μM/l is a steady-state level achieved after four days of oral therapy and is very close to the dose of quinidine used *in vitro* which bring about near maximal reversal of MDR (6.6μM).

These findings with the use of quinidine in patients are consistent with the toxicity obtained when quinidine and other modulators were administered to mice bearing a MDR-positive xenograft (Chapter 5). Although a transient, reversible toxicity was seen due to quinidine alone there was no increase in toxicity due to epirubicin. In contrast 2 mice treated with epirubicin and verapamil died within the first 24 hours after treatment. Whether this was due to the acute toxicity of verapamil alone or to the combined toxicity of epirubicin and verapamil is not known. The use of cytotoxic drugs themselves relies on the existence of a therapeutic window such that drug concentrations that are toxic to the tumour are lower than those toxic to the normal tissues. Likewise it is possible that the amount of the modulator required to sensitize tumour cells is less than that required to sensitize normal tissues. On this basis modulators may increase the therapeutic window of the drug.

In conclusion these results indicate that oral quinidine can be used as a modulator of MDR in patients with breast cancer without increasing patient systemic toxicity. It remains to be seen whether oral quinidine at the dose used can improve the response to chemotherapy with epirubicin and these results of this clinical trial are awaited.

CHAPTER 8

GENERAL DISCUSSION

The two main aims of this thesis were firstly to confirm the presence of P-gp in human breast cancers and secondly to establish an *in vivo* model of MDR with which to test known *in vitro* modulators of MDR to determine whether P-gp is a functional target in human solid tumours. These aims have both been achieved and they have led to some important findings regarding potential modulation of MDR in human breast cancers. P-gp has been confirmed in human breast cancers and has been localised not only to malignant cells but to breast cancer stromal cells also. Although the expression in malignant cells predicted a worse disease-free survival it was not related to other known prognostic factors. By establishing a solid tumour model of MDR it was possible to demonstrate that two modulators of MDR, quinidine and RO11-2933, could cause a significant growth delay when given in combination with epirubicin. This led to an appraisal of the effect of one of these modulators, quinidine, on epirubicin distribution in human breast cancers with no apparent effect. The work in this thesis however does provide a sound basis for a clinical trial of quinidine in patients with breast cancer in an attempt to improve the response rate to epirubicin in patients with locally advanced or metastatic breast cancer.

The use of chemotherapy in the treatment of breast cancer is compounded by the development of drug resistance in a large number of cases. Although several mechanisms of cellular drug resistance have been identified *in vitro* little is known about the contribution these mechanisms make to the overall clinical drug resistance of human breast cancers. One mechanism of drug resistance

multidrug resistance (MDR), characterised by expression of a 170kDa transmembrane protein (P-gp) which acts as a drug efflux pump, has been identified in several human tissues and tumours but previous studies have failed to provide clear evidence of its presence in breast cancer.

The detection of P-gp by immunohistochemistry using two monoclonal antibodies (C219 & MRK16) is described in Chapter 2. This allowed confirmation of P-gp, at low but variable levels, in the majority of 29 primary, untreated breast cancers. This suggested that a population of resistant cells were present in breast cancers before any exposure to cytotoxic drugs. The use of immunohistochemistry in tumour sections however led to two other important findings in addition to the confirmation of P-gp expression in these tumours. Firstly the distribution of expression was found to be heterogeneous and secondly this expression occurred not only in the malignant epithelial cells, as expected, but in breast cancer stromal cells, thought to be myofibroblasts, also.

One advantage of this study was that the tumour samples were historical and this allowed assessment of P-gp expression in relation to patient survival. Although the numbers are small, and do not reach statistical significance, the results suggest that those patient with the highest level of expression in tumour cells (>5% of tumour cells) have a worse prognosis in terms of disease-free survival (p=0.09). In contrast to this finding there was no similar association between P-gp expression in stromal cells and prognosis.

As mentioned previously two recent studies have identified a correlation between P-gp expression and a poor response to chemotherapy in patients with advanced breast cancer (Verrelle et al, 1991; Ro et al, 1990). Unlike these studies the poor prognosis associated with P-gp expression in the patients described in Chapter 2 is apparently unrelated to a poor response to chemotherapy since only three of six patients who received adjuvant chemotherapy were exposed to MDR-related drugs. It is possible that P-gp expression may be a biological marker of tumour aggression in breast cancer and this may explain both a poor response to chemotherapy and a worse disease-free survival. This hypotheseis would be supported by a recent study of colonic carcinomas which found that P-gp positive colon cancer cells have an increased potential for dissemination (Weinstein et al, 1991).

If P-gp expression in breast cancer is associated with local tumour invasion and dissemination then measurement of P-gp, together with other known prognostic factors may help to identify patients who would benefit from adjuvant therapy. Alternatively measurement of this protein in patients with locally advanced or metastatic breast cancer could select MDR-positive tumours such that these patients could receive appropriate treatment. Either anthracyclines can be avoided or modulating agents such as quinidine could be incorporated into their treatment.

The findings reported in Chapter 3 suggest that the immunoreactivity in breast cancer stromal cells represents true expression of P-gp. The use of peptide blocking of the monoclonal antibody prior to incubation with tumour sections, which confirmed genuine P-gp expression in these stromal cells, provides a more

specific negative control when using C219 and should be used routinely instead of omission of the primary antibody or substitution by an irrelevant antibody. Although the function of P-gp in these stromal cells is not yet known it is conceivable that it has a secretory role in these cells and may even secrete stromal products which are important in promoting tumour invasion or metastasis eg. Stromelysin 3 (Basset et al, 1990). If this hypothesis can be proven then these stromal cells, or their products, may become specific targets for anti-cancer therapy.

