MULTIPLE DRUG RESISTANCE IN HUMAN TUMOR CELL LINES

by

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ABSTRACT

Seven new multidrug resistant variants of six established cell lines were developed with the drug adriamycin, HEP-2A, HEP-2B, DLKP-A, OAW42-A, SKMES1-A, SKLU1-A and DLRP-A. All the cell lines, except for DLRP-A were found to cross resistant to a range of drugs. There is a consistent rank order correlation between resistance to the selecting drug and cross resistance to other drugs. The detailed cross resistant profile varies markedly among cell lines selected with the same drug. Heterogenity in the level of drug resistance was a feature of all the MDR variants tested. The MDR variants were generally found to have altered biophysical properties, with sensitivity to standard freezing and standard subculture procedures noted. Additionally, the MDR variants were found to be more resistant to sonication, indicating possible altered lipid composition of the cell membranes. The P-170 mechanism of resistance was investigated by Western Blotting, Immunofluorescence and antisense transfection, and was found to be involved in resistance in CHrC5, DLKP-A, HEP-2A, HEP-2B, SKMES1-A and OAW42-A. There are however indications of other mechanisms of resistance, (e.g. cross resistance to 5-Flurouracil and cis-platin). Additionally incomplete reversal of resistance by Antisense oligomer transfection is suggestive of alternative mechanisms of resistance to P-170 present in a number of the cell lines. SKLU1-A was negative in all the P-170 studies so alternative mechanisms of resistance must be causative reason of MDR in this cell line. Cytogenetic manifestation of MDR in the form of DMs was evident in DLKP-A, HEP-2A and HEP-2B. Drug resistant cell lines were generated by transfection with a complete murine cDNA for mdr1. The drug resistance pattern observed were

significantly different from those of the variants obtained by adaptation from the corresponding parental cell line. It was possible to circumvent adriamycin resistance with clinically relevant doses of a number of compounds, verapamil, quinine, quinidine, nifedipine, chloroquinine, caffeine, generic and standard aspirin.

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION.

Multidrug resistance (MDR) is a phenomenon whereby cells exhibit resistance to a broad range of structurally unrelated cytotoxic drugs. This is found to be an inherent property of certain cancers which never respond to chemotherapy eg. colon carcinoma. Other cancers are intially responsive to a given chemotherapeutic regime but eventually acquire resistance not only to the drugs that have been administered but a broad range of other compounds. The chemicals involved are the Anthracyclines (Adriamycin, Daunorubicin), Vinca alkaloids (Vinblastine, Vincristine), epipodophyllotoxins (VP-16, VM-26), Actinomycin D, and Taxol (all these compounds being hydrophobic natural compounds). There are many systems involved in drug resistance but the best understood is the decreased accumulation of drug in resistant cells when compared to their sensitive counterparts. The resistant cells express a gene for multidrug resistance which encodes a 170 KDa membrane glycoprotein termed P-glycoprotein, or P-170. The procedures used to clone the mdr1 gene, which codes for P-glycoprotein have been described by Croop et al, (1989); Endicott and Ling,(1989); Gottesman and Pastan (1988), and Bradley et al.(1988). It has been found (using transfection experiments) that overexpression of mouse and human mdr1 cDNA is sufficent to confer the MDR phenotype (Gros et al.,1986b; Ueda et al.,1987a). Although P-170 is believed to be a pump that actively effluxes drugs from resistant cells (Dano et al.,1973; Safa et al.,1987) the actual mechanism for this activity is not understood. It has also been proven that the P-170 can transport a wide range of organic chemicals as well as anticancer drugs (Ichikawa et al.,1991). This could suggest that one of the physiological functions of P-170 is the secretion of organic

substances. It has been suggested that the M.W. of the drugs is a important determinant in the MDR phenotype. Selassi et al.(1990) have suggested that current chemotherapy regimes may be improved by treating resistant cells with antineoplastic agents displaying physiochemical characteristics opposite to that of the original inducing agent.

It is now thought that P-170 acts as a flippase (C.Higgins, personal communication) which actively shunts drugs out of the cell. This would explain the lack of substrate specificity that is the hallmark of MDR. The involvement of the P-170 in MDR of tissue cultured cells is now quite clear, however the direct role of this protein in cancer chemotherapy has been more difficult to determine. Since there is differential expression of P-170 in normal tissue (Section 1.5) it seems that P-170 acts as a pump in normal cells as well.

1.2 Protein Organisation and Function.

The ATP binding cassette (ABC) superfamily of transport systems now includes over 30 proteins that share extensive sequence similarity and domain organisation (Hyde et al.,1990). The MDR protein designated P-170 glycoprotein is included in this super family. P-170 is approximately 1280 amino acids in length and consists of 12 transmembrane domains and two nucleotide binding sites (figure 1.1); There are two homologous halves plus a variable linker region. According to the current model of P-170 orientiation in the membrane, the highly charged amino acid termini are both intracellular, the nucleotide binding sites are cytoplasmic, and there are three potential glycosylation sites in the first

extracellular loop. This model is supported by the fact that antibodies to specific regions of P-170 recognise extracellular or intracellular determinants as predicted (Bradley et al.,1990). Also ATP binding sites in the cytoplasm would be expected if the protein functions to pump drugs out of cells. Finally at least one of the putative extracellular glycosylation sites contain carbohydrate residues since treatment with endopeptidase F allows an antipeptide antibody specific for the sequence at this site, to bind there. There are striking similarities in sequence and apparent structure between P-170 and a number of procaryotic transport proteins (Chen et al.,1986, Higgins et al.,1989) and a number of the bacterial transport proteins. The strongest homology is with HLYB, the ATP-binding hemolysin export protein in E.Coli (Felmlee et al.,1985; Blight et al.,1990); NDV-A a protein possibly involved in ß-(1-2)glycan transport in Rhizobium Meliloti (Stanfield et al.,1988);Ikt ,a leukotoxin secretion determinant in Pasteurella Haemolytica, analogous to HLYB (Strathdee and Lo,1989). Also the extracellular secretion of the antibacterial toxin colicin V is mediated by a signal sequence independent process which requires the product of two linked genes: cvaA and cvaB. The nucleotide sequence of the cvaB reveals that its product is a member of ABC subfamily of proteins, (Gilson et al.,1990). Also the amplified H circle of Leishmania tarentolae contains a novel P-glycoprotein gene (Ouellette et al.,1990). Although these proteins are most similar to P-170 in their ATP binding domains, homology does extend to other regions. P-170 is also very structurally similar to a number of eukaryotic proteins thought to be also involved in cellular transport processes. There is at least one homologue of the MDR gene in Malaria parasites pfMDR, Plasmodium falciparum Multidrug resistance gene (Foote et al.,1989). Structure and

sequence homologies have also been found in the brown and white protein pigments in Drosophila, (Dressen et al.,1988) and in a yeast protein which is thought to be involved in the secretion of mating factor-α. The product of the STE6 gene shows almost 57% amino acid sequence homology with human P-170 (Kuhler et al.,1989, Mc Grath,J.P.,1989) Also , the product of the cystic fibrosis gene (CFTR) has also been classified into the ABC group of transporters, (Hyde et al.,1990, Riordan et al.,1989). In addition to the 4 domains present in all the other proteins mentioned above, CFTR has another domain designated as the regulatory domain R. Because the best homologies have been found for pumps involved in protein efflux (HYLB and STE6), it has been suggested that one function of the MDR system may involve efflux of proteins or peptides (Mc Grath et al.,1989). However, none of the substrate binding sites for these transporters have been identified, and until such information is available it is not possible to predict substrate specifity from sequence data.

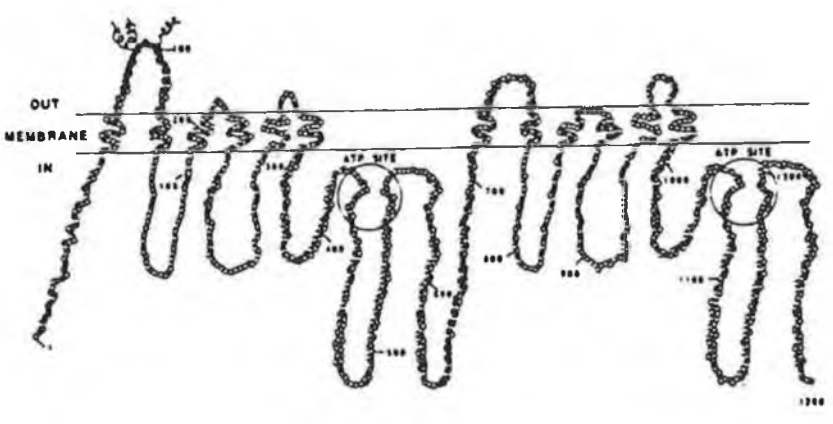


Figure 1.1: Model of the MDR Transporter.

1.3 Gene Structure and Organisation.

MDR is the result of overexpression and amplification of multidrug resistant genes, designated mdr1 and mdr2 (or mdr3) in human, and mdr, pgp or pgy in non human species, (Croop et al., 1989; Endicott and Ling., 1989, Raymond et al., 1990a). (There are discrepancies in the terminology used for the human and mouse MDR genes, see table 1.1 for details). In human, mouse and hamster these make up a small multi-gene family, with two human (Roninson et al., 1986, Van der Bliek et al., 1986) and three rodent genes, (mdr1a, mdr1b, mdr2) (Ng et al., 1989), see table 1.2 for interspecies and intergene homology at the amino acid level. Transfection experiments with full cDNA from the mdr1 gene have unambigously shown that increases in P-170 expression are sufficent to cause the MDR phenotype. Monoclonal antibodies have proved to date to be the most exact method for determining the expression of different P-glycoprotein genes in normal and human tissue. P-glycoprotein specific monoclonal antibodies have been used to isolate P -glycoprotein cDNA clones from libraries prepared in the expression vector Lambda gt11 (Riordan et al., 1989). A P-glycoprotein cDNA sequence pCHP-1 was first isolated from the CHrB30, a resistant clone of CHrC5. The clone pCHP-1 was proven to code P-170 by its detection with three monoclonal antibodies (C219, C494 and C32), its hybridisation to an mRNA of 4.7kb and its hybridisation to amplified genomic DNA sequences in different MDR cell lines. It was found on hybridisation of the pCHP-1 probe to hamster resistant DNA cut with ECOR1, that 12 differentially stained bands resulted. This was the first indication that P-170 may be encoded by a multigene family. P-glycoprotein sequences are

amplified in MDR cells. This has allowed P-glycoprotein expression to be analysed by the technique of differential hybridisation or by gel renaturation (Roninson et al., 1989). These methods were advantageous to use because other genes coamplified with P-glycoprotein have also been isolated. The presence and the analysis of these genes may contribute to our understanding of the MDR phenotype.

Using the technique of differential hybridisation to labelled single stranded cDNA from CHOCHrC5 (the chinese hamster ovary MDR cell line) and to cDNA from the parental drug sensitive cell line AuxB1, five different classes of transcript (Classes 1-5) distinguished on the basis of their hybridisation to mRNAs of different lengths on northern blot analysis, were initially isolated (Roninson et al., 1983 ; Van der Bliek et al., 1986). Since then, a sixth sequence (Class six) has been isolated (Borst et al., 1988). The class 2 cDNA clones were found to hybridise to pCHP-1 and to an mRNA transcript of 4.7kb. This transcript was the only one found to be consistantly overexpressed in MDR cells (De Bruijn et al., 1986). Mapping experiments using the technique of pulse field electrophoresis have shown that there are three class 2 genes in mouse; these correspond to the three pgp genes found in hamster. The two human class 2 genes mdr1 and mdr3 are linked within 330kb of each other on chromosome 7, band q21.1 (Chin et al., 1989). These two genes encode proteins that are 80% homologous at an amino acid level, (Van Der Bliek et al., 1986), suggesting that the genes arose from a duplication event. See figure 1.2 for comparison of deduced amino sequences of the three MDR proteins and the human mdr1 protein and figure 1.3 for comparison of the putative start site of transcription. A function for the mdr3 has

not yet been determined, however it has been found that it undergoes differential splicing (Hsu et al., 1989). The transcript for the mdr3 gene is found to be consistently shorter (4.1Kb Vs 4.5Kb), partly as a result of differences in the lengths of the 3'-untranslated region. Three different transcripts for mdr3 were isolated from cDNA libraries from human liver. The predominant transcript isolated aligned with the mdr1 sequence. The other two variant sequences have either an in frame 21-base pair insertion between the region encoding the highly conserved 'A' and 'B' nucleotide-binding consenus sequences in the C-terminal cytoplasmic domain of P-glycoprotein, or alternatively a deletion that removes sequences encoding the fifth potential transmembrane region from the C-terminal domain. Differences in 3'-untranslated region processing have also been reported for both hamster pgp1 and pgp2 transcripts (Endicott et al.,1989). The biological significance of these observations is not known.

The fact that the P-170, and therefore MDR genes, are composed of two homologous halves has suggested that the gene itself was generated by a duplication event before mdr1 and mdr3 divergence. On the analysis of the mouse, hamster and human MDR sequences there appears to be a greater relationship between entire MDR class molecules of different species (i.e. mdr1 Vs mdr1a or mdr1b) than between the two halves of any single gene, (Hsu et al., 1989). Futhermore, a very surprising finding has come from the intron /exon structural analysis of human mdr1 and mouse mdr1b genes (Chen et al., 1990; Raymond et al., 1989). It has been found for both these genes, that even though each half contains about the same number of introns and exons most of the introns occur at different places in the two halves of the gene. It is possible that this

gene structure is the result of a primodrial gene containing few introns, with subsequent insertion of introns, giving the resultant gene (Raymond et al., 1989). However, it has been suggested that the gene for P-170 arose by fusion of two structurally similar genes which had evolved separately and not by gene duplication (Chen et al., 1990).

On cloning and transfection of the mouse mdr1, mdr2 and mdr3 (Devault et al., 1990) it was found that mdr1 and mdr3 conferred the MDR phenotype with overlapping but distinct substrate specificities. Cells transfected with and expressing the mdr1 showed a 2-3 fold preferential resistance to colchicine and adriamycin compared with cells expressing an equivalent amount of mdr3. Conversely cells transfected and expressing mdr3 showed a preferential resistance to actinomycin D. Transfection of full cDNA of the mdr2 into sensitive cells did not confer the MDR phenotype. This situation is probably unique to rodent , unlike human in which only full cDNA from the mdr1 can confer the MDR phenotype.

Roninson et al. (1988), Devine et al. (1991) and Croop et al. (1991) have provided direct evidence in the human mdr1 gene (Glycine-185) that alterations probably occuring during the course of drug selection, can modify the profile of drug resistance in human cells. Gekeler et al., (1990) undertook a study to determine if alterations in the nucleotide sequence would result in alterations in the cross resistance profile. Using PCR technology, the sequences around codon 185 which codes for an amino acid residue possibly influencing the drug binding function of the P-170 were cloned and sequenced. Altogether, only 2 single nucleotide

differences in an intron were found in 2 out of 40 recombinants each harbouring a 209 bp genomic or a 269bp cDNA fragment of the mdr1 / P-glycoprotein gene. Results conclude that clustered mutations around this region are not a causative reason for different multidrug resistant profiles.

Additionally in a murine system with J774.2 (Lothstein et al., 1989), the mdr1a and the mdr1b encode the 120-125kda P-glycoprotein precursors, a switch of gene product was noted from mdr1b to mdr1a which was associated with an approximate 4-fold increase in resistance to vinblastine, taxol and doxorubicin, with no dectectable changes in immunoreactive P-glycoprotein. It was suggested that the mdr1a may encode a more efficient P-glycoprotein. Although the mechanism governing the gene switching event is not understood, it is likely to occur within a single cell. These findings suggest that differential expression of distinct MDR genes which encode unique P-glycoprotein isoforms is a possible mechanism for generating divergence in the phenotype.

Table 1.1. Mammalian P-glycoprotein genes.

P-glycoprotein Class	Human	Mouse	Hamster	Involved in MDR
1	mdr1	mdr3 (mdr1a)	pgp1	Yes
11		mdr1 (mdr1b)	pgp2	Yes
111	mdr3 (mdr2)	mdr2	pgp3	Unknown

Alternative designations are given in parenthesis.

Table 1.2. Relationship between the mouse genes and their hamster and human homologues based on sequence comparison of their linker regions.

	Mu-mdr1a	Mu-mdr1b	Hu-mdr1	Ha-mdr1b	Hu-mdr2
Mu-mdr1a	—	47%	68%	45%	20%
Mu-mdr1b	47%	—	48%	67%	13%
Mu-mdr2	25%	20%	23%	23%	60%

The percentages indicate the degree of optimised amino acid homology. Mu= Mouse, Ha= Hamster, Hu=Human.

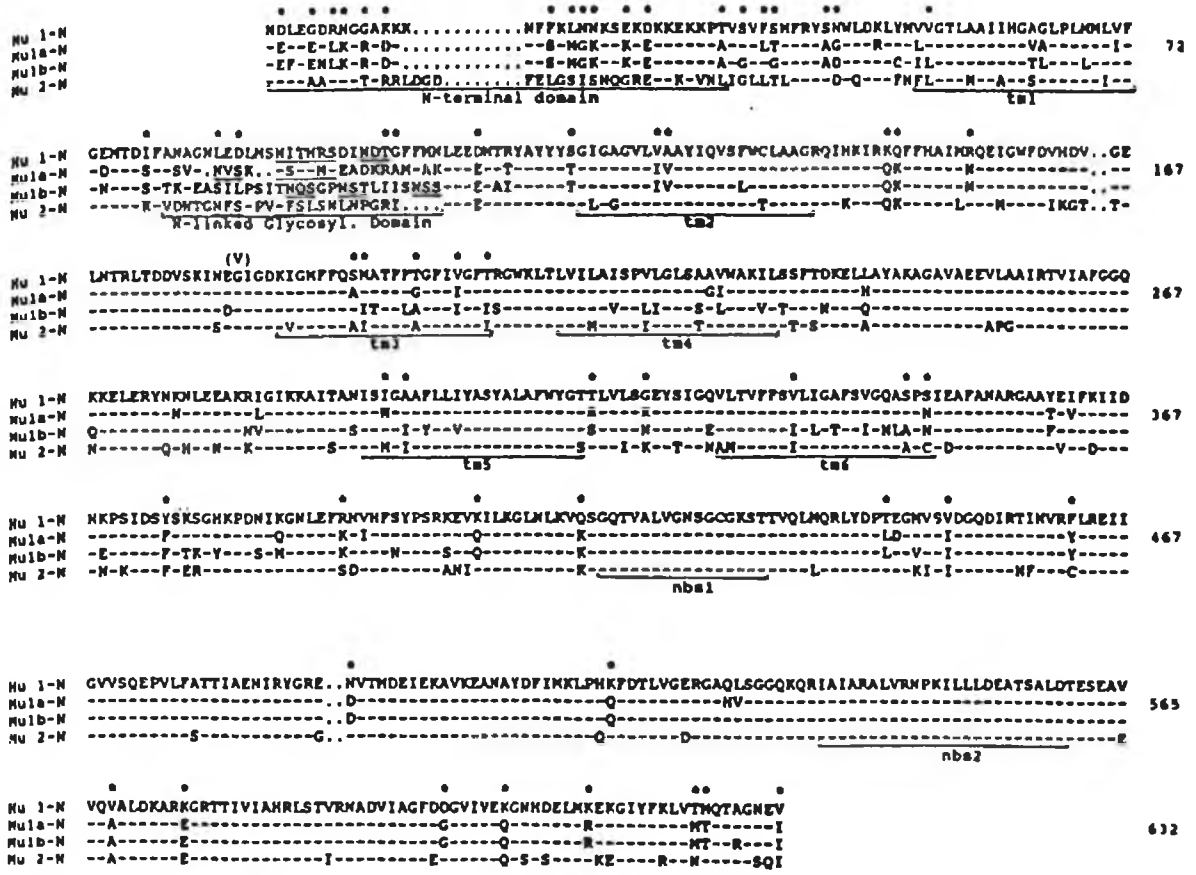


Figure 1.2. Comparison of deduced amino acid sequences of the mouse mdr proteins and the human mdr1 protein. For the mouse mdr proteins, only the residues which differ from the human mdr1 sequence are shown; identical amino acids are marked with a dash, asterisks mark positions where at least two distinct mouse mdr proteins differ from the human mdr1, predicted transmembrane domains (tm) and nucleotide binding sites (nbs) are as determined for the human mdr1 sequence.

mdr1 - Human TTGGTGGCCGTTCCAAGGAGCGCGAGGTCGGGATGGATCTTGAAGGGGACCGC

mdr2 - Mouse AAGGAAACCCGGAGGTGGCACGTGAGGTGGTGATGGAGTTTGAAGAGAACCTT

mdr3 - Human CCTGCCAGACAGACACGCGCGAGGTTCGAGGCTGAGAGATGGATCTTGAGGGG

Consensus sequence GccGcc ccATGG
A
G

Figure 1.3. Alignment of nucleotide sequences flanking the putative start site of translation for human mdr1, mdr3 and mouse mdr2. The consenus sequence is given below with the most common nucleotide in capital letters, the ATG start codon is underlined.

1.4 Cytogenetic Alterations in MDR Cells.

The P-Glycoprotein amplicon maps to chromosome 7q21-31 (Jongsma et al.,1990). Five gene classes that are co-amplified with P-glycoprotein have been isolated and appear by a number of investigations to map with the P-glycoprotein amplicon (Van der Bliek, 1986, Borst, 1988). By the use of pulse field gradient electrophoresis, it has been shown that 1100kb of DNA can be co-amplified with P-glycoprotein sequences, and that this DNA encodes for five additional genes (termed gene classes 1 and 3-6). Only the gene product of class 4 gene and class 2 has been identified to date. The class 4 gene encodes sorcin, a cytosolic calcium binding protein, that is homologous to calpain (Van der Bliek et al., 1986). This protein has been identified in increased amounts in vincristine resistant chinese hamster and mouse cells (Meyers et al., 1981). In one study (Meyers et al., 1985), increases in sorcin expression paralled changes in DNA amplification, suggesting that increased sorcin expression resulted from increased gene expression. Four other RNA transcripts are also overexpressed by being part of the six gene amplicon. It is not known what role, if any, these additional proteins may play in the modulating the MDR phenotype, but it is interesting to note that the overall organisation of the genes identified within the P-glycoprotein amplicon are conserved among human, mouse and hamster cells. The mdr1 and mdr3 genes (The two class 2 genes) have been localised to 7q 21.1 approximately 300 bases apart.

Double Minute (DM) chromosomes and Homogenously Staining Regions (HSRs) are the most common manifestation of amplification present in MDR cells (Bell et al., 1987). In metaphase chromosome spreads

DMs appear as small paired usually spherical chromosome-like structures. Characteristically, DMs vary in number and size from cell to cell and can accumulate in certain cell populations. Their features include varying ability in the uptake of stain, and the lack of a functional centromere (Barker et al., 1982). The resistance of DMs to loss by classic mechanisms might result from the abnormal replication or segregation. An unexplored additional possibility that might explain the retention of DMs is extramitotic transfer of DMs between cells. Ruiz et al.(1989) have offered another explanation, it has been shown that DMs can be formed from submicroscopic circular precursors referred to as episomes. A variety of methods including electrophoresis of undigested DNAs in high voltage gradients, Not1 digestion, and the production of double stranded breaks by gamma irradiation; were used to distinguish between mdr1 sequences amplified on submicroscopic circular molecules and those amplified within DMs or chromosomal DNA. The gamma irradiation procedure provides a new method for detecting and determining the size of circular molecules from 50kb to greater than 1000kb. These procedures revealed that some of the amplified mdr1 genes in vinblastine-resistant KB-V1 cells are contained in supercoiled circular molecules of between 600 and 750kb. It was found from Meselson-Stahl density shift experiments that these molecules replicate approximately once per cell cycle. Ruiz et al.(1989) has added further support to a model for gene amplification in which DMs are generally formed from smaller, autonomously replicating episomes. Chromosome banding applied to tumor cells led to the discovery of HSRs which are distinctive regions representing amplification of uniformly stained regions on a chromosome. HSRs have been identified in a variety of tumor cells from man and experimental animals (Barker et al., 1982;

Barker et al., 1980) and frequently as with DMs ,in neuroblastomas (Brodeur et al., 1980). The presence of HSRs is not restricted to cell lines: Kovacs et al. (1979) showed HSRs in chromosome preparations made from solid tumors. A HSR present in a cell line derived from a breast carcinoma patient was also present in a subsequently obtained pleural effusion from the same patient (Lau et al., 1986). In a number of studies it has been found that the number of DMs and the length of the HSRs correlate well with the degree of resistance. In a study of the mouse cell line J774.2 it was found the DMs were absent in revertant cells (Sandberg et al., 1983), also in the colchicine resistant variant the number of DMs correlated very well to the degree of resistance (Lothstein et al., 1986). Biedler et al.,(1983) noted in vincristine resistant chinese hamster lung (DC-3F) that the length of the HSR (localised to chromosome 7) studied in a range of resistant cells correlated proportionally to the degree of resistance. Jongsma et al.(1990), also noted that a decrease in the length of the 7q HSR correlated with the decreases in relative colchicine resistance. A most interesting study involved four neuroblastoma cell lines (Sandberg et al., 1983), in which with banding techniques, all the cell lines were shown to contain a marker with a long HSR. This HSR-containing chromosome differed in each cell line. One line contained two classes of cells: one with an HSR marker and the other with DMs. It was suggested that DMs are derived from the HSR, with the location and the size of the HSR differing from one cell line to the next. This phenomenon, whereby either HSRs or DMs are present, has been observed in a number of cases , (see table 1.3 for details). The presence of both HSRs and DMs concomitantly has been only noted in three cases (Quinn et al., 1979; Meyers et al., 1989; Slovak et al., 1987). Table 1.3

summarises the cytogenetic observations in MDR cell lines. It is interesting that HT1080/DX4 is the most resistant population studied, with DMs been only noted in the less resistant cells. This would suggest that HSRs are predominantly found in the more resistant cells and DMs are found at earlier stages of resistance (Riordan et al., 1985). In addition there have been a number of suggestions that DMs are related to the de novo appearance of unusual chromosomes or markers with long HSRs (Balaban-Malenbaum et al.,1977). These investigators described a neuroblastoma population cell line that had two populations. In one cell population, DMs were observed. In the other cell population, no DMs were evident, but marker chromosomes with HSRs were present. Since there were marker chromosomes common to both cell populations it is possible that both cell populations were derived from a common precursor. A similar phenomenon was noted in COLO-320 and COLO-321. In both cases DMs were observed from the earliest cytogenetic anaylsis. A number of subcultures lost DMs and gained HSR markers after 1 to 1.5 years in culture. It was noted that the number of DMs were drastically reduced in 99% of the cells with HSR markers. The basis for the sudden appearance of a new marker chromosome with a HSR and concurrent dissappearance of DMs is not clear.

Table 1.3. Cytological Evidence for Gene Amplification in Cell Lines and Tumors.

Cell Line	Cytological Evidence for Gene Amplification / Comments	Reference
DC-3F/VCR MAZ/VCR-U SH-SY5Y/VCR	HSRs DMs, absent in revertants. DMs, absent in revertants.	Meyers et al., 1985.
HA-A Melanoma Cell Line	Less than 1% of cells had HSR alone; greater than 90% had only DMs present	Trent et al., 1984.
CHOB30	Presence of a number of large HSRs	Riordan et al.,1985.
SH-SY5Y/VCR	100% of cells has 10-100 very small DMs	Meyers et al., 1989.
CEM/MTXR3 Leukemia	DMs in 10% of cells analysed	Kavallaris et al.,1990.
CEM/VLB.100	HSR present on chromosome 1	Hill et al., 1988.
MCF7/ADR	HSR present on chromosome 7 Translocation on chromosome 11	Fairchild et al.,1987.
Primary Human Neuroblastoma	HSR present on chromosome 13p	Balban et al., 1982.
WiDR/R Colon Carcinoma	Two versions of a HSR predominant on the short arm of chromosome 7	Dalton et al., 1988.
1GROV1/VCR	Del short arm of chromosome 11 del (6)(q16)	Benard et al., 1989.
Human Leukemia	Presence of ABR (Abnormal band region) on chromosome 13. Not sure if it an amplified gene.	Beck et al., 1987.
NCI-H249P Human NSCLC	DMs present ,unstable lost in the absense of selection	Curt et al., 1983.
KB/ADR KB/VLB KB/COLCH	DMs present in all resistant lines, found to be very unstable in absence of drug	Fojo et al., 1985.
Colo-320 Colo-321	At low levels of resistance 100% DMs; in latter stages of resistance the number of HSRs increased	Quinn et al., 1979.
MC-IXC	DMs and HSRs CHR19p and 22q.	Meyers et al., 1989.
HT1080/DX4	DMs and HSRs present in the most resistant, DMs only present in less resistant.	Slovak et al., 1987.

1.5. P-170 Function in Normal Cells.

Expression of P-170 has been detected in a number of normal tissues, both as mRNA and protein. Using immunohistochemical staining of normal tissues, P-170 is found in liver, adrenal glands, pancreas, kidney, colon and jujenum (Fojo et al.,1987). Localisation within these tissues appears to be extremely specific; it is on the apical surface only of epithelial cells in liver, kidney, colon and jujenum; on the bilary canalicular front of hepatocytes; on small bilary and pancreatic ductules; and on the adrenal cortex (Thiebaut et al.,1987, Sugawara et al.,1988b). P-170 has also been detected in human placenta (Sugawara et al., 1988a) and is expressed on specialised endothelial cells in the brain and testis (Cordon-Cardo, 1989 and 1990). Preliminary results suggest that in the brain it is on the luminal surface of endothelial cells of some post capillary venules. Gene specific RNA analysis indicates that most P-170 in normal tissue results from mdr1 expression. Expression of human mdr3 gene is at lower levels, with highest expression in the liver (Van der Bliek et al., 1986; Chin et al.,1989; Arias et al.,1990). It has been shown in a study by Teeter et al.(1990) that the over expression of mdr3 gene in mouse liver tumors does not requir exposure of the animals to a carcinogenic agent and suggests that its overexpression is associated with a general pathway of hepatic tumor development. The overexpression of the mdr3 gene in hepatocellular tumors may be responsible for the poor response of the tumors to chemotherapeutic agents. From the above data, it is evident that P-170 has a normal function. The localisation of P-170 to specialised cells in human tissues indicates that it might be involved in transepithelial secretion of toxic substances or unknown cellular metabolites into

the bile or the lumen of the GI tract. The fact that the mdr1 gene can be co-induced with the P4501A1 gene possibly supports this (Burt et al.,1988). The discovery of P-170 in the endometrium of mouse gravid uterus (Yang et al., 1989) also suggests it might be involved in secretion of steroid hormones. In a MDR mouse line binding of [^{3}H] azidopine or [^{3}H] vinblastine to P-170 is inhibited by steroid progesterone. Also reports by Kacinski et al.,(1989) and McGuire et al.,(1990) offer evidence that in cancer specimens, overexpression of P-170 is inversly correlated with the expression of the progesterone receptor. Using in situ hybridisation Kalinski et al.(1989) showed that in 9/16 untreated breast cancer patients, there was a strong inverse correlation between increased mdr1 expression and expression of progesterone receptors. This study also raises the possibility that in-situ hybridisation may be a more sensitive technique for detecting mdr1 expression in solid tumors. From both these studies it can be seen that an endogenous steroid hormone is at least capable of interacting with P-170 and might represent one class of natural substances for the pump activity. Another possible function is that P-170 dependent efflux represents an alternative pathway for the secretion of proteins and peptides which have no formal signal sequences (McGrath et al., 1989). This concept was thought to explain the broad range of drugs transported by P-170 itself, since they could be bound to different proteins, which were in turn transported by the pump (Gerlach et al., 1986). Although it has been suggested that host substrates interact directly with the transporter itself, hence no carrier proteins would be necessary, other data suggests that certain proteins may be substrates for P-170. One necessary requirement noted for P-170 substrates is hydrophobicity. There appears to be a direct correlation between hydophobicity and

ability to serve as a drug substrate for P-170, such that the ability of steroids to inhibit P-170 in mouse cells, increases with increasing hydrophobicity (Yang et al., 1990a). Further proof of this point is that the yeast mating factor-α, a natural substrate for the STE6 gene product, is a dodecapeptide containing a lipophilic farnesyl group at its carboxyl terminus. In addition multidrug resistant cells are resistant to the hydrophobic cytotoxic peptides,gramicidin D and valinomycin. One possible substrate for P-170 according to the mechanism of protein secretion is interleukin-1 (IL-1) (McGrath et al.,1989), a secreted protein that is not handled in the classical manner. Like yeast mating factor-α, IL-1 has no signal sequence and the mature peptide is modified by the lipid myristic acid (Bursten et al.,1988). In the eventuality of mdr1 (P-170) not being responsible for protein transport, a more distantly related transporter may be found to play the role.

The mode of regulation of the mdr genes has been investigated extensively. It has been found in the rat that liver damage caused by hepatectomy (Thorgeirsson et al., 1987) or treatment of the animal with cytotoxic drugs, results in large increases in MDR (Gottesman et al., 1988). The mechanism of regulation is unknown, but it could involve RNA stabilisation, since new transcription as determined by nuclear run-off assays is not seen (Marino et al.,1989). The large number of tissue culture models available has proven to be a valuable source of material for the study of the regulation of the mdr genes. Current examples include heat shock or arsenite induction of the mdr1 gene in human renal adenocarcinoma (Chin et al.,1990) and increased expression in human neuroblastoma cells after retonic acid treatment (Bates et

al.,1990). Regulation of the mdr1 gene also appears to involve differential promoter utilisation. In a study on human KB cells transcription initation starts at one of the two major promoters (Ueda et al.,1987a), whereas in most normal tissues only the downstream promoter is used. The significance of the activation of the upstream promoter is unclear, but it could possibly be related to the increased expression of the mdr1 gene after drug selection, since human childhood acute Lymphocytic Leukemias can show activation of this promoter (Rothenburg et al.,1989). In the mouse mdr1 gene, Raymond et al,(1990b) have cloned the 5' end of the gene in an attempt to define cis acting elements implicated in transcriptional regulation. Sequences located between nucleotides -93 and +84 were able to confer basal promoter activity and cell specificity to the reporter gene. The addition to the basal reporter of sequences upstream of position +141 was found to up-regulate or down regulate the basal level of expression of the reporter gene in a tissue specific manner. This suggests that the tissue specific expression of mdr1 is controlled by the combination of positively and negatively cis-acting 5' regulatory elements. This suggests a basis for the identification and characterization of the trans-acting factors mediating, this regulation both in normal tissues and in MDR cells. Another particularly interesting study by Cornwell et al.,(1990) found that deletion of sequence 5' and 3' of the transcriptional initiation site modulated the level of transcription. Deletion of exon 1 sequence +5 to +127, completely inhibited accurate initiation of mdr1 transcription. Replacement of just the +5 site , used for initation in vitro and in vivo did not reverse inhibition. mdr1 transcription was specifically inhibited by an oligonucleotide corresponding to sequences +46 to +58. This again proves that sequences upstream

and downstream of the transcription initiation site affect the efficiency of the mdr1 proximal promoter. A similar case is noted in the mouse J774.2 cell line (Hsu et al.,1990), who showed that transcriptional initation from the putative upstream promoter correlated with a 70% to 85% decrease in steady state mdr1a protein levels relative to transcript levels. In addition, the identification of putative AP-1 and AP-2 promoter elements suggest a possible role for protein kinase A and protein kinase C in the regulation of mdr1a. It has been suggested that the mdr1a gene may be regulated by transcription factors which mediate the signal transduction pathways involving both protein kinase A and protein kinase C.

It has been noted also, that a decrease in Protein Kinase A (Abraham et al.,1990, Staats et al.,1990) leads to an increased drug sensitivity. A lower level of expression of the MDR gene and protein has been described in cells that carry the dominant regulatory subunit gene of the cAMP dependent protein kinase (PKA) gene (Abraham et al.,1990). This suggests a direct effect of PKA on maintaining levels of the P-glycoprotein in these cells, probably through control effects on gene expression. However, in a study by Schwartz et al.(1991), using MCF7-ADR and resistant MOLT cells, it was found that the PKC activity can be increased or decreased in MDR cells. Both staurosporine and phorbol ester activation failed to produce changes in drug resistance, PKC in MCF7-ADR and MOLT3 seems to be unrelated to mdr1 levels. To further define the role of PKC-catalysed phosphorylation P-glycoprotein function, it will be necessary to inhibit phosphorylation, and studies utilizing PKC inhibitors are in progress. An alternative approach, whereby PKC is depleted by

prolonged exposure of cells to phorbol 12-myristate 13-acetate (PMA), was precluded, since KB-V1 PKC was relatively resistant to PMA down-regulation (Chambers et al.,1990b). Evidence for the role of PKC in numerous secretion and transport processes has been well documented. PKC activation is associated with release of neurotransmittors, hormones and other agents, and with the transport of glucose and ions such as Ca^{++}, H^{+}, Na^{+}, K^{+} and Cl^{-} (Nishizuka et al.,1986). The transporter proteins responsible for some of these processes have been identified and in some cases phosphorylation in vivo noted eg. glucose transporter (Sardet et al.,1990). However, the modulation of transport activity via PKC phosphorylation of P-170 glycoprotein appears to be unique,and is possibly a important factor in the regulation of P-170 glycoprotein function.

1.6 Structure Function Relationship of P-170.

If the structural homologies between P-170 and other transport proteins are significant, it appears that a motif of six transmembrane portions and a nucleotide binding site is important to function. The proof that P-170 exists as a dimer (Boscoboinik et al., 1990) could suggest that for conformational reasons there may be a need for such transport proteins to be present in at least two subunits. One suggestion is that a large structure may be necessary to form a membrane channel or pore big enough to transport high M.W. substances. Alternatively each unit of transmembrane domain may contribute separately and uniquely to substrate binding specificity, such that P-170 must be thought of as a whole molecule rather than two halves. This observation is substantiated by the finding that neither half can function alone (Currier et al., 1989). Independent evidence shows that the binding sites in each half of P-170, are extremely important in drug resistance. Inactivation of either site alone leaves no activity, inactivation of one site only leaves only a small amount of residual activity (Azzaria et al., 1989). On mutation of the nucleotide binding site, the patterns of residual resistance are different. This would suggest that both halves of P-170, and possibly the 2 nucleotide binding sites are contributary to the specificity of drug recognition or binding. Also, slight changes in amino acid sequence have been shown to alter drug sensitivity patterns or totally abolish them. It is evident that the determinants of substrate specificity, interaction and transport function will be complex and probably encompass the entire structure of these proteins. Intermolecular and interspecies chimeras might be interesting in terms of dissecting the

relationship between structure and function.

1.7. Clinical Relevance of the MDR Genes and P-170 Protein Levels.

Levels of the P-170 glycoprotein and the mdr1 specific mRNA have been anaylsed in a large number of tumors. In general, expression of the mdr1 gene is high in those tumors which derive from tissues that naturally express high levels of P-170. Tumors derived from the colon, kidney, liver and the pancreas usually have such high levels. In poorly differentiated kidney and colon tumors the levels are often lower because of the altered differentiation state of these tumors. In most differentiated tumors of the kidney, high levels of expression of P-170 were noted (Kanamura et al.,1989). Human tumors of this type are often intrinsically resistant to standard chemotherapy, therefore the response rate is normally very low. Expression of the mdr1 gene is sometimes high in leukemias, lymphomas, and some other tissues that do not normally express the mdr1 gene. This phenomenon is difficult to explain, it is thought that one of the biochemical or genetic events that occur in carcinogenesis could possible activate the mdr1 gene. For example when chronic myeloid leukemia goes into blast crisis, the mdr1 gene is often activated (Pirker et al.,1991). Several kinds of tumors have low levels of the mdr1 gene and are known to be drug sensitive. These include Wilm's tumor and cancer of the ovary and the breast. However a number of tumors exhibit low levels of mdr1 expression but are resistant to chemotherapy, for example adenocarcinoma of the lung and non small cell lung cancer. This indicates that mechanisms of resistance other than P-170 are prevalent, for example involving Topoisomerase 11 or GST. Such alternative mechanisms of resistance could also be partially

responsible for intrinsic drug resistance in colon carcinomas or hepatomas. These tumors are derived from tissues that are involved in detoxification of exogenous and endogenous toxic compounds, and such alternative detoxifying systems could contribute to the intrinsic drug resistance of these cancers. A list of the tumors in which mdr1 expression is usually high, sometimes high, or usually low is shown in Table 1.4.

Table 1.4. MDR1 Expression in Untreated Human Cancer.

Usually High	Sometimes High	Usually Low
Colon	Acute Leukemias	SCLC
Kidney	Lymphomas	NSCLC
Liver	NSCLC-NE	Gastric
Adrenal	CML-blast crisis	Eesophageal
Pancreas	Neuroblastoma	Ovary
		Wilm's
		Head and Neck
		Myeloma
		Breast

(Pastan et al.,1991)

Many groups are now investigating mdr1 expression in patients with acquired drug resistance. Increased expression has been noted in drug resistant leukemias, myelomas, ovarian cancer, breast cancer, sarcomas and neuroblastomas (Goldstein et al.,1989; Bourhis et al.,1989; Golstein et al.,1990; Dalton et al.,1989a and 1989b). In most cases an increase in mdr1 expression was predictive of failure to respond to chemotherapy. Elevated levels of P-170 (both mRNA and protein) have been noted in some solid tumors following chemotherapy, however the relationship between clinical resistance and elevated levels of P-170 have been very difficult to ascertain, (see Table 1.5 for details).

Table 1.5. Cancers In Which MDR1 Expression has been Found to Increase After Chemotherapy.

Acute Leukemias	Ovary
Breast cancer	Pheochromocytoma
Lymphomas	Rhabdomyosarcoma
Multiple Myeloma	Sarcomas
Neuroblastoma	

(Pastan et al.,1991)

A number of case studies from different tumor classifications and origins will be now highlighted.