By examination of paired tumour and "normal" breast specimens from the same patient it was possible to show that stromal cells in normal breast do not express P-gp. Thus P-gp expression would appear to be specific to breast cancer stromal cells and future studies should address how it it may arise in these tumours. P-gp however was expressed in some ductal epithelial cells in normal breast in 2/9 patients and it is of interest that these patients also had the tumours with the highest number of P-gp positive stromal cells.

The finding that the MDR phenotype may be partially reversed *in vitro* by specific compounds which interact with P-gp is not new. It was however important to confirm that known circumventing agents could modulate a breast carcinoma cell line (MCF7^{ADR}) *in vitro* (see Chapter 4) prior to attempting modulation of drug resistant solid tumours and ultimately human breast cancers. By using the MTT microtitration assay it was possible to demonstrate a modulating effect by tamoxifen, verapamil and quinidine and furthermore quinidine also enhanced doxorubicin uptake in the same cells. Quinidine was therefore selected for further study since it has been used for many years as an

anti-arrthythmic agent and there is already evidence to suggest that plasma levels suitable for modulation are non-toxic. The studies described in Chapter 4 also highlighted the finding that the combination of quinidine (6.6µM) and epirubicin produced a much higher increase in chemosensitivity (22.5-fold) than the combination of quinidine (6.6µM) and doxorubicin (7-fold).

Although there are now many compounds, including quinidine, which modulate MDR cells *in vitro* it is much more difficult to show a similar effect *in vivo*. A pre-requisite for attempting to modulate MDR cells *in vivo* is the attainment of tumour, as well as plasma, levels adequate for MDR modulation. Since many MDR modulators are highly protein-bound in plasma it was therefore extremely encouraging to find that quinidine levels, in the range known to modulate MDR cells *in vitro*, could be achieved both in an animal solid tumour model of MDR and in human breast cancers (Chapter 4).

The paucity of effective *in vivo* modulator studies almost certainly is a reflection of the difficulty in establishing a good solid tumour model of MDR cells with which to test potential modulators. The use of the 2780AD xenograft in nude mice was not without difficulty since intraperitoneal epirubicin proved extremely toxic to the mice (Chapter 5). In this same model quinidine did not increase tumour or tissue epirubicin concentrations and this result is consistent with recent recognition that it may be a redistribution of drug within the cell which is important in MDR modulation rather than small increases in total intracellular drug levels (Schuurhuis et al, 1989).

The most encouraging result from the xenograft studies was the finding that quinidine, in combination with epirubicin, caused a significant growth delay in the 2780AD xenograft at day 2 when compared with an untreated group ($p < 0.001$) and the group treated with epirubicin alone ($p = 0.01$). Although this significant growth delay appears to be maintained at days 5 and 7, when depicted graphically (Figure 19), the enhancement of the growth delay does not reach statistical significance due to the size of the standard errors within the groups at these time points.

This improved growth delay with quinidine appears to represent true MDR modulation since P-gp expression was confirmed in the 2780AD xenograft and cells derived from one of the untreated group could be modulated in a MTT microtitration assay. Furthermore the use of confocal laser microscopy has shown that quinidine can cause a redistribution of intracellular epirubicin in this model with increased nuclear fluorescence in the quinidine-treated group compared to the group treated with epirubicin alone.

The combination of oral quinidine, at doses known to be adequate for MDR modulation (Chapter 4), and intravenous epirubicin can be given to patients without any apparent increase in systemic patient toxicity (Chapter 7). Quinidine at this dose however did not increase total tumour epirubicin levels in human breast cancers nor did it appear to affect intracellular epirubicin distribution (Chapter 6). A more definitive test of the hypothesis that MDR-positive cells in human breast cancer can be modulated with quinidine will be the outcome of the clinical trial described in Chapter 7. In this trial the effect of oral quinidine

is being assessed on the response to chemotherapy and patient survival in patients with locally advanced or metastatic breast cancer treated with epirubicin. If this trial shows an improvement either in response to chemotherapy or patient survival in the quinidine arm of the trial then this will be an exciting advance in the use of chemotherapy in breast cancer. If on the other hand there is no difference with or without quinidine then there are several reasons why this may arise.

Although data on P-gp expression is available for a small number of patients in the trial one concern is that any benefit to P-gp positive tumours may be hidden when all tumours, P-gp negative and positive, are considered together. In addition it is possible that the number of P-gp positive cells in the MDR-positive tumours may be too small to have a significant effect on the whole tumour when modulation is attempted.

The most likely scenario is that several mechanisms of resistance will be present in any one tumour and may include for example alterations in Topoisomerase enzymes or alterations in glutathione-S-transferase levels. The first of these mechanisms may well be extremely important in breast cancer following the recent recognition that alterations to Topoisomerase loci in breast cancer is a relatively frequent event (Keith, personal communication). Since different resistance mechanisms may be more or less important from tumour to tumour future attempts to combat clinical drug resistance may have to combine stategies to overcome several different mechanisms of resistance. This may involve the use of so called modulators which interact with each mechanism, in the same way as quinidine and P-gp, or by development of new cytotoxic agents which are

designed to be unaffacted by known resistance mechanisms. In this way the morpholinyl anthracyclines, which are not substrates for the P-gp pump and which can restore drug-resistant cell lines to nearly the same sensitivity as the drug-sensitive parent lines, have been developed (Coley et al, 1990b; Watanabe et al, 1991).

In summary the confirmation of P-gp expression in human breast cancer together with the finding that quinidine can modulate P-gp positive cells in a solid tumour model of MDR provide a sound basis for attempts to modulate MDR-positive cells in human breast cancers. A clinical trial is currently ongoing to assess this attempted modulation of MDR cells *in vivo* and the results are awaited with interest.

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