Hematological Maligancies:

Expression of P-170 and mdr1 RNA has bee found in untreated hematological maligancies in which the intrinsic levels of P-170 would not be apparent e.g. Myelodysplastic syndromes (Holmes et al.,1989; Weide et al.,1990) and in chronic myelogenous leukemia in blast crisis, but not in chronic phase (Goldstein et al.,1989). In some Lymphomas, high levels of expression of mdr1 mRNA have been noted with 7 of 12 patients with indolent cancers possesing moderate levels, but only one of the more aggressive lymphomas having comparable levels of expression (Moscow et al.,1989a). However high incidences of recurrences have been reported. Overexpression of mdr3 mRNA has been reported in many cases of polylymphocytic leukemia Nooter et al.,1990b), mdr1 overexpression was noted in one patient with this disease. The patient in question had prior chemotherapy; this was the factor that attributed to the increased levels of mdr1. Ma et al.,(1987) used western blotting procedures to show P-170 overexpression (following initial chemotherapy) in sequential samples from patients with progressive chronic myeloid leukemia during chemotherapy. Similar findings of a relationship between P-170 overexpression and clinical resistance has been reported by Carulli et al.(1988) in hematological maligancies.

Table 1.6. The clinical relevance of the expression of the MDR1 gene in hematological maligancies.

Tumor Type	Number of Patients with mdr1 expression	
	Treated	Untreated
Secondary acute myeloblastic leukemia (Holmes et al.,1989)	9/13	2/8
Acute Myeloblastic Leukemias (Notter et al.,1990a)	6/10	1/6
Chronic Lymphoblastic leukemia (Holmes et al.,1990)	11/17	2/15
Acute Lymphoblastic leukemia, salvage chemotherapy (Rothernburg et al., 1989)	3/15	1/9
Acute myeloblastic leukemia Acute Leukemia (Ito et al.,1989)	0/14 0/5	
Acute myeloblastic leukemia (Sato et al.,1990a,b)	45/65	28/36
Acute lymphoblastic leukemia		4/11
Acute Myeloid Leukemia (Kumazuru et al.1990)		9/17
Acute Myeloid Leukemia (Pirker et al.,1991)		50/63

In another study by Herweijer et al.(1990) expression levels of mdr1 and mdr3 were ascertained using an RNase protection assay on peripheral blood from 69 patients with acute and chronic leukemias. Increased mdr1 levels were noted from patients with acute lyphocytic leukemia (13/17), CML chronic phase (10/10), blast crisis (3/4), ALL (8/11), B-CLL (17/17) and Hairy Cell Leukemia

(1/2), but not at all in B-cell prolymphocytic leukemia; expression of mdr3 was only not noted in B-cell lymphocytic leukemias. This study did not specify whether the samples were taken before or after chemotherapy.

70% of patients with multiple myleoma are normally treated with the VAD (Vincristine, Doxorubicin, Dexamethasone) regime of therapy, however nearly all relapse. It has been established that most patients who relapse after this regimen express high level of P-170 (Dalton et al.,1986). The percentage of cells expressing P-170 in these treated patients, quantified by immunohistochemistry, was significantly greater than found in untreated patients (Epstein et al.,1989) Results from an in vitro study by Carulli et al.,(1990a) have also supported the implication of P-170 as a mechanism of resistance in multiple myeloma. Partial circumvention of resistance to the VAD regimen has been possible with verapamil (Dalton et al.,1989a).

Malignant Melanoma :

P-glycoprotein expression was measured, using Northern blotting for mRNA analysis and immunohistochemistry with MAB C219, in a number of primary and metastatic melanoma, (Fuchs et al.,1991). Substantial expression was noted in only 1/37 primary melanoms and only 1/27 melanoma metastases. None of the patients with negative metastases responded to chemotherapy. However, a complete remision of metastatic growth was observed in the patient in whom metastasis significantly expressed the P-glycoprotein. Sequential studies revealed no significant increases of P-glycoprotein during and after chemotherapy. It was concluded that P-glycoprotein is not a mechanism of resistance in malignant melanoma metastases.

Colon Carcinoma :

P-glycoprotein was noted in 65 of 95 primary colon carcinomas (using RNA and protein analysis) which were stage B1 or greater (Weinstein et al.,1991). An interesting observation was that P-170 positive invasive colon cancer cells appear to have an increased potential for dissemination, suggesting that P-170 may influence cellular properties other than drug resistance.

Childhood Sarcoma:

In general, the high level of expression of P-170 appears to be an adverse prognostic factor for tumor response to chemotherapy. P-170 was elevated in nine patients with childhood sarcoma, all of whom relapsed after an initial response, whereas only one of 20 initially negative P-170 patients, relapsed after clinical response (Chan et al.,1990).

These workers used a markedly improved immunohistochemical technique for P-glycoprotein detection that can be applied to formalin fixed, paraffin embedded tissue sections. In the 9 positive samples,small patches of P-glycoprotein positive cells were detected. These would have been probably missed in using bulk techniques and they appeared to be of crucial importance in the development of MDR phenotype.

Kaposi's Sarcoma:

Significant P-170 expression has been found in untreated tumors from 10-12 skin biopsies in patients with Kaposis sarcoma (Schwartsmann et al.,1989).

Neuroblastoma:

Goldstein et al.(1990) reported high levels of mdr1 expression in 5/18 patients with neuroblastoma, but only 3/31 untreated patients with the disease. Corrias et al., (1990) showed no amplification in 34 patients with neuroblastoma, using southern blotting procedures and in gel renaturation.

Ovarian Carcinoma:

Advanced nonresponsive ovarian cancer was found to overexpress P-170 in 2/5 cases (Bell et al.,1985; Bourhis et al.,1989). Another study (Bell et al.,1985b) reported 3/15 tumors expressing P-170, the P-170 positive patients failed to respond to chemotherapy, as opposed to none of the 35 untreated ovarian carcinomas were positive for P-170. Similarly, Sikic's group found 11/33 Vs 8/50 untreated ovarian carcinomas, as well as 5/15 previously treated breast cancers expressing high levels of P-170 mRNA. Two MABs (C219 and JSB1) were used to identify P-170 in frozen tissue from the genital tract of 14 women with benign gynecological conditions (Finstad et al.,1990). P-170 was noted in half of the analysed samples. It was suggested from this study that if epithelial ovarian cancers are derived from the surface epithelial cells of the ovary, a small proportion of the cancers might be expected to retain the phenotype found in non-cancerous tissues and to express P-170.

Breast Carcinoma:

Significant P-170 expression has been detected by immunohistochemical staining in three samples from patients with breast cancer, who had either received an anthracycline or a vinca alkaloid in treatment. However, patients who had received either

alkylating agents, antimetabolities, or hormonal agents showed little or no expression of P-170 (Schneider et al.,1989). Significant MDR1 mRNA expression was also detected in 25/49 primary breast samples from untreated patients using Northern and Dot Blotting procedures, (Keith et al.,1990). Also, Verelle et al.(1991) confirmed the presence of P-170 in 17/20 tumors; 3 of these patients had confimed metastases in which the highest levels of P-170 were noted.It was concluded from this study that pretreatment evaluation of P-170 expression may be of prognostic value in patients with locally advanced breast cancer. However, Jungsil Ro et al., (1990) and Merkel et al.(1989) found no significant levels of P-170 in breast cancer at any stage. It was concluded that it is possible that acquired resistance may play an important role in the failure of locally advanced breast cancer.

It can be noted from the above data on P-170 expression in breast cancer, that the results are not consistent from one investigator to the next. In order to evaluate fully the role of P-170 in clinical resistance it is necessary to develop sensitive reproducible assays which also must be able to discrimate between normal and neoplastic tissue (Dalton et al.,1991).

1.8 Alternative Mechanisms Involved In MDR.

Although the study of MDR has mainly focused on P-170 expression, an increasing number of biochemical and molecular mechanisms have been described in MDR selected cell lines. These findings have resulted in the designation of MDR phenotype as "classic" (denoting P-170 mediated MDR), or "atypical" MDR (At-MDR) in which cells appear to possess other mechanisms of MDR. The two best characterised additional mechanisms observed in cells that are multiply drug resistant are changes in the expression of activity of enzymes involved in detoxification pathway (in particular Glutathione-S-Transferase, GST) and alterations in the nuclear enzyme Topoisomerase 11 (Topo 11). Several MDR lines which do not express P-170 show patterns of cross resistance significantly different from the classical MDR phenotype. (Haber et al.,1989 ; Weber et al.,1989). GST is a multifunctional phase 11 detoxification enzyme that catalyses the conjugation of electrophilic substances and endogenous xenobiotics to the tripeptide thiol Glutathione (GSH), forming stable secretable metabolites, and prevents oxidative damage through intrinsic, organic peroxidase activity. Alterations in the activity of GST and GSH-related enzymes have been identified in several cell lines associated with cross resistance to alkylating agents. (Tew et al.,1986; Lewis et al.,1988; Saburi et al.,1989 ;Durse et al.,1990). Certain isoenzymes of GST may protect cells against the damage caused by free radicals, which may be an important determinant in the cytotoxicity of anthracyclines (Bradley et al.,1988). Comparison of cloned MDR P388 mouse leukemia cells with cloned wild type parental cells showed increased expression of GST, and more rapid onset of repair, but no evidence for enhanced drug

efflux in the resistant variant (Deffie et al.,1988 a,b). In addition, the doxorubicin selected MDR human breast cell line, MCF-7ADRr, possesses an amplified and overexpressed MDR1 gene (Fairchild et al.,1987). It also shows a seven-to twelve-fold increased selenium dependent-GSH peroxidase activity and a 45 fold increased GST activity , the latter resulting from the increased expression of a single, anionic(π)isoenzyme of GSH (Mimnaugh et al.,1989). This could possibly suggest that GSH peroxidase or GSH may have independent activity in conferring cellular resistance to adriamycin. Transfection and expression of cDNA clones of the GSTπ isoenzyme in drug sensitive MCF-7 cells that do not overexpress P-170, conferred extremely low level (1-2 fold) resistance to alkylating agents, but no increase in resistance to natural products such as adriamycin (Moscow et al.,1989b). Thus, the role of GST in MDR involving non-alkylating agents is not very clear. However, work involving the gamma-glutamylcysteine synthetase inhibitor buthionine sulfoximide (BSO) suggests that the GSH system may have a role in addition to that of P-170 in the conferring of MDR. The addition of BSO results in a five to seven fold increase in cellular sensitivity to adriamycin MCF-7ADRr cells (Durse et al.,1989); an effect was also noted, but to a lesser extent by Ford and Hait (1989) where only a 1.5 fold increase in the sensitivity of 200 fold doxorubicin resistant MCF7-ADRr cell line was seem. A particularly interesting study has been reported in which three distinct cell lines were established from a patient with small cell lung cancer (De Vries et al.,1989) at different stages of disease, (from sensitive to completely resistant to chemotherapy). Progressive increases were seen in levels of glutathione, GSH, and catalase; cell surface P-170 immunofluoresence staining was only evident in the most resistant

of the in vitro cells. In these studies, BSO enhanced adriamycin and cis-platin cytotoxicity (but not X-ray induced cytotoxicity). However, in the SCLC cell line H69AR a marked decrease in the level of GSH was noted. The activity of three enzymes (GSH-S-transferase, GSH-reductase and gamma-glutamyl transpeptidase) were elevated while the selenium -dependent and independent GSH -peroxidase activities were unchanged. The consequences of these changes resulted in an 6-fold reduction in H69AR cellular GSH levels. This pattern of resistance is different to that reported for other SCLC cell lines, however it has not been conclusively proved that thes changes link with the mechanism of resistance (Cole et al.,1990). These researchers established 8 small cell lung cancer lines, from treated and untreated patients. None of the cell lines showed detectable levels of P-170. There was however a 50 fold range in the total activity of glutathione-S-Transferase (8-409nm CDNB/min/mg) and a four fold range in the activity of glutathione peroxidase (10-40 nm cumene H_2O_2/min/mg). The activity of glutathione-S-transferase measured is mainly due to the π isoform. The effect of ethacrynic acid, a potent inhibitor of GSH was investigated and no modulation of MDR was noted. In tranfection studies by Fairchild et al.(1990) it was shown that transfection of GSTπ in an expression vector (pMTP) is insufficent to confer resistance to adriamycin in cells that lack P-170. Another study was undertaken to acertain if GSTπ could be acting in a synergistic effect with P-170 to alter MDR. Using the MCF7ADRr cell line and vectors expressing full cDNA for GSTπ and mdr1, it appeared that GSTπ does not alter patterns or levels of MDR in MCF7ADRr cells. In a study by Keith et al.(1990) all breast tumor samples with the exception of one showed low or undetectable levels of GSTπ. Also, the leukemia samples generally had low

levels of GSTπ.However, both colon tumors and a large proportion of head and neck tumors had high levels of GSTπ mRNA levels. Expression of mdr1 and GSTπ genes in 35 advanced neuroblastomas have been reported by Bourhis et al.(1990). No co-ordinate expression between the two genes was found. GSTπ and MDR1 expression failed to be significantly related to the response of the primary tumor at the first induction treatment, however in subsequent studies and on the occasion of metatasis, a significant relationship was noted between combined expression of MDR1 and GSTπ genes and tumor response in neuroblastoma patients. It is interesting to note that the high levels of GSTπ have been apparent in tumors that are intrinsically resistant. This could be a possible indication that GSTπ is an important factor in the development of intrinsic resistance. To date GSTπ has been mainly implicated in the MDR phenotype, Recently a number of reports have emerged that show elevated levels of other isoforms. Evidence of over expression of GSTμ class subunits in human breast carcinoma have been found (Schisselbaurer et al.,1991), and GSTπ in addition to GSTα and GSTμ in patients have been found with malignant lymphoma (Ringborg et al.,1991).

One specific atypical MDR phenotype involving cross resistance to a variety of natural product antitumor drugs, but not to Vinca-Alkaloids has been the reduction of DNA Topoisomesase 11 (Topo11) (Beck et al.,1989). The topoisomerases are enzymes that catalyse the breaking and rejoining of DNA required for genomic unwinding and are necessary for DNA replication. Much attention has been focussed on the role of topoisomerase 11 in drug resistance because it is believed to be a target for many DNA

intercalating and non-intercalating drugs such as doxorubicin, mitoxantrone, etoposide, tenoposide and 4'-(9-acridinylamine)-methanesulfona- m-aniside (Chen et al.,1984). Alteration in the quantity or function of Topoisomerase 11 has been suggested as a possible mechanism for cellular resistance to this group of drugs (Ross et al.,1988). Several MDR cell lines are characterised by decreased levels of Topisomerase 11 activity in the absense of P-170 expression or changes in drug accumulation, (Pommier et al.,1986; Charcosset et al.,1988; Deffie et al.,1989; Zwelling et al.,1989;De Jong et al.,1990). The decreased enzyme level could be due to a number of factors. During the enzymatic modification of the topological state of the DNA, Topo11 binds to the DNA to form a enzyme DNA reaction intermediate. Many of the chemotherapeutic compounds are DNA intercalative drugs and they interfere with the action of the action of the enzyme by trapping the enzyme, covalently bound to the 5' end of the DNA break, thus reducing intracellular concentration of Topo 11. Also, a number of investigators have suggested that the enzyme in resistant cells is of a much higher activity, despite the reduced quantities (Capranico et al.,1990). There has also been a suggestion by Cunningham (1991) that DNA Topoisomerases might be involved in oncogenesis. Inherited susceptability to a wide range of neoplasia (Li-Fraumeni syndrome), was shown in studies of one cancer prone family,whereby an intriguing association with an abberent c-raf-1 gene and the inheritance of a radioresistant phenotype in their non cancerous fibroblasts was found. Additionally a p53 germline mutation is evident in Li-Fraumeni syndrome. Also the fact that topoisomerases are preturbed by <u>in vitro</u> serine/threonine kinases similar to raf encoded proteins (which conveys radioresistance), indicates a possible role for topoisomerases in the advent of

carcinogenesis. It has been found that topoisomerases can introduce error into the DNA,particularly but not exclusively, when perturbed by activators. This malfunction has been implicated in mutation, sister chromatid exchanges, chromosome stickiness, fragmentation of tumor DNA and tumor promotion. The fact that it has now been established that Topoisomerase 11 is required for chromosome condensation (Adachi et al.,1991), this implies any alterations in levels of Topo 11 could have a drastic effect on the natural function of the cell. Multifactorial resistance tends to be a common enough finding. Ganapathi et al.(1989) characterised a series of L1210 cells selected for progressively incresaed resistance to doxorubicin. These cell lines displayed increased expression of P-170 in addition to reduced Topoisomerase 11 mediated clevage of DNA. Reduced levels of DNA Topoisomerase 11 have been evident in a number of multidrug resistant cell lines, FM3A breast carcinoma (Hong et al.,1990), A549/CPT and HT29/CPT (Sugimoto et al.,1990), CCRF-VM1 human leukemic (Danks et al.,1988), SCLC and Non SCLC (Danks et al.,1988) P388 Leukemia cell (De Isabella et al.,1990) and SCLC GLC4/ADR (De Jong et al.,1990). Much emphasis in this area is now given to models of topoisomerase gene regulation, such as altered rates of transcription. Kaufman et al.(1991) added additional confirmation of the presence of hypermethylation in atypical MDR cell lines, this could open new avenues for therapeutic intervention. For example, treatment with azacytidine which inhibits DNA methylation, might up regulate the expression of Topoisomerase 11 resistant (presumably hyper methylated) cells sensitive to antineoplastic agents. However, the clinical use of Topo11 directed agents may be limited, particulary in the treatment of intrinsically resistant tumors (Kaufmann et al.,1991). Screening of

bacterial gyrase inhibitors has identified quinolone analogues that induce Topo11 mediated strand breaks in eukaryotic cells by enhancing the rate of Topo11 -DNA aduct formation. Given their unique structure and novel mechanism of action, it will be interesting to see whether these agents have any unique antitumor activity. The concept of tailoring drugs against Topo 11 mediated MDR has been also highlighted by Baguley et al.(1990) and Beck et al.(1989).

Many other mechanisms of resistance have been suggested as important features of MDR cells. Certain of these changes appear to occur only in conjunction with mdr1 gene expression. This is an indication that the other genes which may be part of the six gene amplicon localised to chromosome 7q21-31, are well associated with MDR cells. Amplification been a hallmark of MDR cells, the overexpression of other genes that are part of this amplicon is not surprising. For example, Sorcin, a 22 Kda anionic calcium binding protein, has been shown to be overproduced in vincristine resistant mouse cells (Meyers et al.,1985, 1987) and in L1210 cells selected for MDR with VP-16, VM26, adriamycin , dactinomycin or vincristine (Roberts et al.,1989). However it is unclear if sorcin itself plays a role in the establishment, maintenance or patterns of MDR. Other low molecular weight cytosolic proteins are uniquely expressed in MDR, such as a 21Kda protein found in colchicine resitant KB cell lines (Shen et al.,1986) and a 20Kda protein phosphorylated to a greater extent in MCF7-ADRr cells exposed to phorbal esters (Fine et al.,1988). The significance of these proteins in MDR is unknown. Similarly, several membrane proteins unique to MDR cells have been observed in addition to P-170, such as an 85-Kda glycoprotein in K562/ADM cells (Hamada et al.,1988)

and a 150 Kda protein immunologically distinct from P-170 (Mc Grath et al.,1988). Numerous biochemical changes have been implicated in MDR. Decreased levels of epidermal growth factor were reported in an MDR variant of human KB cells with reduced tumorigenicity (Takano et al.,1989). Alterations in the monophosphate shunt have been reported (Yeh et al.,1987) in an adriamycin resistant variant of a human breast carcinoma, MCF-ADRr. In this same cell line alterations in the phase 1 cytochrome P450 enzymes such as aryl hydrocarbon anhydroxylase (Ivy et al.,1988), and an increased activity of the drug activating enzyme DT-diaphorase and Glucuronyl transferase (Cowan et al.,1986) have been observed. Induction of the enzyme Thymidylate Synthase (Chu et al.,1991) has been noted in Human breast MCF-7/ADR cell line and in the colon cancer cell line DLD-Ad, this was following exposure to adriamycin ,with concomitant resistance to 5-Flurouracil noted. A number of studies are now noting the alteration of a number of varied enzyme systems in resistant cells. Redmond et al.,(1991) in the study of human colorectal tumors noted alterations in glutathione, glutathione transferase and peroxidase, and the DNA repair enzyme O^6-alkylguanine-DNA-Alkyltransferase. All of these showed significant increases in tumor tissue levels when compared to normal tissue from the same patient. The significance was highest for glutathione peroxidase. Increased expression of O^6-alkylguanine-DNA-alkyltransferase has been noted in two other systems also,a human melanoma cell line (Egyhazi et al.,1991) and human brain tumors and biopsies (Tuma et al.,1991).

It is assumed that these changes have a significant regulatory or functional role rather than being simply a coincidental result of the selection procedure. The large number of changes highlighted

give an indication of the complexitity of the MDR phenotype.

The permutations and combinations of mechanisms of MDR are indeed extensive. It may be of clinical importance to ascertain the normal levels (of the above stated enzymes) in various tissue types, and whether these levels change significantly from tumor tissues to normal tissue. These changes, if highlighted could be exploited to prevent the emergence of resistance by the development of drugs that would act as first line targets against these enzymatic alterations. Therefore, numerous avenues exist to further establish the regulatory mechanisms for MDR and for exploring the molecular and biochemical basis for alternative forms of MDR.

1.9. Pharmacological Reversal of MDR.

A major goal in experimental as well as clinical studies in the MDR area,is that of reversal of MDR with compounds that are not too toxic *in vivo*. Therefore, many investigators have focused on the pharmacological reversal of MDR. Through the identification of agents that can reverse resistance it is hoped to introduce these agents to clinical situation and to gain a further understanding of the mechanisms of MDR. The chemosensitizers described to date can be grouped into nine categories :

1. Calcium Antagonists.
2. CaM antagonists.
3. Non toxic antracyclines and Vinca Alkaloid analogues.
4. Steroid and Hormonal analogues.
5. Miscellaneous cationic compounds.
6. Cylosporins.
7. Antisense oligonucleotides.
8. Anti-MDR antibodies conjugated with toxic agents.
9. Agents that can alter At-MDR, Topoisomerase 11 resistance.

(See section 4.9. and 4.10. for more comprehensive details)

Although these compounds only share broad structural similarities, most are extremely lipophilic, and those in the first five groups are heterocyclic, amphipathic substances. This suggests that there is more than one specific receptor site for circumvention agents, which have unique, although as yet poorly defined, structural requirements for efficent binding and circumventing ability (Ford et al.,1990). MDR cells are not normally resistant to cis-platin,

but it is interesting to note that calcium channel blockers (Onada et al.,1989; and Ikeda et al.,1987) as well as hyperthermia may reverse cis-platin resistance (Bates et al.,1990; Manouri et al.,1989).

Verapamil,the compound that has been most used to date in in vitro and in vivo studies has been found to be extremely cardiotoxic. Plasma concentrations of verapamil have not been monitored, because the cardiovascular therapeutic effects are easily measurable and titratable. Usual plasma concentrations for treatment of angina are 150-500μg/ml (0.4-1.2μM) (Anderson et al.,1982). Clinical toxicity begins to occur with concentrations greater than these and are characterized by atrioventricular blocks and hypotension. The plasma concentration required to produce an MDR sensitizing effect is in the 2-6μM range. Thus, the concentration that is required to produce an effect in vivo possesses potentially life threatening cardiovascular effects. Therefore, more potent and less toxic chemosensitizing agents than verapamil were investigated in this thesis. Verapamil was used as a 'standard' chemosensitizing compound, also because it has been proven to be effective in the reversal of P-170 mediated resistance. A number of other compounds were studied to ascertain their circumvention effect on a panal of MDR cells, nifedipine, chloroquinine, quinine, quinidine, caffeine and aspirin. Nifedipine is a calcium channel blocker, which has less calcium antagonizing ability than verapamil, but its potency would be higher, it has been found that it is possible to obtain clinically non-toxic levels of nifedipine and it has been proven effective in a number of in vitro systems (Ramu et al.,1984 ; Tsuruo et al.,1983a). Chloroquinine, a lysosomotropic agents, has been tested on a number of in vitro systems and proven to be

effective (Shiraishi et al.,1986; Zamora et al.,1988; Beck et al.,1988). It has as yet not been studied in vivo but its structure, pharmacology and metabolic pathways indicate that it may be suitable. Quinidine and Quinine have been shown to be non-toxic in vivo and both have used in in vivo studies (Tsuruo et al.,1984; Zamora et al.,1988). Caffeine another lysosomotropic agent has not been investigated as a circumvention agent previously, it was chosen for study due to the large volume of data available on achievable in vivo levels. Finally, aspirin was chosen because it fits into the mould that has been outlined as the essential requirement for a circumvention agent. It is an extremely lipophilic, cyclic amphiphathic substance and it has also been shown to have an effect on intracellular calcium levels (Flescher et al.,1991). It has been noted however by a number of investigators that the calcium levels do not alter in resistant cells during the circumvention process (Naito et al.,1989). Despite this, due to the diversity of compounds already tested and proven to wholly or partially reverse resistance, aspirin was investigated on a number of MDR cell lines. There would be enormous clinical implications if a compound like aspirin was to prove effective in reversing resistance, due to the volume of data available on its mode of action and its synergistic effects.

A tremendous amount has been learnt about MDR at the molecular, biochemical, and cellular levels, but little yet has been translated into clinical gains. However much of the data now emerging from studies of the clinical expression of P-170 and its relevance to drug resistance in humans, suggests that there will be a role for chemosensitizers in cancer chemotherapy. Such circumvention strategies may be effective in intrinsic or acquired

drug resistance. However even if P-170 associated MDR proves to be a relevant and reversible cause of drug resistance, numerous questions remain to be answered before effective clinical chemosensitization may be achieved. Such factors as absorption, distribution, metabolism and toxicity, the effect of the chemosensitizing agent on chemotherapeutic drug toxicity to normal tissues expressing high levels of P-170, and the most effective regime, all still remain to be studied in vivo. When to administer the chemosensitizing agent in a regime of therapy is another important consideration, should it be administered with first line chemotherapy the ward off the development of resistance or should it be administered in cases of relapse in second line chemotherapy. Clinicans are very wary of the former regime because if a standard chemotherapy has proved effective in the treatment of a certain tumor type, there is a certain conservatism in the readiness to change. However, the addition of a chemosenstizing agent in first line therapy in the treatment of Ehrilich ascites (adriamycin resistant) in mice, has been proven to increase the life span rather than treatment of a recurring tumor with chemotherapy and chemosensitizing agent (Tsuruo et al.,1985). Clinical drug resistance however, may be so complex that the likehood of a drug that effects only one mechanism of resistance is small. It must be noted, that renal cell carcinoma and colonic carcinoma express high levels of P-170 and these tumors are resistant to virtually all classes of chemotherapeutic agents, including those not classified as MDR drugs. Indeed, multiple mechanisms of resistance are much reported (See section 1.9 for details). This would should be taken into account in the design of regimes of therapy incorporating chemosensitizing agents. Future regimes may need to include combination chemosensitizers or the

rational use of drugs to circumvent the likely causes of resistance. This approach is definitely not simple. For example, Kramer et al.,(1988) used a combination of buthioine sulfoximide (BSO) a potent inhibitor of GSTπ, with verapamil (a known chemosensitizing agent of P-170 mediated resistance), on the MCF7-ADRr cell line (That is known to have altered P-170 and GSTπ levels in its resistant variant). It was found that the combination of the two agents could completly reverse resistance, whereas each agent individually could not. It was found that the combination of BSO and verapamil in the absence of adriamycin was extremely toxic to tumor cells. Futhermore otherwise non-toxic doses of verapamil were found to be lethal to animals receiving BSO (at non toxic levels) in their drinking water. These finding emphasize the complexity involved in reversal of multiple mechanisms of resistance. Findings to date definitely provide promise for potential clinical applications in the future. The further study of circumvention agents in animals an man and the rational search or design of novel chemosensitizers with improved activity should define the importance of MDR to clinically resistant cancer.

1.10. Aim of This Thesis.

The aim of the research described in this thesis was to select a range of Multiply-Drug-Resistant human tumor cell lines (by adaptation in progressively increasing concentrations of adriamycin) and to characterize these cell lines using pharmacological, biochemical, biophysical and genetic criteria. Cross resistance profiles would then be ascertained for each of the cell lines, as this can be a first indication as to the mechanisms

of resistance. An assessment was also undertaken of clonal variation in drug sensitivity; sensitivity of the cells to standard subculture procedures; sensitivity to standard freezing procedures; and resistance above the parental cell line to sonication. Possible involvement of overexpression of P-170 in the variants was investigated by the techniques of Western Blotting, Immunofluorescence and by transfection with antisense oligonucleotides. An antisense oligonucleotide, (18mer), which was specific for 18 bases downstream of the mdr1 gene promoter was used to test the inhibition of P-170 expression, with an adriamycin toxicity assay as the endpoint to the assay. Transfection techniques were investgated and optimized with a view to transfection of the complete cDNA for the mdr1 gene. The aim of this aspect of the study was to compare the characteristics of an "adapted" cell line to that of a transfected "cloned" cell line. Due to the long time period involved in the development of "adapted" cell lines development of resistant cell lines by transfection procedures is certainly less troublesome. Whether or not transfected cell lines are as adequate of the in vivo situation as lines adapted to resistance by exposure to drugs in vitro remains to be determined.

HSRs and DMs are a common cytogenetic manifestation in MDR cells. A number of the adapted cell lines were studied for the presence of DMs to ascertain if the number of DMs is representative of the degree of resistance.

Several of the cell lines chosen were derived from human non small cell lung carcinomas (DLKP, SKMES-1, SKLU-1, and DLRP) since

resistance mechanisms in these tumors are relatively poorly studied. This research also covered cell lines of ovarian (OAW42) and cervical origin (HEP-2). A particular emphasis in this thesis is investigation of "circumvention agents" which might be useful in the clinical reversal of resistance at pharmacological doses.

The investigation of the biochemical, biophysical and genetic characteristics highlighted above will add to our knowledge of the basic biochemical alterations and the mechanisms of resistance in MDR cells and could be relevant in the design of new regimes of therapy and circumvention.

CHAPTER 2

MATERIALS AND METHODS

2.1. Ultrapure H_2O.

A Milli-Pore milliQ ultrapure water system or an ELGA UHP system was used to purify all water used for preparation of media and reagents. These systems consist of a reverse osmosis system, MilliQ or double distilled apparatus with two prefilters to remove ionic and non-ionic solutes, followed by two-ion exchange filters, a carbon filter and a 0.22μm cellulose acetate filter. The quality of water is monitored by an on line conducting meter, with the specifications of 10-18 megaohms/cm resistivity as the acceptable standard.

2.2. Glassware for Cell Culture.

Media and reagent glassware, plus bottlecaps (separately) were soaked in a non-toxic detergent RBS-25, at 2% (v/v) in warm water. 1-2 hours later bottles were scrubbed manually and rinsed thoroughly in warm water, rinsed three times in reverse osmosis or double distilled water followed by a final rinse in ultrapure H_2O. Glassware containing waste medium from cells was autoclaved and rinsed with tap water, and washed and dried as above. All glassware was dried and autoclaved before use.

2.3. Sterilisation.

Water, glassware and solutions of thermostable compounds were sterilized by autoclaving at 120°C for 20 min. at 15 p.s.i. pressure. Temperature labile chemicals (eg. chemotherapeutic drugs, enzymes) were filter sterilized through 0.22μm sterile disposable

filters (millipore millex-gv, for low binding of protein). Growth medium was sterilized via a Bell filter (Gelman G.1423S).

2.4.1. Growth Medium.

Liquid DMEM (Gibco 042-02501) media were prepared from concentrated 10X stock solutions of basal media and were diluted before use with ultrapure H_2O. MEM α (Gibco 041-24561M) was purchased in 1X form and HAMs F-12 was made up from powdered media. HAMs F-12 contains many labile components, thus the stability of the components is greatly increased on lyophilization. Powdered HAMs F-12 (Gibco 074-1700) was reconstituted in 95 % total volume with ultrapure H_2O and 1.176g/L sodium bicarbonate added, the pH was adjusted 0.3-0.2 pH units below the desired pH (pH 7.5). The total volume was brought to 5 litres and filter sterilized through a Gelman Sterivex unit equipped with a peristaltic pump. Medium was collected in 500ml sterile bottles and stored at 4°C for up to 3 weeks. All medium was supplemented with 2mM L-glutamine and other components (see table 2.1 for details). All media were supplemented with FCS or NBCS at a concentration of 5-15 % depending on the cell line (see Table 2.2 for details). Before use, all media were sterility checked. 5-10 mls of medium was dispensed into sterile universals and incubated at 37°C for 3 days. Antibiotics were not used during routine cell culture. 1 ml Penicillin/Streptomycin (Gibco 043-05140H) and 250μl Fungizone Gibco (043-05290) per 100 mls of growth medium were used for all large scale animal cell culture.

TABLE 2.1. Growth Medium and Supplements

(per 500ml basal media)

Media Component	DMEM	MEM-α	DMEM/HAMs:F12 (50:50)
2mM L-Glutamine Gibco 043-05030	5ml	5ml	5ml
1M Hepes * Sigma H-9136	10ml	5ml	10ml
7.5% NaHCO3 BDH 30151	6ml	6ml	6ml

* Hepes :4- (2-hydroxyethly) -piperazineethanesulfonic acid.

2.4.2. Assay Medium.

Assay medium was prepared by the mixing of DMEM and Ham-F12 in the proportion 50ml:50ml, measured out exactly using a 10ml pipette,the addition of 5% FCS, 1ml of L-Glutamine, 1ml Penicillin/Steptomycin and 250μl Fungizone.

2.5.1. Cell Lines.

All cell culture work was carried out in class II downflow recirculating laminar flow cabinet (Microflow, Holten or Cytoguard - which is specially designed for handling chemotherapeutic agents). See table 2.2 for details of cells cultivated. Cell lines were grown in 25 cm^2 flasks (Cell Cult 32025) or 75 cm^2 flasks (Costar 307S) in the recommended medium. Routinely cells were fed every 2-3 days, or when a pH change was observed, (i.e. phenol red color change in media); on reaching confluency cells were subcultured.

Table 2.2. CELL LINES.

Cell Line	Growth media % FCS	Details of Cell Type	Source
HEP-2	DMEM/HAMs F12 5% FCS	Human Larynx Carcinoma	ATCC CCL 23
OAW42	DMEM 10% FCS	Human Cyst-Adenocarcinoma Ovary	ECACC 8507310
SKMES-1	DMEM/HAMs F12 5% FCS	Human Lung Squamous Cell Carcinoma	ATCC HTB 58
SKLU-1	DMEM/HAMs F12 5% FCS	Human Lung Adenocarcinoma	ATCC HTB 57
DLKP	DMEM/HAMs F12 5% FCS	Human Lung Squamous Cell Carcinoma	G.Grant. NCTCC,DCU
DLRP	DMEM/HAMs F12 10% FCS	Human Lung Squamous Cell	B.Gregory NCTCC,DCU.
CHOKI	HAMs F12 5% FCS	Chinese Hamster Ovary MDR-Sensitive	ECACC CCL 61
CHrC5	MEM-α 5% FCS	Chinese Hamster Ovary MDR-Resistant	V.Ling.
LTK$^-$	DMEM 5% FCS	C3H/Mouse Areolar Fibroblast	ECACC 85011432
NIH3T3	DMEM 5% NBCS	NIH Swiss Mouse Embryo Fibroblasts Clone D4.	ECACC 85111801

2.5.2. Large Scale Cell Culture.

Cells required for RNA and protein analysis were required in numbers in excess of 10^8 cells. For this purpose Roller bottles were used. Bellco roller bottles were used with an internal surface area of 670 cm^2 were washed, (section 2.2) dried and autoclaved. Firstly roller bottles were rinsed with about 10mls of growth medium (containing 5% FCS and 1ml of penicillin streptomycin) and and then innoculated with $5X10^5$ cell/ml of the required cell type. The roller bottle was then left at 0.25 r.p.m. for 24 hours and then increased to 0.75 r.p.m. The roller bottle was fed every 2-3 days until confluency.

2.5.3. Subculture of Cell lines.

Animal cells grow in monolayer attached to the bottom of culture vessels. Upon reaching confluency, cells were enzymatically removed using 0.25% Trypsin (Gibco 043-05090) 0.01% EDTA in PBS-A (Oxoid BR14a). Solutions were prewarmed to 37°C. Waste medium was removed from the cells which were then rinsed with PBSA or Trypsin/EDTA , for every 10mls of growth medium on the cells 2mls of trypsin solution was used. 2ml of Trypsin/EDTA was added and incubated with the cells for 10-15min, at 37°C. Once a single cell suspension was obtained, medium containing FCS, which contains an inhibitor of trypsin, was added (An equal volume of the trypsin originally added). The cell suspension were then transferred to a sterile universal (Sterlin 128A) and centrifuged at 1,000rpm for 5min. The cell pellet was then resuspended in 5ml of growth medium and a count obtained (section 2.5.4) Cells were then diluted to 10^3-10^4

per ml of growth medium, this figure is dependent on the cell line. 10ml of cell suspension was added to 25cm^2 flasks and 20ml to 75cm^2 flasks. Cells were then incubated at 37°C.

2.5.4. Cell Counting.

A sample of a single cell suspension was applied with a Pasteur pipette to a Weber haemocytometer such that it was held in the counting area between the slide and the coverslip. The enclosed area below the coverslip and the 16 squares is 0.1mm^3. All those cells found in the 16 squares of the four courner grids were counted, cells lying on any two of the four outer lines were also counted, and the total number, X, was estimated, an average of the four corner grids was calculated and multiplied by 10^4. This gives the number of cells per ml of solution. Cell viability was estimated by the addition of an aliquot of cell suspension to trypan blue dye (Gibco 525) ratio 4:1, Those cells that take up the blue dye are non-viable.

2.5.5. Mycoplasma Detection.

Mycoplasma examination of cell lines was carried out using a fluorescent staining method, specific for DNA (Chen,1977). An indicator cell line NRK was used and medium from cells was added for testing. NRK cells were grown overnight on sterile coverslips in 1ml of medium at 10^3 cells/ml, at 37°C, 5%CO_2. 2ml of growth medium from cell lines to be tested were then added to individual coverslips and incubated for a further 4 -5 days at 37°C and 5% CO_2. Cells were then rinsed twice in PBSA, rinsed once in Carnoys

fixative (Appendix of solutions 6.1.1) /PBSA (1:1),and then fixed in Carnoys fixative for 10 min. The coverslips were removed and rinsed thoroughly in PBS. Hoescht 33258 (Sigma B2883) at 0.05mg/ml in PBSA were added to the cells so that they were completly covered, and left for 10 min in a light-proof sealed container at room temperature. Excess stain was removed by rinsing in water. The coverslips were then mounted and sealed onto a glass slide (with the cells between the two surfaces). The cells were then studied under oil immersion using 405nm light. Definite pinpoints of fluorescence in the cell cytoplasm indicated the presence of mycoplasma contamination. Contaminated stocks were disposed of and fresh cultures were grown up from stock.

2.5.6. Long Term Storage of Animal Cells.

New cell stocks received from the ECACC (European Collection of Animal Cell Cultures), ATCC (American Type Culture Collection) or any other source were immediately grown up to high cell number and then frozen in liquid nitrogen for long term storage. Cells in subconfluent exponential phase were suitable for long term storage. 1ml of a 10% (v/v) solution of DMSO (BDH 282166) in growth medium, with 25% FCS, was added to 1ml of cell suspension (at least $2X10^6$ cells/ml) in a slow dropwise manner with continuous mixing. This method of freezing was only suitable for the parental cell lines see section 2.7.3 for details of the freezing procedure for the mutliple drug resistant variants. This was then transferred to sterile cryotubes (sterlin 35001). The cells were then slowly frozen in the vapour phase of liquid nitrogen for three hours, then stored in liquid nitrogen storage containers until required. It is

necessary to take a number of precautions in the handling of vials of cells stored in liquid nitrogen. It is recommended to ware full face mask in addition to protective gloves during any contact with liquid nitrogen.

2.5.7. Cell Thawing.

The required vial was removed from liquid nitrogen and thawed rapidly at 37°C. The thawed cell suspension was transferred to a sterile universal with growth medium and centrifuged at 1,000rpm for 5min. The cell pellet was resuspended in 5ml of growth medium and transferred to a $25cm^2$ flask. The cells were then incubated at 37°C for 24 hours. The cells were then refed with 10ml fresh culture medium.

2.6.1. Safe Handling of Cytotoxic Drugs.

There are two principal risks in the handling of cytotoxic drugs-one definite and one potential. The definite risk results from the fact that a number of cytotoxics are extremely irritant and produce harmful local side effects after direct contact with skin or eyes. The potential risks stem from the knowledge that some cytotoxic drugs are proven carcinogens. Cytotoxics drugs must be treated with the utmost care in the laboratory in the initial stages of reconstitution and the handling of drug containing culture medium. Generally in handling cytotoxic drugs, double gloves, and face masks are worn, and all work involving cytotoxics is performed in a 'Cytoguard' laminar air flow cabinet. All liquid waste is treated with hypochlorite followed by autoclaving, all plastic disposable waste that had come in contact with cytotoxics

are rinsed three times, and subsequently autoclaved. Any reconstitution vials or syringes that would have been in contact with concentrated cytotoxics drugs were incinerated through 'Bioburn 'limited. Initially it was noted that the reproducibility of some of the toxicity assays was particularly bad, indicating instability problems with the drugs. The stability of each drug was ascertained by storing a number of aliquots in different ways and comparing these to the results of a toxicity assay preformed with the drug on the day of reconstitution of the original vial. In general, dissolving the drug from powder formulation with water for injection is advisable, followed by further dilution in medium containing 10% FCS (The protein concentration present is thought to stabilise the drug). Secondly, any of the drugs stored frozen at -20°C can only be thawed five times, activity of the drug on subsequent freezing is markedly reduced. Table 2.3 summarizes the finding of these results.

Table 2.3. Storage of Cytotoxic Drugs.

Drug Presentation	Storage	Special comments
Adriamycin 10mg/50mg powder Farmatalia	-20°C,2mg/ml or 20μg/ml	Can be stored at -20°C for 6 months.Light sensitive.
Cis-platin 10mg/50mg powder Lederle	room temp. for 6 months 1mg/ml,5mg/ml quantities.	Do not refrigerate or freez as a ppt.forms, Light sensitive.
VP16/VM26 100mg powder Lederle	Store 3 months at -20°C, 6 month at-40°C 1mg ,10mg quantities.	
5-Fluorouracil 250mg/10ml powder Farmatalia	Store between 10-30°C, 25mg/ml,2.5mg/ml quantites	Sensitive to light stable for 12 months.
Vincristine 1mg/2mg vials of solution Lederle	-20°C in 10μg/ml or 20μg/ml quantities.	

2.6.2. Adaptation of MDR Variants.

Initally, a toxicity assay (section 2.7.1) with Adriamycin was preformed to ascertain the concentration of drug at which there was 5% survival of each cell line. ADR was added at the relevant concentration,cells were grown at that concentration until they appeared healthy and attaining high numbers, the drug concentration was then doubled. This process was continued, doubling drug concentrations where the cells appeared fit enough to do so. See table 2.4 for details of adaptation.

2.6.3. Obtaining of MDR Variants by Mutagenesis followed by Adaptation.

It was cited in the literature that mutagenesis was a suitable method for the adaptation of MDR variants (Debenham et al.,1982). This method involved the treating of the cells with a point mutagen Ethyl Methanesulfonate (Sigma M8024) at a concentration of 25μg/ml for 18 hours followed by three days preselection period, the cells were exposed to ADR at 1μg/ml. The selection then proceeded as in section 2.6.2. Only one cell line was adapted by this method, HEP-2B.

Table 2.4. Cell Lines Adapted To Resistance.

Cell Line	Initial ADR Concentration (μg/ml)	Final ADR Concentration (μg/ml)
HEP-2A	0.05	1.65
HEP-2B	0.1	1.55
DLKP-A	0.05	2.45
SKMES1-A	0.025	0.95
SKLU1-A	0.05	0.525
OAW42-A	0.05	0.85
DLRP-A	0.025	0.1

2.7.1. Toxicity Assays - 24 well plate.

The cells were pretreated as follows : Cells growing in 10 mls of media (Table 2.2) in 25 cm^2 flasks were trypsinised and set up at 10^6cells /75cm^2 flask, 2 days prior to the assay and fed the following day. Cells were plated at a concentration of $2X10^3$ cell/ml in 500μl per well. Exactly 24 hours later drug dilutions were added (2 X final concentration in 500μl) to triplicate wells (Costar 3524). The plates were then incubated for 7 days and the cell growth was determined by two procedures. Both procedures involved firstly removing drug medium, rinsing three times in PBSA and staining for exactly 10 min with 500ul 0.25% crystal violet (Sigma C 3886) (Matthews et al.,1987). The stain was then removed and the plates washed five times in distilled water, the plates were then allowed to dry for 24 hours. Procedure 1 involved reading the plate by image analysis, Yielding number of colonies and the area covered by the cell in mm^2. Procedure 2 involved the elution of the crystal violet with glacial acetic acid (500μl per well), transfer to quaduplicate wells of a 96 well plate and reading at 570nm (using 620nm as reference wavelength) in an ELISA reader. It is necessary to include a control to allow for non specific binding of the crystal violet to the plastic. This involved adding the crystal violet (500μl/well) to four wells, not containing cells. After 10 min the plate was rinsed five times in H_2O and dried overnight: 500μl of glacial acetic acid was then added and O.D. were measured as described above.

2.7.2. Toxicity Assay - 96 well plate.

Cells were plated into 96 well plates (Griener 6621160),5,000 cell per well in 100μl assay medium. To one column of the plate only medium was added. This acted as the blank in the subsequent reading of the plate. After 24 hours incubation at 37°C,5%CO_2, 100μl of drug dilution (2X final concentration) was added to each well, in replicates of eight, for each drug dilution. Control wells had 100μl of medium added only. Plates were incubated for a futher 6 days with drug after which the end point was determined by the acid phosphatase method (Connolly et al.,1986 ; Martin et al.,1991). Medium was removed from the wells and rinsed with 100μl PBSA. 100μl of p-nitrophenyl phosphate substrate containing buffer (Appendix of Solutions 6.1.2.) was added to each well. It was found necessary to make up the solution freshly each time before use, Air bubbles were eliminated. The plate was then incubated at 37°C in 5% CO_2 for the standard incubation time of 2 hours, after which it was read in a dual beam ELISA plate reader at 405nm (reference wavelength 620nm). If the colour obtained was slight the plate could be reincubated for a further time to give increased sensitivity. Otherwise the reaction could be stoped by the addition of 50μl of 1N NAOH to each well, in addition to stopping the reaction the color is further developed by the addition of NAOH.

2.7.3. Cell Freezing Experiments.

It was found that when the MDR variants were frozen in the conventional manner that the cell recovery after thawing had decreased,in addition to a loss in resistance. Cell freezing experiments were preformed by the 96 well plate - toxicity assay

(section 2.7.2). Cells were pretreated at a density of $2X10^6$ cells/$75cm^2$ flask and allowed to grow to 75% confluency trypsinized and counted, a toxicity assay was carried out on the cells before freezing. The cells were then frozen at varying cell densities (10^6, $2.5X10^6$, $5X10^6$, $1X10^7$), varying serum (10%, 20%, 50%, 100%) concentration, varying concentraton of cryoprotectant agent DMSO (10%, 20%) (Sigma D8386) or Glycerol (10%, 20%) (Sigma G9012). The cells were frozen for six weeks. On thawing a viability count was ascertianed by Trypan blue viability. The cells were allowed to grow for seven days in the absence of drug, then a toxicity assay was preformed to estimate the level of resistance. The optimum procedure found suitable for all the MDR variants was 10^7 cells, 10% DMSO, and 50% FCS.

2.7.4. Trypsin Sensitivity Assay.

Two days before the experiment, the cells were removed from $25cm^2$ flasks by incubation with 0.05% EDTA (Sigma E9884) for 7min, and 25 cm^2 flasks were set up at a concentration of $5X10^5$cells/flask. The cells were fed the next day and the assay was set up on day three. Five different procedures of cell removal were investigated: Scraping in PBSA; 0.025%EDTA for 7 min; 0.05%EDTA for 7min; Trypsin(0.25%)/EDTA(0.02%) and Trypsin (0.25%). Adriamycin sensitivity assays were the preformed as in section 2.7.1 and 2.7.2.

2.7.5. Cloning Efficiency Assay.

Pretreated cells were plated at 100cells /500μl per well in a 24 well plate, adriamycin at varying concentrations were added 24 hours later. The plates were incubated for 10 days at 5%CO_2 at 37°C, then stained for exactly 10 minutes with 0.25 % crystal violet, dried thoroughly and colony number quantified by image analysis.

2.7.6. Circumention Assays.

Pretreated cells (Section 2.7.1) were plated at $2X10^3$ cells/100μl per well and then adriamycin and circumvention agent (CA) added 24 hours later. All circumvention assays were carried out in the 96 well plate assay (section 2.7.2). Both adriamycin and circumvention agent were added at 4X final concentration in a final volume of 25μl. Verapamil (Sigma V4629), quinidine (Sigma Q3625), caffeine (Sigma C1778), quinine (Sigma Q1625) and nifedipine (Sigma N7634) were made up in PBSA (1mg/ml stock), chloroquinine (Sigma C6628) in 0.9% NaCl (1mg/ml stock), nifedipine and aspirin (100mg/ml) were made up in water for injection (10mg/ml stock). Both generic aspirin ("Soluble Anadin" International Chemcial Co.Ltd., Island bridge, Dublin 8) and Standard Aspirin (Sigma A3160) were studied. The assay was incubated for 6 days , cell growth quantified and the resistance modulating ratio (RMR) of each agent calculated.

$$RMR = \frac{\text{IC50 of Adriamycin alone}}{\text{IC50 of Adriamycin + CA}}$$

2.8. Immunofluorescence.

P-170 is recognised by a number of monoclonal antibodies (See tables 2.5. and 2.6.). C219 (Centocor) a monoclonal antibody to the internal moiety of the P-170, was used in this study. Cells were trypsinized and diluted to 10^5 cells/ml. 50μl of this suspension was added to each well of a Dynatech multi-well slide and incubated at 37°C, 5% CO_2 overnight. The slides were incubated in 100mm petri dishes (Griener 633185). The following day slides were rinsed three times, with 2ml of PBSA, fixed and permeabilised in acetone (pre cooled at - 20°C) for 20min. Slides were then air dried. 20μl of primary antibody, C219 (5μg/ml) was added. C219 was incubated overnight at 4°C. After the incubation time slides were rinsed three times in PBSA (10 minutes per rinse). 20μl of FITC-labelled second antibody (Sigma F3008) was added to each well and incubated in a dark, 37°C,5% CO_2 enviroment for one hour. Slides were then rinsed three times in PBSA, excess PBSA was removed with filter paper. Slides were then mounted in glycerol/DABCO (Aldrich D2,780,-2)(1mg/ml)(Johnson et al.,1982) , sealed with nail varnish and viewed under a Nikon fluorescent microscope.

Table 2.5. P-170 Specific Monoclonal Antibodies

Antibody	Cell line used in immunisation	Epitope	Reference
Hamster			
C219 C494 C32	CHOCHrC5 and CEM/VLB(500)	Recognises internal epitope	Kartner et al., 1985
265/F4	CHOCHrC5	Recognises external epitope	Lathan et al., 1985
JSB1	CHOCHrC5	Recognises internal epitope	Scheper et al., 1988
Human			
MRK16 MRK17	K562/ADM	Recognises internal epitope	Hamada et al.,1986
32G7 9A7 1F10	CEM/VLB(100)	Recognises internal epitope	Danks et al.,1985
HYB-612 HYB-241 HYB-195	SH-SY5Y/VCR and P3X63Ag8.653	Recognises external epitope	Hamada et al.,1986
MS.2	K562/DOX	Recognises external epitope	Marie et al.,1991
MDR-AB1	N.K.	N.K.	Krishan et al.,1991
Murine			
Moab17F	Murine 3T3 transfected with human *mdr1* gene	Recognises external epitope	Dr. D. Ring Cetus Corporation 1991.

Table 2.6. Description and source of cell lines used for MAB production.

Cell Line	Description/Comments	Reference
CHOCHrB30	Chinese Hamster Ovary Colchicine - resistant	Kartner et al., 1983.
CHOCHrC5	Chinese Hamster Ovary Colchicine - resistant	Kartner et al., 1983.
K562/ADM	Human Myelogenous Luekemia - Adriamycin Resistant	Tsuruo et al., 1985.
CEM/VLB.100 CEM/VLB.500	Human leukemia Vinblastine resistant 100 and 500 fold from parental cell line	Beck et al., 1979.
SH-SY5Y/VCR	Human neuroblastoma Vincristine Resistant	Biedler et al., 1988.
P3X63Ag.653	Human Myeloma cell line	Kearney et al., 1979.

2.9. GENETIC METHODOLOGY.

2.9.1. Table 2.7. Listings of Plasmids

Plasmid	Source	Selective Marker
pSV2NEO	Beatson Inst. Glasgow	Neomycin/Geneticin
PLW4	Beatson Inst. Glasgow	Chloroamphenicol
pHaMDR1a (Full MDR1 cDNA)	Ira Pastan	AMP

2.9.2. Calcium Chloride Transformation of Bacterial Hosts with Recombinant Plasmid DNAs.

Transformation is the process of uptake of DNA by bacteria. The calcium chloride method of bacterial transformation was described by Mandel et al.,(1970), and the bacterial host for all transformation was E.Coli C600. From an overnight culture of C600 in LB medium, 10ml was innoculated into 100ml of fresh LB medium and incubated at 37°C until an O.D. of 600nm of 0.4 - 0.5 was attained. Cells were then centrifuged at 9,000 r.p.m. 5 min ,4°C (in a GSA rotor head of a Sorvall RC-5B refrigerated centrifuge) resuspended in 10ml cold 30mM $CaCL_2$ and chilled on ice for 20min. To 100μl 'competent cells', 0.1μg of recombinant (covalently closed circle) plasmid DNA was added and the mixture chilled on ice for 60min. The cells were heat shocked for 5 min at 37°C, and removed immediately to ice. To each 100μl 'transformed cells', 1ml of LB medium was added and the cells incubated at 37°C for 1-2 hours. This period was allowed for the expression of the antibiotic resistance encoded by the recombinant plasmid. Samples of 100μl were then plated on LB agar containing the selective antibiotic (Concentration 100μg/ml). The plates were allowed to dry, and

incubated overnight at 37°C. The efficiency of transformation was calculated and was usually $1X10^6$ transformants per μg DNA.

2.9.3. Small Scale Purification of Plasmid DNA.

10ml cultures of the plasmid containing strains were grown in LB medium for 18h at 37°C with shaking. The selective drug for the plasmid in question (see Table 2.9. for details) was included at a concentration of 100μg/ml in the LB medium. The cells were harvested in 30ml Sorvall tubes by centrifugation at 10,000 revs/min in an SS-34 Sorvall rotor. The cells were washed once with TE (10mM Tris, 1mM di-Sodium EDTA, pH 7.4) and centrifuged in the same manner. The supernatant was discarded and the pellet drained. The pellet was resuspended in 0.25ml of 25% (W/V)sucrose in 0.01M Tris-HCl, pH 8.0 and transferred to an Eppendorf tube. To this was added 100μl of freshly prepared Lysozyme (Sigma L6876) solution (25g/L in 0.25M Tris-HCL, pH 8.0) and 0.25M EDTA, pH 8.0. The mixture was kept on ice for 15min and the 500μl of Triton lysing mix (2% Triton X 100 in 0.05M Tris-HCL, 0.0625M EDTA,pH 8.0) was added. After a further 15min on ice, centrifugation was preformed at 26,000 revs/min at 4°C in an eppendorf tube and 0.75ml of phenol- chloroform (25:24) was added. This was then mixed and spun in a microfuge for 3min at 2,000 rpm. Upper Aqueous phase was removed to a new tube and to this was added 1/10 of a volume of 20% sodium acetate firstly, followed by 2X volume of 100% ethanol. This was kept at -70°C for 20 min. This was then spun at 2,000 r.p.m. in a microfuge for 15 min. The supernatant was discarded and the pellet was dried under a 60 watt lamp for 20 min, then dissolved in 100μl TE buffer. This was then passed through a Sephadex G50

fine column to further purify the plasmid. This DNA suspension was then suitable for transfection experiments or transformation.

2.9.4. Large Scale Plasmid Isolation-Cleared Lysate Procedure.

Large amounts of recombinant plasmid DNA (1-2mg) was obtained using the cleared lysate procedure of Maniatis et al,(1982). Selective agent was added to both the starter culture and the large scale preparation to maintain selective growth of only those bacteria harbouring the required recombinant plasmid. 2.5ml of the overnight starter culture (10ml) was innoculated into 500ml of LB medium (contained in a 1L baffled flask) and incubated at 37°C 200 rpm, for 18 hours. The cells were harvested by centrifugation in 250ml tubes for 10min at 16,000 r.p.m. in a GSA Sorvall (RC-5B) rotor. The supernatant was discarded and the pellet thoroughly drained of excess liquid, it was then resuspended in 3ml of a solution containing 25% (w/v) sucrose in 0.05M Tris-HCl, pH 8.0 and transferred to a 30ml Sorvall centrifuge tube. To this was added 1ml of a freshly prepared Lysozyme solution (25g/L in 0.25 M Tris-HCl, pH 8.0) and 1ml of 0.25M di-SODIUM-EDTA, pH 8.0. This mixture was allowed to stand on ice for 15 min, then 7ml of triton lysing mix was added and the mixture allowed to stand on ice for 15 min. Cell debris was removed by a clearing spin at 20,000 r.p.m. in a SS-34 Sorvall rotor for 45 min at 4°C. 9.5ml of supernatant was decanted into a silanized glass universal. 1g of Cesium cloride (Sigma C3032) was added per ml of cleared lysate, together with 500μl of ethidium bromide (10mg/ml). When the cesium chloride had fully dissolved, the mixture was centrifuged at 5,000 r.p.m. for 15 min, this causes any proteinaceous material to collect at

the surface, this material was removed and the remaining lysate was transferred to heat sealable Beckman ultracentrifuge tubes and centrifuged at 42,000 r.p.m. for 40 hours at 15°C in a 70iTi fixed angle rotor. Chromosomal (upper) and the plasmid (lower) DNA bands were visualised using ultraviolet light. The plasmid band was extracted with a syringe and transferred to an acid washed glass universal. The ethidium bromide was extracted from the recovered fraction by addition of 1 volume of CsCl- saturated isopropanol. The upper, isopropanol was then removed and the procedure repeated until the lower layer was colourless. The plasmid fraction was then dialysed against TE buffer for 4 hours to remove the CsCl. The plasmid fraction was then extracted with phenol, saturated with 1M Tris-HCL (pH 7.6) shaken vigorously for 30 sec. The aqueous layer was recovered using a pasteur pipette and transferred to a 30ml corex tube (DuPont). A 10% volume of sodium acetate and two volumes of cold absolute ethanol were added and the mixture stored at -70°C for 2h to precipitate the plasmid DNA. DNA was recovered by centrifugation at 20,000r.p.m. for 30 min in a SS-34 Sorvall rotor at 4°C. The pellet was washed in 80% ethanol to remove residual salt and then air dried under vacuum. The pellet was dissolved in TE and stored at -20°C until required.

2.9.5. Quantification of DNA by Spectrophotometric Methods.

An aliquot of the DNA to be determined was diluted in TE buffer and the O.D. at 260nm and 280nm taken. An O.D. of 1 at 260 nm wavelength corresponds to 50 µg/ml for double stranded DNA. The ratio of 260nm to 280nm gives an estimate of the purity of the DNA,and should be 1.8 for pure DNA. The concentration of DNA in µg/ml was given by the reading at O.D.260nm X dilution factor X 50.

2.9.6. Agarose Electrophoresis of Purified Plasmid and Genomic DNA.

Isolated plasmid and genomic DNA were checked for quality and purity using 0.8% agarose gel, 5μg of sample was placed in loading buffer (Appendix of buffers 6.1.3.) and run in TBE buffer (89mM Tris-HCl, 89mM Boric acid and 2mM EDTA pH 8.0) at 75 V for 4 hours. The resultant gel was stained with ethidium bromide, 100μg/ml and visualised under U.V. illumination.

2.10. Cytogenetic Analysis.

Cytogenetic analysis was carried out on HEP-2, HEP-2A, HEP-2B, DLKP and DLKP-A. Cells were analysed 2 days out of drug. Cells were innoculated at 6 X 10^6 cells per 75 cm^2 flask 21 hours hours prior to harvesting. For HEP-2 and DLKP colcemid at a final concentration of 0.02μg/ml was added two hours prior to harvesting, while with HEP-2A, HEP-2B, and DLKP-A colcemid was added ten hour before harvesting. This was due to the altered growth pattern of the drug resistant cell, it is much more difficult to get a good metaphase yield. Cultures were not trypsinised as a satisfactory mitotic yield was achieved by vigorous shaking of the flasks. The resultant cell rich supernatant was decanted, centrifuged and resuspended in 0.075M KCL, 37°C, parental lines for 25 min, HEP2-A and HEP2-B for 35min, and DLKP-A for 45 min. The varying time is differential of the ease at which different metaphases of different cell lines spread. The cell suspension was centrifuged and the cell button gently resuspended in cold Methanol-acetic acid fixative (3:1 (v/v)) for a minimum of 1 hour. A protocol involving

several washes in fixative was found to improve chromosome spread. Air dried preparations were prepared by applying one or two drops of concentrated cell suspension to a clean glass slide. Staining was with either conventional Giemsa or Giemsa-Trypsin (Seabright, 1971).

2.11. Transfection Techniques.

2.11.1. Calcium Phosphate Transfection Technique.

Cells were pretreated as described in section (2.7.1) to ensure the cells were in exponential stage of growth. Cells were trypsinized and replated at a density of $1X10^6/25cm^2$ flask and incubated at 37°C for 24 hours. Day 2 involved the preparation of the $CaPO_4$ precipitate (Graham et al.,1977). To 40μg/ml of donor DNA in 900μl 0.1mM EDTA, 1.0mM Tris-HCL, pH 8.0 100μl of 2.5M $CaCL_2$ was added and mixed thoroughly. This DNA solution was added slowly with continuous mixing to 1ml of 2 X HBS (See appendix of buffers 6.1.4.). This solution was mixed immediately by vortexing and allowed to stand at room temperature for 30 minutes to allow time for the DNA/$CaPO_4$ preciptate to form. Then 500μl of this solution was added to each flask containing 5ml of growth medium. It is necessary to ensure the DNA precipitate is spread evenly throughout the flask, the flask was then incubated at 37°C for 3 hours and then gently mixed again and then left incubating for 20 hours without disturbing, this time period allows for absorption of the DNA calcium phosphate precipitate. After the 23 hours total incubation time the precipitate was washed from the flask and selection free medium added (see section 2.11.6 for selection details). In all experiments a control transfection was preformed by the replacement of the DNA by T.E. buffer, this allows for the quantification of spontaneous mutants for the particular selected phenotype.

2.11.2. Calcium Phosphate Transfection Procedure with Facilitators.

The effect of an number of facilitators on the efficiency of the $CaPO_4$ assay was investigated, to try and increase transfection frequency. These included chloroquinine, PEG 6000 and DMSO. The transfection technique was as described in section 2.11.1, but an additional agent was added either pre or post addition of the calcium phosphate precipitate.

It was necessary to optimise the concentration of each of the facilitators, this involved preforming a toxicity assay with the relevant agent (as described in section 2.7.1). It was noted from literature that a certain amount of cell kill was required with treatment of the cells with facilitators to increase the transfection frequency. The cell kill required for each of the agents to be effective as a transfection agent was as follows: Chloroquinine -30% kill, DMSO -10% kill, Peg 6000 -10% kill (see section 3.9 for further details). Pretreatment with Peg 6000 for two minutes was used prior to the addition of the $CaPO_4$ precipitate. The concentration varied depending on the cell line in question (see section 3.9.4). Pretreatment with chloroquinine for 3 hours prior to addition of the $CaPO_4$ precipitate was investigated, the concentration of chloroquinine varied with the cell line under study (see table 3.9.2a for details) DMSO exposure for 4 minutes was investigated both pre and post addition of the $CaPO_4$.

2.11.3. Polybrene/DMSO Transfection Procedure.

This procedure was highlighted by Morgan et al.(1986). As in the case of the facilitators highlighted in section 2.11.2 approximately 10% kill of the combined effect of polybrene/DMSO is necessary before an efficent transfection occurs. Thus toxicity assays were set up (as highlighted in section 2.7.1) with three different concentration of each agent (10/20/30 μg/ml polybrene (Sigma H9269) and 10/20/30% DMSO) to ascertain the optimum combined affect of Polybrene and DMSO necessary to achieve the necessary 10% kill reqiured for transfection. The transfection was preformed by the addition of the appropriate Polybrene concentration, in the presence of the DNA to be transfected to the cells for 6 hours at 37°C, the cells were then washed twice in PBSA followed by the addition of the appropriate DMSO concentration for 4 minutes. The cells were washed twice in PBSA and replaced in growth medium (see section 2.11.6 for details of selection procedures).

2.11.4. Electroporation Toxicity Assay.

Survival curves for any cell line to be electroporated were set up varying voltages and μF to observe the effects of electroporation (Potter et al.,1984) on healthy cells in the absense of DNA. It has been observed that a 30% kill is required for successful transfection. Pretreated cells were trypsinised and washed twice in sterile PBSA and counted. To each cuvette 200μl TE buffer and $5X10^6$ cells /600μl PBS were added. The cuvettes were kept on ice for 5 minutes prior to electroporation. One control cuvette was kept on ice while the other was kept at 37°C to account for cell death due to drop in temperature. Electroporation was carried out

on each cuvette at a different set of conditions. After electroporation the cells were kept on ice for a further 10 minutes and a viability assay preformed (see section 3.7.1 and 3.7.2) on the cells to ascertian the affect of the varying electroporation conditions.

2.11.5. Transfection of Plasmid and Genomic DNA By Electroporation.

Cell in exponential growth phase were trypsinized, washed 2X in PBS to remove and residual medium and counted. 200μl DNA/TE and 600μl cells in PBS ($5x10^6$ for $75cm^2$ flask) were added to each cuvette and placed on ice for 5 minutes. The suitable electroporation conditions, as decided from section 2.11.4., were set (μF and voltage) and the pulse discharged. After electroporation the cells were left on ice for a further 10 minutes and then added to the culture flask with fresh culture medium (see section 2.11.6 for details of selection period).

2.11.6. Selection Procedures for Transfected DNA.

Most gene transfer experiments are monitored by the transformation of cells from one phenotype to another. This usually involves the use of selective medium for a specific biochemical marker. It is thought that in many cases, a period of expression in non-selective conditions is necessary prior to selection. This is known as the preselection time. It varies markedly depending on the gene been transfected.

Table 2.8. Selection Procedure for Transfected DNA.

Donor DNA	Preselection Time	Selection agent Concentration μg/ml
PLW4	3 days	Chloroamphenicol
pSV2NEO	1 day	Geneticin 400-1000μg/ml (Over four days)
pHAMDR1A	3 days	Colchicine 20ng/ml increase to 40/50ng/ after 3 weeks
A9/NIH3T3	24 hours	Ouabain 10^{-6} M
CHrC5	3 days	0.075 μg/ml Adriamycin

2.11.7. Transfection of Antisense Oligonucleotides.

The sequences of the oligomers used in this study were

d5'(GTC CCC TTC AAG ATC CAT)3' Antisense oligomer.

d5'(ATG GAT CTT GAA GGG GAC)3' Sense oligomer.

These sequences represent the first 18 bases in the human mdr1 coding sequence. Oligomers were made and purified by Dr.Mike Powell, Delta Biotechnology. I acknowledge the co-operation of Dr. Becky Shaddaux for the computer search showing uniqueness of the above nucleotides for P-170. Cells were set up in 25cm^2 flasks at a density of 4X10^6 cells in 3 mls of culture medium. All culture medium was made up with 10% FCS that had been previously heat treated at 65°C for 15 minutes to elimate serum nucleases. Oligomers were added for three days to the culture medium at a dose of 80μg on the first day and 40 μg on the second and third days. After pretreatment the cells were trypsinized and plated at a density of 2X10^3 /100μl in 96 well plate. An adriamycin toxicity assay was preformed as in section 2.7.2., using a four day incubation instead of 7 to test for the down regulation of P-170. 5 assays were set up for each cell line, a control adriamycin toxicity profile, antisense and sense without oligomer present in the toxicity assay, and antisense and sense with oligonucleotide present in the toxicity assay. The assays were set up in the presence and absense of oligomer to ascertain the stability of the down regulation event. Oligomers were added at a concentration of 40μg each of the four days of the toxicity assay.

2.12. Protein Analysis by Western Blotting.

2.12.1. Purification of Cell Membranes.

Cell membranes were prepared by sonication followed by differential centrifugation, (Ronchi et al.;1989) 10^8 cells were trypsinized and washed three times in cold PBSA. The cell were then placed in lysing buffer (appendix of buffers, 6.1.5.). The cells were then sonicated as highlighted in section 3.5.2., sonication time is dependent of the cell line in question. The cells were checked microscopically for the appearance of cell lysis. The cells were then spun at 7,000 r.p.m. for 15 min. The supernatant was then recovered, with care been taken not to dislodge the pellet. This supernatant was the loaded onto a sucrose cushion, 15 ml (appendix of buffers 6.1.6.), and spun at 37,000 r.p.m. in an SW28 rotor of a Beckmann ultracentrifuge, for 90 min. The membrane enriched fraction collects at the interface of the sucrose and the lysis buffer. This was removed carefully, volume was raised to 10ml with washing buffer (appendix of buffers 6.1.7.) and centrifuged at 36,000 r.p.m. for 40 min at 4°C. The pellet was then resuspended in storage buffer (appendix of buffers 6.1.8.) and frozen at 70°C. The samples were then freeze dried.

2.12.2. Quantification of Membrane Protein - Pierce Bincinchoninic Acid (BCA) Protein Assay.

This is a highly sensitive reagent used for the determination of protein concentration. It combines the well known reaction of protein with Cu^{++} in an alkaline medium (yeilding Cu^{+}) with a highly sensitive detection reagent for Cu^{+} namely bincinchonic acid (Smith et al.,1985).

Two pre-formulated reagents were supplied :-

Reagent A :Base reagent which containes sodium carbonate ,sodium bicarbonate, BCA detection reagent and sodium tartarate in 0.2N NaOH.

Reagent B :4% (w/v) copper sulphate.

The BCA protein assay working reagent was made up by mixing 50 parts of reagent A with one part reagent B. The working reagent was stable at room temperature. Two different protocols were followed; one involved incubation at 37°C for 30 min for expected protein concentrations of 0.2-1.2 mg/ml and the other used incubation at 60°C for 30 min for expected protein concentrations of 0.05-0.25 mg/ml. In both cases a standard BSA stock solution 2mg/ml was prepared and appropriate dilutions were made to give the range of standards required. Then 0.1ml of each standard or unknown protein sample was pipetted into a testube and 0.2ml of BCA working reagent was added. For blanks, 0.1ml of diluent was used. The tubes were incubated at the selected temperature and absorbance readings taken at 562nm. All samples and standards were made up in

duplicate. This method has several advantages over other protein assays such as the Lowry assay. These include the very small amount of sample required for this assay ; there is only one step involved in the assay, which reduces operational error ; and the working reagent can be stored for 1 week at room temperature.

2.12.3. Western Blotting of Membrane Proteins.

Following protein analysis of the cell membranes by the BCA method Smith et al.(1985) suitable quantities of membrane proteins 0.6μg/μl were separated of 7.5% SDS polyacrylamide gels in a discontinuous method, according to the method of described by Lamelli (1970). Using the "midget" system of vertical electrophoresis a $7cm^2$ X $8cm^2$ gel was made as follows. 15ml of resolving gel was made by mixing 5ml acrylamide stock (Appendix of buffers 6.1.9.), 6.8ml distilled H_2O and 3.0ml 1.875M Tris-HCL, pH 8.8. To this solution of 10% (W/V) SDS, 7.5μl TEMED, and 50 μl 10% (W/V) ammonium persulphate was added. The gels were cast immediately in two gel cassetes, each comprising a plastic plate, two $0.75cm^2$ spacers and a aluminium oxide plate mounted against the vertical core of the unit, and allowed to set, (appromiately 30 min). A stacking gel, 5ml, was prepared by mixing 0.8ml of stock acrylamide, 3.6ml distilled H_2O and 0.5ml 1.25 M Tris-HCL buffer pH 6.8, 50μl 10 % (W/V) SDS and mixed well. To this was added 5.0μl TEMED and 17μl 10% (W/V) ammonium persulphate. This was carefully poured on top of the resolving gel and a comb of the appropriate thickness inserted. The amounts of resolving and stacking gel stated above were sufficent for two $0.75mm^2$ thick gels.

Separated proteins were visualised by staining with Commassie Blue Brillant Dye R-250. Western blotting of separated proteins was achieved by the method of Towbin et al.(1979) using a Biorad Transblot apparatus. Membrane proteins were transferred to Hybond-C (Amersham,PLC). The Western blots were placed in blocking buffer (3% (w/v) BSA, 15mM NaN_3 in PBSA) overnight at 4°C. Test antibody, (monoclonal antibody C219, Centicor diagnostics or polyclonal antibody from mouse immunised with CHrC5 cells) was diluted with 2% (w/v) BSA, 0.1 % (v/v) Tween 20 in PBSA and incubated for 2 hours at 37°C. Western blots were washed in washing buffer (10mM Tris; 150mM NaCL; 0.5% (v/v) Tween 20, pH 7.5) and second antibody, alkaline Phosphatase-conjugated anti-mouse IgG (Sigma A-1902) was added. Western blots were again washed and developed in substrate consisting of 0.1% (V/V) Nitroblue tetrazolium, NBT (Sigma N-5514), in 10mm Tris-HCL, pH 8.9 5mg/ml 5-bromo-4-chloro-3-phosphate (BCIP), in N,N' -dimethylformamide (Sigma D-8654), 1.0M magnesium chloride in distilled H_2O and 0.5M Tris -HCL,pH 8.9. The addition of substrate gave rise to the development of blue/purple dots for positive antibody recognition. In all case a negative control strip untreated with test antibody was included, to ascertain any backround staining.

2.12.4. Determination of Protein Size.

Protein size was ascertained by the running of prestained molecular markers (Sigma MW SDS-Blue, and Sigma MW SDS-200) these are 200Kd, 116kd, 96Kd, 66Kd, 45Kd, and 29Kd.

SECTION 3

RESULTS

3.1. Establishment of 7 Novel Multidrug-Resistant Human Cell Line Variants, and Investigation of their Cross Resistant Profiles.

Seven new multi-drug resistant variants of six established human cell lines were established, as described in section 2.6.1 and 2.6.2. To ensure the adriamycin selected cell lines were collaterally resistant to other drugs and representative of the *in vivo* situation, they were assayed for sensitivity to chemotherapeutic agents from a number of different drug classifications. The drugs included in this study were adriamycin, an anthracycline antibiotic; VP16 and VM26 both semisynthethic derivatives of podophyllotoxin; Vincristine (Oncovin) a vinca alkaloid; Cis-platin, a nonclassical alkylating agent which consists of a complex of chloride and ammonium ions with platinum; colchicine an antimitotic agent and 5-Fluorouracil, an antimetabolite (See appendix 6.2 for drug structures). Resistance to 5-fluorouracil is not a usual observation in MDR cells. The instability of a number of the chemotherapeutic drugs was evident from initial toxicity assays, it was necessary to optimise storage procedures for each drug to ensure reproducibility of experimental data. (See section 2.6.1. for correct handling of chemotherapeutic drugs). The following tables represent the IC50 (nM) of the MDR variants and are the mean of two experiments, the assay procedure used was the acid phosphatase (unless otherwise stated). In the acid phosphatase procedure, 8 replicate wells were studied and in the 24 well assays, 3 replicate wells were studied; the results presented in the following tables are the IC50nM ascertained from a toxicity profile for each agent (See appendix 6.3 for sample calculation of IC50 nM). Data presented are the mean of two experiments ± standard derviation.

3.1.1. Cross Resistance Patterns of DLKP and DLKP-A at Different Levels of Resistance.

DLKP-A, a squamous cell carcinoma of the lung, was the cell line that adapted to resistance most readily. Subsequent experiments designed to investigate the clonal variation of parental cell line DLKP (Section 2.7.5) showed that a resistant population was evident that accounted for approximately 4% of the total population. DLKP-A resistant variants were analysed at three different levels of resistance, 0.8μg/ml, 1.75μg/ml and 2.45μg/ml of adriamycin.

The time period for selection was as follows :

0.05μg/ml - 0.8 μg/ml ADR November 1989 - September 1990.
0.8 μg/ml - 1.75μg/ml ADR October 1990 - December 1990.
1.75μg/ml - 2.45μg/ml ADR January 1991 - March 1991.

DLKP-A was found (Table 3.1.3, 3.1.2) to be cross resistant to all the agents tested, with highest cross resistance noted to the podophyllotoxins and vincristine. A very interesting phenomenon is noted in the most resistant variant of DLKP-A (2.45) whereby a lower fold resistance to vincristine is noted. With all the agents (other than vincristine) an increase in resistance to the test agent is noted as the adriamycin selected concentration increases. A surprising finding is the resistance to 5-FU, which is not normally noted with the classical MDR phenotype.

Table 3.1.1. Cross resistance patterns of DLKP and DLKP-A at different levels of resistance.

IC50nM Drug	DLKP	DLKP-A (0.8)	DLKP-A (1.75)	DLKP-A (2.45)
ADR	16.55±3.68	2216±82.5	4995.5±320.5	6834±377
VP16	42.47±6.8	1444±112.1	1521.0±127	1486±76.5
VM26	84.76±7.17	708±54.13	1035.0±127	1256±98
VCR	0.83±0.01	56±2.6	88.0±029	33±3.54
C-PT	1433±200	3499±233.65	5500.0±300	7510±550
5-FU	6571±499.5	13064±1614	15372.5±768.5	19215±1537
COLH	17±0.32	45±2.376	70.7±5.885	114±7.14

n=2 Data= Mean + S.D.

Table 3.1.2. Fold resistance of the DLKP-A MDR variants relative to the control cell line DLKP.

Drug	DLKP-A (0.8)	DLKP-A (1.75)	DLKP-A (2.45)
ADR	133.9	301.84	412.93
VP16	34	35.81	35.00
VM26	8.35	12.21	14.82
VCR	67.46	106.02	39.75
C-PT	2.44	3.8	5.24
5-FU	1.98	2.34	2.92
COLH	2.64	4.15	6.405

3.1.2. Cross Resistance Patterns of OAW42,OAW42-A, SKMES-1 and SKMES1-A.

The SKMES1-A selection at the initial stages proceeded very slowly. Looking at the clonal population of the parental cell line (section 3.4. figure 3.4.4) a resistant population is not as evident as in the case of DLKP.

The time period for selection was as follows:

0.025μg/ml - 0.30μg/ml November 1989 - September 1990.

0.3 μg/ml - 0.55μg/ml October 1990 - December 1990.

0.55 μg/ml - 0.95μg/ml January 1991 - March 1991.

At the later stages of selection the cells adapted very well to increases in drug concentration, it is presumed that a more resistant population had become dominant. As with the case of SKMES1-A, OAW42-A was initially very slow to adapt to resistance. No clonal variation was noted in the parental cell line (Figure 3.4.2).

The time period for selection was as follows:

0.05μg/ml - 0.25μg/ml November 1989 - September 1990.

0.25μg/ml - 0.65μg/ml October 1990 - December 1990.

0.45μg/ml - 0.85μg/ml January 1991 - March 1991.

Both OAW42-A and SKMES1-A were selected to almost the same level of adriamycin resistance (0.85μg/ml and 0.95μg/ml ADR) respectively, but varying patterns of cross resistance are very evident. SKMES1-A shows a greater diversity in cross resistance , with highest levels of resistance to podophyllotoxins and vincristine. Low levels of

resistance were noted to both C-PT and COLH with no cross resistance noted to 5-FU. OAW42-A cross resistance patterns are unusual in that highest level of resistance is noted to Cis-Platin with low level resistance to VP16, VM26 and VCR.

Table 3.1.3. Cross resistance patterns of OAW42, OAW42-A, SKMES-1 and SKMES1-A.

IC50nM Drug	OAW42	OAW42-A (0.85)	SKMES-1	SKMES1-A (0.95)
ADR	26.36±1.7	1812±138	24.8±0.9	3026±156
VP16	33.10±4.5	187±21.2	66.0±3	1514±46.74
VM26	82.99±9.9	233±17.41	54.1±11.42	759±34.3
VCR	1.23±.09	3.5±0.3	1.6±0.235	28±2.99
C-PT	583.3 ±16.7	5349±283.35	1333±166.7	6800±200
5-FU	6917.2 ±614	7095±84.55	4188±730.5	7801±576.48
COLH	31.7 ±3.9	33±2.54	21±3.37	82±13.77

Table 3.1.4. Fold resistance of OAW42-A and SKMES1-A relative to the parental cell lines.

Drug	OAW42-A	SKMES1-A
ADR	68.7	122.01
VP16	5.67	22.9
VM26	2.87	14.04
VCR	2.84	17.5
C-PT	9.17	5.1
5-FU	1.025	1.86
COLH	1.04	3.9

3.1.3. Cross Resistance Patterns for SKLU-1, SKLU1-A, DLRP and DLRP-A.

SKLU1 was extremely slow to adapt to even low levels of resistance. The selection was initiated at 0.05μg/ml, it was necessary to restart the selection 4 times as the cells died for no apparent reason. However, after 9 months they reached 0.10μg/ml level of adriamycin resistance. This selection behaved differently in that the cells appeared to senese, the cells were not dividing rapidly but the pH of the medium was changing, indicating metabolic activity.

The time period for selection was as follows :

0.05μg/ml - 0.1 μg/ml November 1989 - July 1990.

0.1 μg/ml - 0.35μg/ml July 1990 - November 1990.

0.35μg/ml - 0.55μg/ml December 1990 - March 1991.

DLRP-A selection was extremely slow, as with SKLU1-A , the selection was restarted a number of times (8 in total), the cells also appeared to remain dormant for long periods, often months at the time. The selection proceeded after 18 months in culture from 0.025μg/ml to 0.1μg/ml. The resistance patterns of these cell lines were different to the others (Tables 3.1.5 and 3.1.6) fold resistance to adriamycin was noted for DLRP-A with slight fold increases in resistance for VP16, VM26 and 5FU. SLKU1-A shows level cross resistance to VCR and colchicine, 5.023 and 3.824 respectively, with very low levels of cross resistance noted for VP16, VM26 and 5FU.

Table 3.1.5. Cross resistance patterns for SKLU-1, SKLU1-A, DLRP and DLRP-A.

IC50nM Drug	SKLU-1	SKLU1-A (0.55)	DLRP	DLRP-A (0.1)
ADR	14.87±1.63	1508±55	6.1±1.48	132.4±5.5
VP16	84.94±11.9	136±13	54.4±8.95	79.9±3.395
VM26	97.44±12.16	147±22.1	50.5±1.423	89.1±3.81
VCR	0.43±0.078	2.16±0.24	0.24±0.05	0.235 ±0.013
C-PT	1450.0 ±150	1946 ±86.4	1266.3±100.3	1214.5 ±86
5-FU	4265.1 ±465	6571 ±576	108.3±0.822	203.7 ±3.803
COLH	15.15±2.11	58 ±5.14	8.5±1.005	9.64±0.624

Table 3.1.6. Fold resistance of SKLU1-A and DLRP-A relative to the respective parental Lines.

Drug	SKLU1-A	DLRP-A
ADR	101.41	21.70
VP16	1.599	1.46
VM26	1.5	1.77
VCR	5.023	0.988
C-PT	1.32	0.958
5-FU	1.54	1.88
COLH	3.824	1.132

3.1.4. Cross Resistance Patterns of HEP-2, HEP-2A and HEP-2B, at Two Resistance Levels.

HEP-2A was selected in a stepwise manner by the increase in concentration of adriamycin over 14 months (September 1988-November 1989) from 0.05μg/ml to 1μg/ml adriamycin. It was subsequently selected to 1.65μg/ml of Adriamycin from December 1989 to March 1991. The selection appeared to slow down and the cells were not adapting well in the presence of drug from October 1990-March 1991. HEP-2B was selected initially by exposure to ethyl methyl sulfonate (25μg/ml for 18 hours) it was selected over 11 months from 0.05μg/ml to 1μg/ml (From December 1988-November 1989). It was subsequently selected to 1.55 μg/ml (from December 1989-March 1991); as with HEP-2A, the cells appeared to go through a crisis stage whereby the cells were not doubling for months at the time, they may possibily have been because they had reached their threshold point as regards levels of adriamycin resistance attainable. HEP-2A and Hep2B were both analysed at two levels of drug resistance. The toxicity profiles for HEP-2A and HEP-2B at 0.65μg/ml ADR were performed using the 24 well plate assay (see section 2.7.1 for details).

Both HEP-2A and HEP-2B show progressive increase in resistance to all agents tested (except 5-FU and colchicine) with increased resistance to adriamycin and generally very similar cross resistance patterns. Highest levels of resistance was noted to the vinca alkaloid vincristine, with lower levels of resistance noted to VP16, VM26, C-PT and COLH. Due to the long period of time required for the HEP-2B selection it cannot be concluded that

initial chemical mutagen treatment on HEP-2B had a role in the development of adriamycin resistance.

Table 3.1.7. Cross resistance patterns of HEP-2,HEP-2A and HEP-2B, at two levels of resistance.

IC50nM Drug	HEP-2	HEP-2A (0.65)	HEP-2A (1.5)	HEP-2B (0.65)	HEP-2B (1.5)
ADR	19.8±2.3	1740±123	3744±137	1650±114	3973±78.1
VP16	62.1±5.3	134±12.5	248±11.9	154±103	260± 7.6
VM26	102.1±3.1	204± 6.7	458±17.5	240± 89	502± 8.3
VCR	1.1±0.1	15.4± 1.3	18.1±2.52	24.1± 2.3	24± 1.2
C-PT	1833.2±145	N.D.	6020±487	2356±216	6151± 314
5-FU	6135.4±380	6900±279	6570±514	7900±645	7686±154
COLH	24.9±11	N.D.	130±11	N.D.	136±13

N.D. = Not done

Table 3.1.8. Fold resistances of HEP-2A and HEP-2B relative to the parental Line.

Drug	HEP-2A (0.65)	HEP-2A (1.5)	HEP-2B (0.65)	HEP-2B (1.5)
ADR	87.87	189	83.33	200.6
VP16	2.16	3.99	2.47	4.18
VM26	1.99	4.48	2.35	4.91
VCR	14.1	16.45	21.9	21.8
C-PT	N.D.	3.28	1.28	3.35
5-FU	1.12	1.07	1.287	1.25
COLH	N.D.	5.22	N.D.	5.46

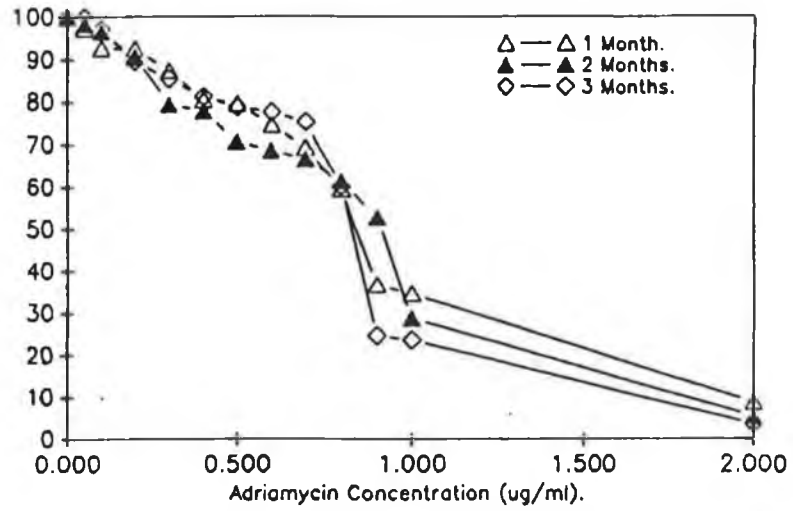
3.2.1. Stability of Drug Resistance in the Selections.

The genetic stability of the MDR phenotype is an important parameter too ascertain, additionally it is a factor that needs to be considered in the design of experimental procedures. The stability of the drug resistant phenotype was studied by removing cells from drug and subculturing in drug free medium for up to three months. The stability of all the MDR variants was studied using the Acid Phosphatase toxicity assay procedure.

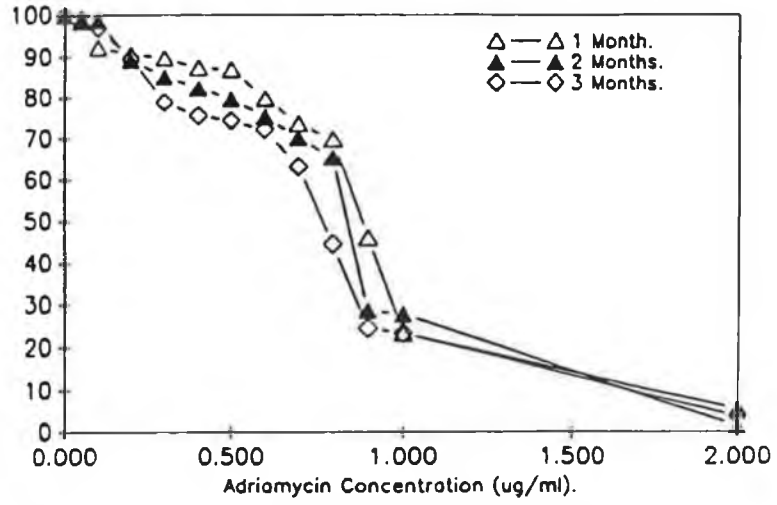
The variants were analysed at the following levels of resistance:

DLKP-A - 2.0 μg/ml ADR.
HEP-2A - 0.85 μg/ml ADR.
HEP-2B - 0.85 μg/ml ADR.
SKMES1-A - 0.75 μg/ml ADR.
SKLU1-A - 0.40 μg/ml ADR.
OAW42-A - 0.65 μg/ml ADR.

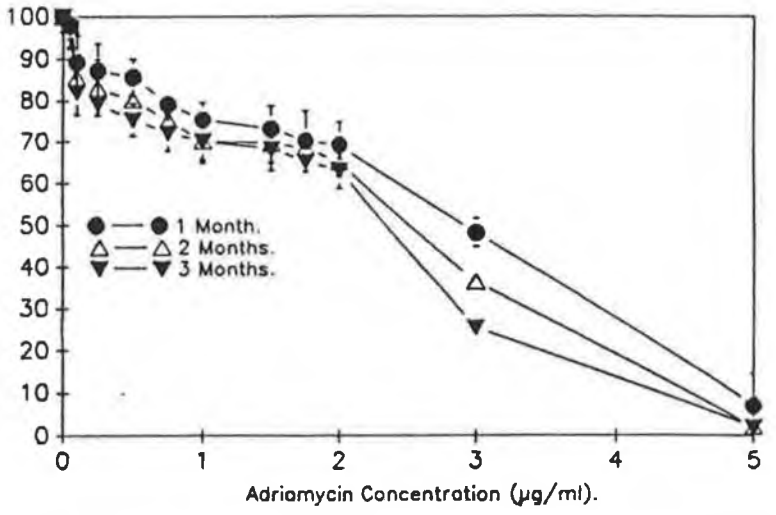
All the variants appeared to be stable after 3 months in the absence of drug, signifying a genetically stable population. In all cases a very slight decrease in IC50 was noted after the 3 month drug free period. An interesting observation was noted in the case of OAW42-A (figure 3.2.5) where the most resistant population (approximately 8%) which was growing at 2μg/ml ADR, at 1 month in drug free medium, was not evident at the 2 month and 3 month assay time. This possibly indicates the genetic instability of a small subpopulation of OAW42-A.



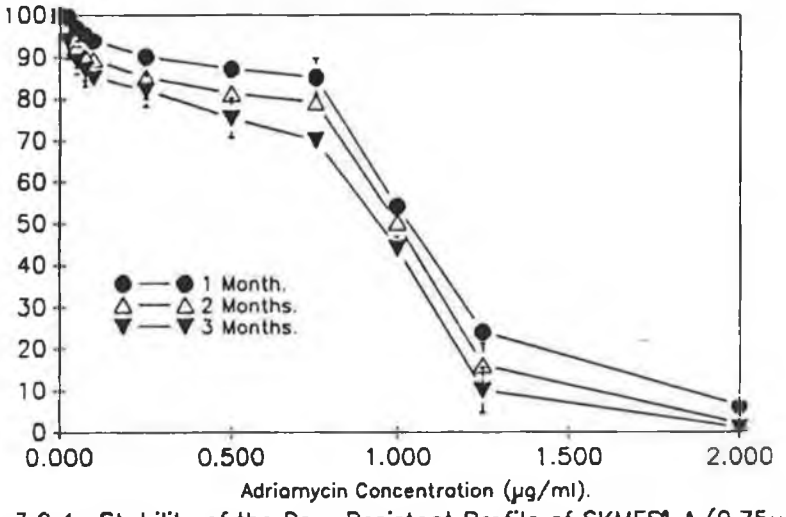
3.2.1. : Stability of Drug Profile of HEP–2A (0.85 ug/ml ADR) in the Absence of drug for 1 month,2 months and 3 months.



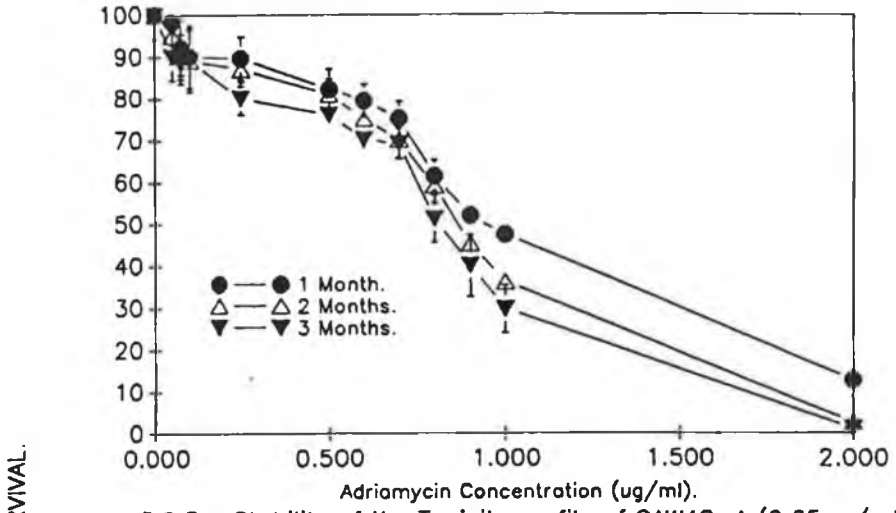
3.2.2. : Stability of Drug Resistance of HEP–2B (0.85ug/ml ADR) in the Absence of Drug for 1 month, 2 months and 3 months.



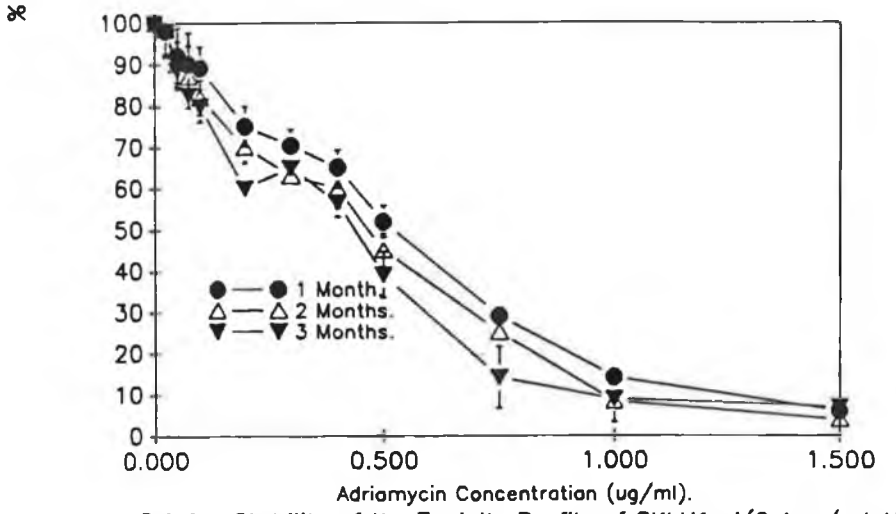
3.2.3. : Stability of Drug Profiles of DLKP–A (2µg/ml) in the Absence of drug for 1 month,2months and 3 months.



3.2.4. :Stability of the Drug Resistant Profile of SKMES1-A (0.75µg/ml) in the Absence of drug for 1 month,2 months and 3 months.



3.2.5. : Stability of the Toxicity profile of OAW42-A (0.65ug/ml) in the absence of drug for 1 month,2months and 3 months.



3.2.6. : Stability of the Toxicity Profile of SKLU1-A(0.4ug/ml ADR) in the absence of drug for 1 month , 2 months and 3 months.

3.3. Sensitivity of the MDR Variants to Standard Freezing Procedures.

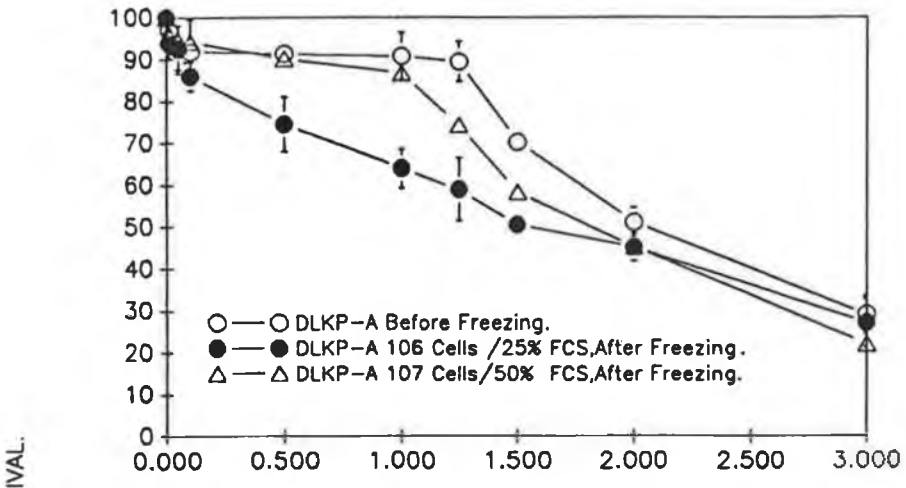
An initial observation that MDR cells were slow to recover from standard freezing procedures and their drug pattern appeared altered, led to the study of various cyropresevation procedures for MDR variants. The conditions altered were cell number, percentage FCS and percentage cryoprotectant. The aim of this experiment was to optimise freezing procedures for the MDR variants that improved percentage viability and recovery of drug resistance after freezing. The acid phosphatase assay was used for all assays (except for those presented in figures 3.3.8 and 3.2.9., where a cloning efficiency assay was set up to look at clonal distribution after freezing).

All the MDR variants were found to be susceptible to standard freezing procedures of 10^6 cells per ml /10% FCS, with HEP-2A and HEP-2B showing the most marked sensitivity (See figures 3.3.1. to 3.3.9.) 10^7 cells per ml/50% FCS proved to be the optimum conditions which favoured high cell viability after revival and maintainance of the drug resistance phenotype. Control freezing experiments were carried out with the parental cells lines, (see graph 3.2.7 for an example) it was noted that viability increased with increased percentage FCS but the drug resistant profiles did not alter depending on freezing procedure. Additionally alteration in the cyroprotectant (10%, 20% and 30%) DMSO and (10%, 20% and 30%) glycerol did not alter viability and drug profile on thawing (Data not shown).

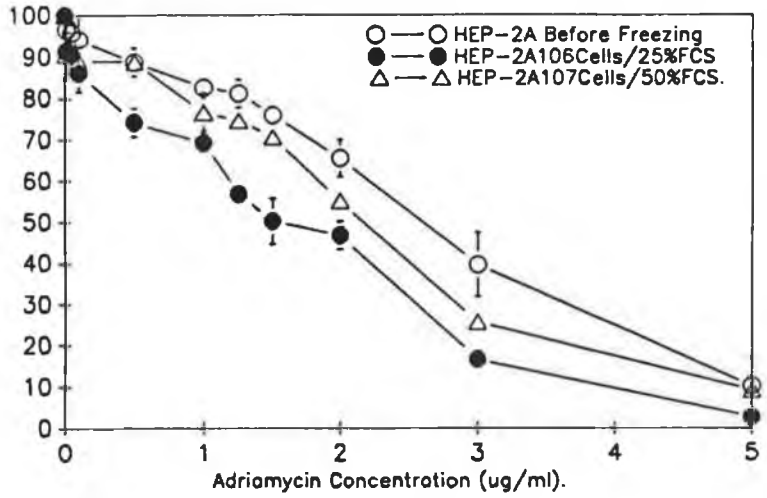
Table 3.3.1. Cell Viability After Freezing

Freezing Conditions	DLKP-A	HEP-2A	HEP-2B	OAW42-A	SKMES1-A	SKLU1-A
10^6cells/10%FCS	69	61	58	72	73	62
10^6cells/25%FCS	75	64	67	79	81	73
10^6cells/50%FCS	84	71	68	83	85	77
10^7cells/10%FCS	76	68	64	75	66	65
10^7cells/25%FCS	81	75	78	85	87	79
10^7cells/50%FCS	91	88	83	90	93	80

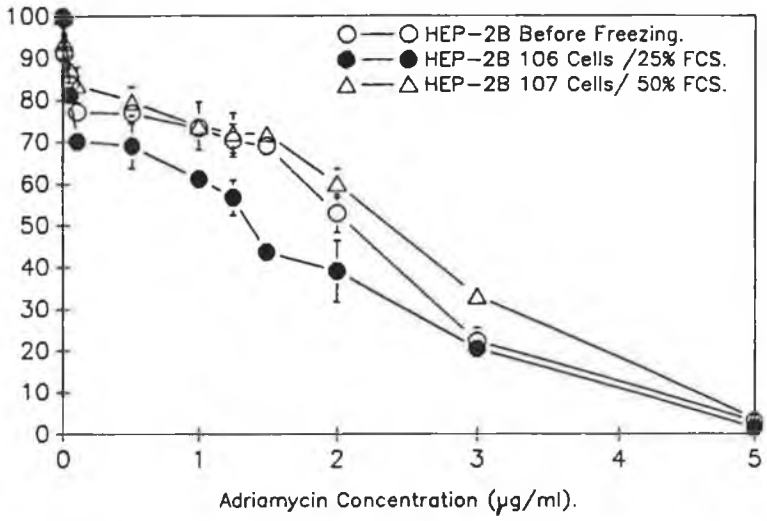
Data = Percentage viability determined by trypan blue vital dye.



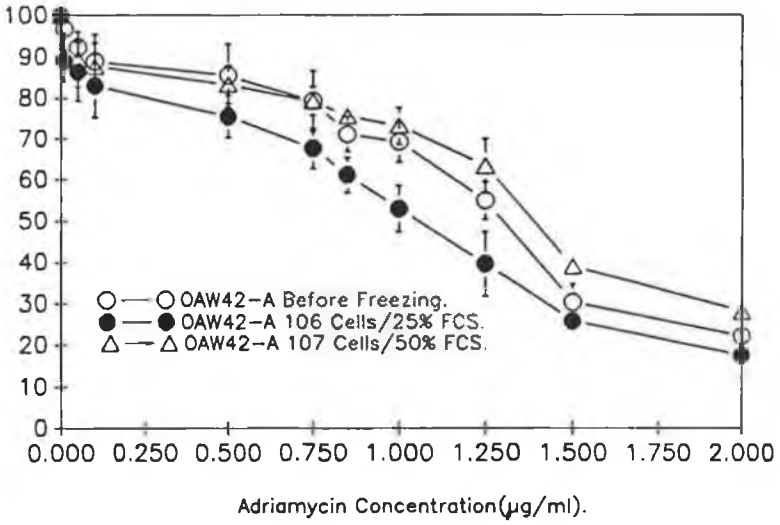
3.3.1.: The Effect of Cell Number and % FCS on Freezing of DLKP-A.



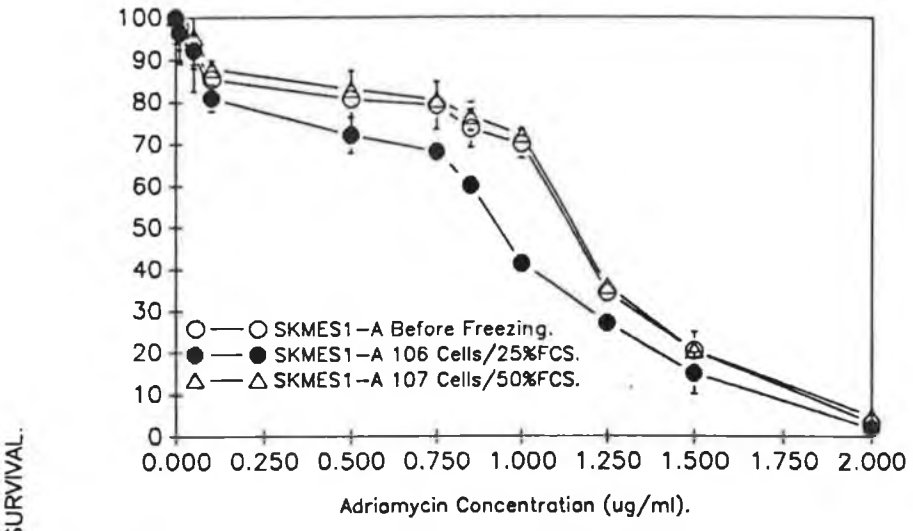
3.3.2. :The Effect of Cell Number and % FCS on Freezing of HEP-2A.



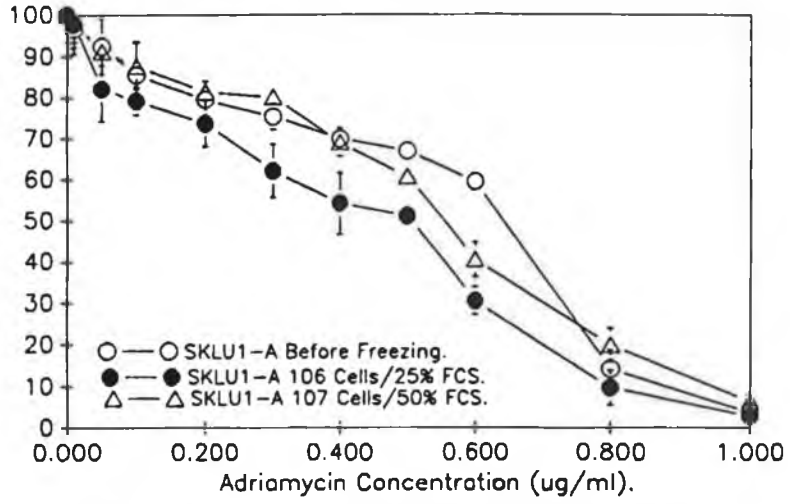
3.3.3. :The Effect of Cell Number and % FCS on Freezing of HEP-2B.



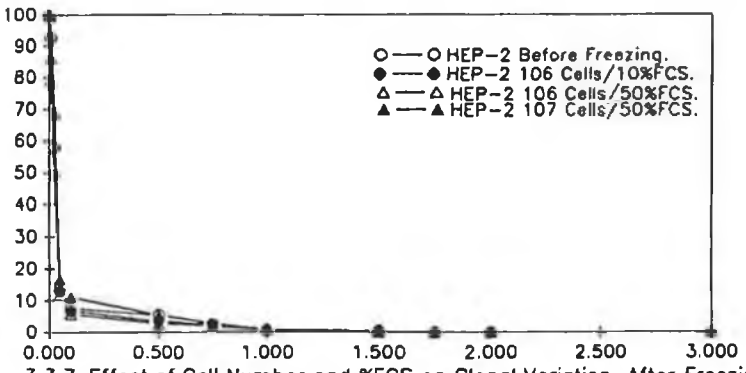
3.3.4. :Effect of Cell Number and %FCS on Freezing of OAW42-A.



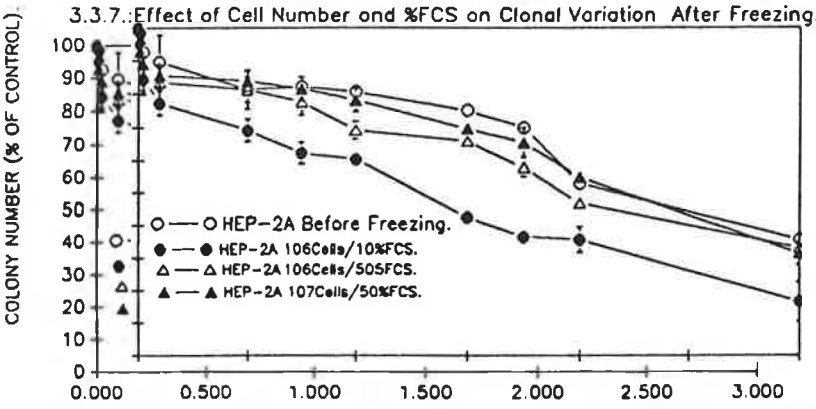
3.3.5. : Effect of Cell Number and %FCS of Freezing of SKMES1-A.



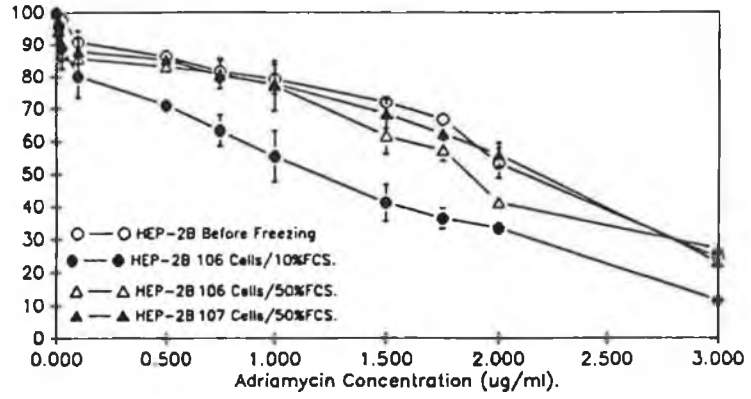
3.3.6. :Effect of Cell Number and %FCS on Freezing of SKLU1-A.



3.3.7.:Effect of Cell Number and %FCS on Clonal Variation After Freezing.



3.3.8.: Effect of Cell number and %FCS on Clonal Variation after Freezing.



3.3.9. : The Effect of Cell Number and %FCS on Clonal Variation of HEP-2B After Freezing.

3.4 Clonal Variation in the MDR variants.

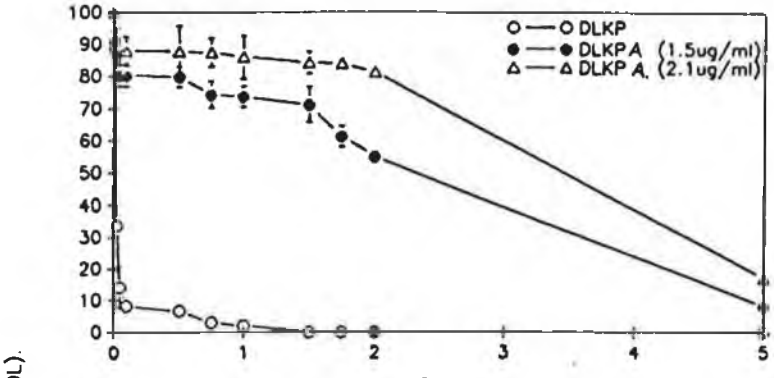
Our observation of reduced drug resistance of MDR variants following freezing by the earlier procedure (10^6 cell/ml 10% DMSO), suggested that our variants since they were not cloned, might contain subclones with different levels of drug resistance, with the more resistant subpopulation being also more sensitive to cryopreservation procedures. Such clonal variation should, if it exists, should be taken into account in assessing results obtained from these variants. We therefore decided to evaluate the possible existence of clonal variation in some of our variants. Clonal variation was estimated by the plating of 100 cells per well in a 24 well plate and exposing to varying concentrations of adriamycin, followed by incubation for ten days. Colony number was counted by image analysis. Heterogenity in the level of drug resistance was a feature of all the MDR variants tested (figures 3.4.1 to 3.4.5). The clonal variation results for DLKP-A and SKMES1-A, indicate the existence of several subpopulations with different adriamycin sensitivity, and also a marked survival above the final selective concentration of adriamycin, indicating the presence of a resistant population.

The presence of two different populations in terms of drug resistance was also implied in OAW42-A, HEP-2A, HEP-2B and SKLU1-A, with a sharp decline in colony survival followed by a plateau in colony survival over a range of higher drug concentrations, indicating the presence of a highly resistant population. In the case of OAW42-A, there is 90%-100% survival up to 0.75μg/ml ADR, between 0.75μg/ml and 1.0μg/ml ADR survival decreases to 50%, the survival then plateaus out to 35% at 1.5μg/ml ADR, 26% survivaal at

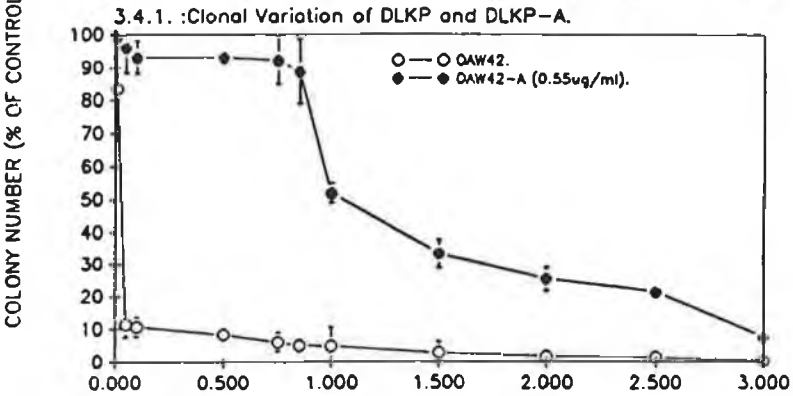
2μg/ml and 8% survival at 3μg/ml. In SKLU1-A an initial decrease in survival to 80% at 0.05μg/ml is evident, this indicates the presence of approximately 20% of the population that is extremely sensitive to drug. The drug profile plateaus at approximately 70% survival up to 0.5μg/ml ADR. A sharp decline from 70% to 10% survival from 0.5μg/ml to 1.0μg/ml ADR is observed, indicating the presence of resistant population in SKLU1-A accounting for approximately 5% of the population. In HEP-2A and HEP-2B there is approximately 90% survival up to 1.5μg/ml of adriamycin, a sharp decline is noted between 1.5μg/ml and 2μg/ml of ADR , with a reduction in survival of HEP-2A to 50% and HEP-2B to 25%. The slighty different results obtained for HEP-2B compared to HEP-2A in the Western blot analysis could possibly be explained by the 25% difference noted between HEP-2A and HEP-2B in the clonal variation at a concentration of 2μg/ml adriamycin. However, we would expect the difference in clonal variation of HEP-2A and HEP-2B at 2μg/ml to make an impact on all our other experimental data, but no other biochemical and pharmacological difference are evident between HEP-2A and HEP-2B.

I believe that it is valid to attribute these results to clonal variation in sensitivity rather than to some trivial explanation eg. Cell cycle specificity ; one reason for this assertion is the quite different individual patterns exhibited by the different variants. However, to show this definitely would require cloning of cells and demonstration of different sensitivity levels. Time did not allow inclusion of this experimental work.

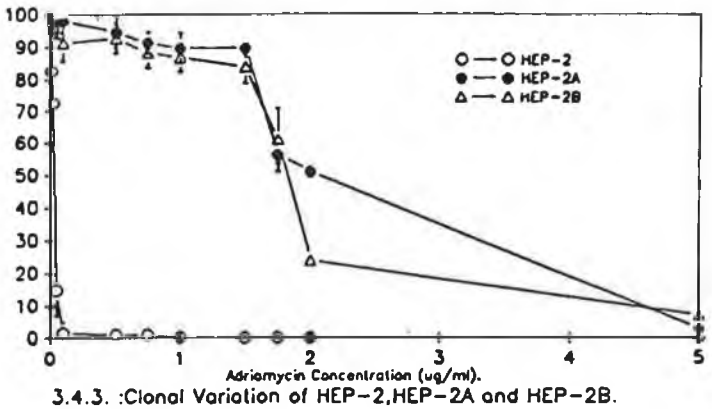
The possible significance of populations with different levels of resistance is addressed in the discussion. I will also propose a hypothesis to explain how the more sensitive population (eg SKLU1-A) cells sensitive to adriamycin at 0.1μg/ml adriamycin) can survive at the selective drug concentration (e.g.SKLU1-A 0.55μg/ml).



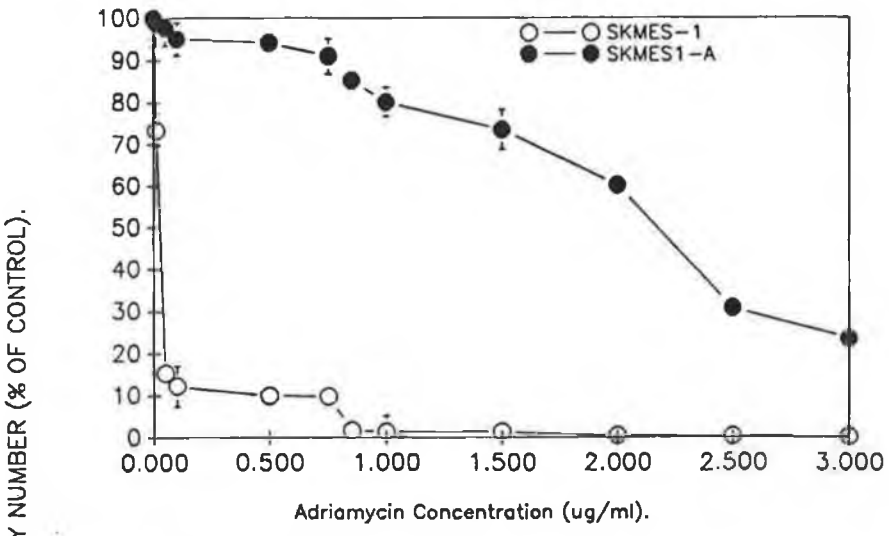
3.4.1. :Clonal Variation of DLKP and DLKP-A.



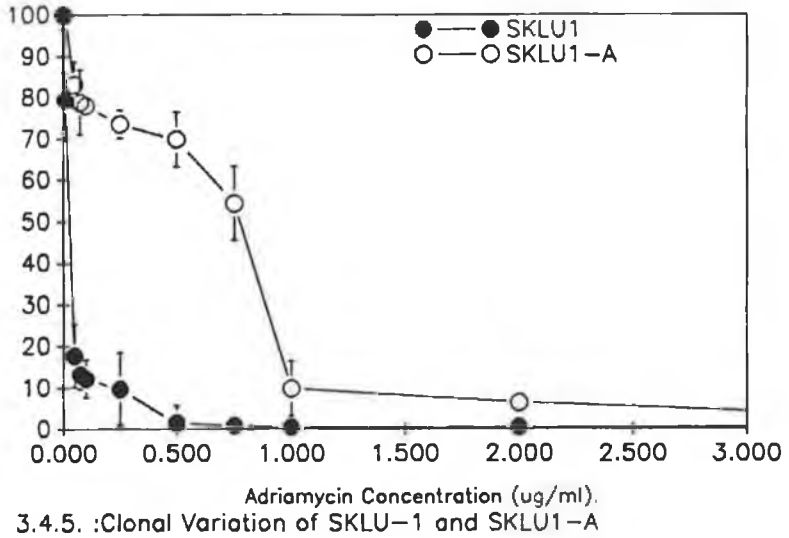
3.4.2. : CLonal Variation of OAW42 and OAW42-A.



3.4.3. :Clonal Variation of HEP-2,HEP-2A and HEP-2B.



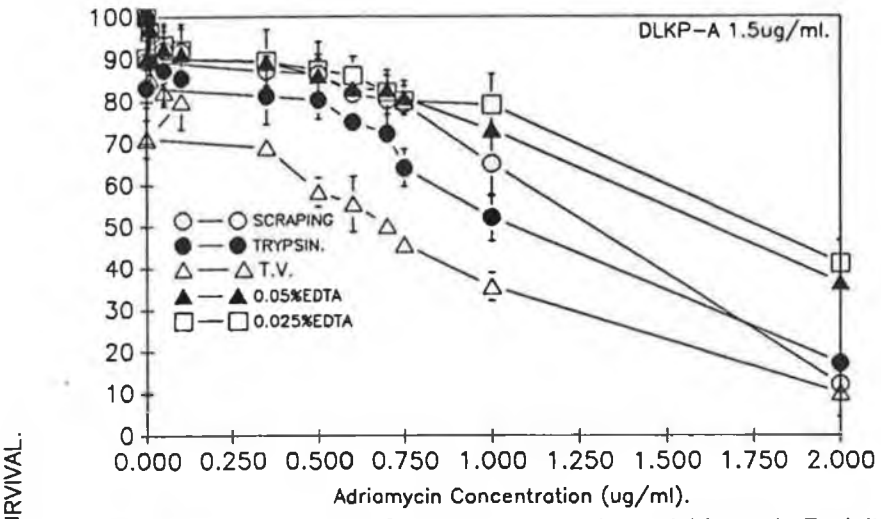
3.4.4. :Clonal Variation of SKMES-1 and SKMES1-A.



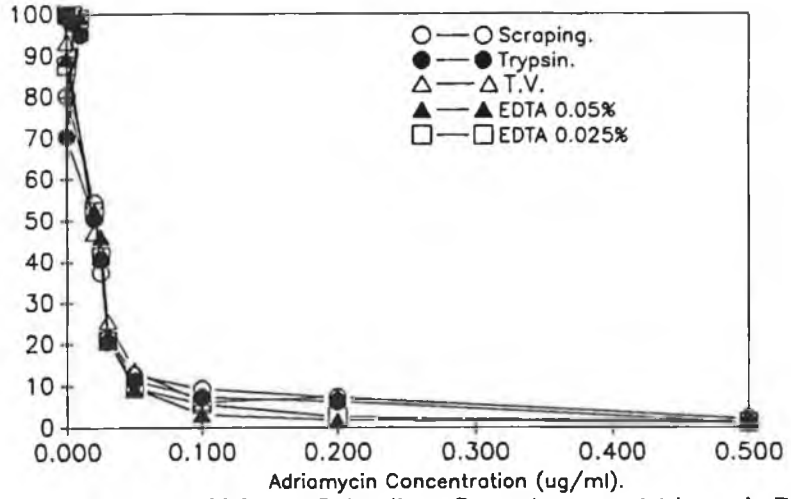
3.4.5. :Clonal Variation of SKLU-1 and SKLU1-A

3.5.1. Sensitivity of MDR Variants to Subculture Procedure.

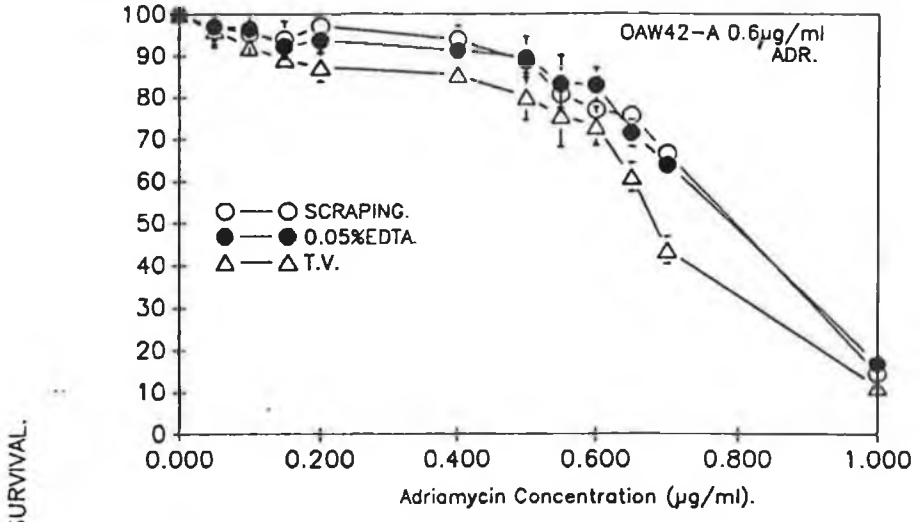
During the adaptation process, it was noticed that the cell being grown in drug took some time to recover from the standard Trypsin-EDTA subculture procedure. When this was replaced by scraping in PBS A, or by a very short treatment with Trypsin -EDTA or EDTA alone, cell growth in drug appeared to improve significantly. To investigate this phenomenon further each of the MDR variants was subcultured by different methods and a toxicity assay performed (Figures 3.5.1, 3.5.3, 3.5.5., 3.5.6., 3.5.7 and 3.5.8.). Additionally, control experiments were performed on DLKP and OAW42 to evaluate if this phenomenon is specific to MDR cells (Figure 3.5.2. and 3.5.4). The adriamycin toxicity curves for the parental cells were independent of subculture procedure, whereas in all the MDR variants tested subculture procedure affected adriamycin sensitivity. The extent of affect noted was very varied with the most pronounced noted in DLKP-A, HEP-2A and HEP-2B and a lesser affect noted with SKMES1-A, SKLU1-A and OAW42-A. Sub culture in 0.05% EDTA or 3 minute exposure to Trypsin/EDTA was the subculture method ultimately chosen for the MDR variants. The sensitivity of the MDR variants to T.V. is a possible indication of degradation of the membrane located P-170 glycoprotein with increased exposure to trypsin.



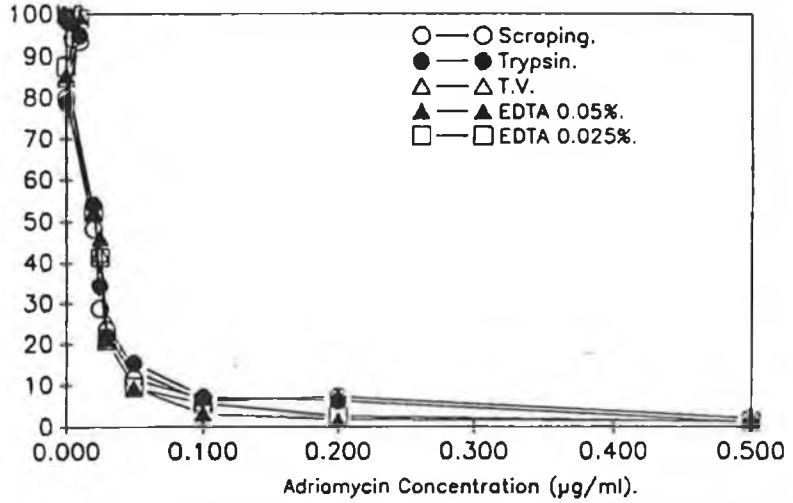
3.5.1.: Effect of Different Subculture Methods on Adriamycin Toxicity to DLKP-A Cells.



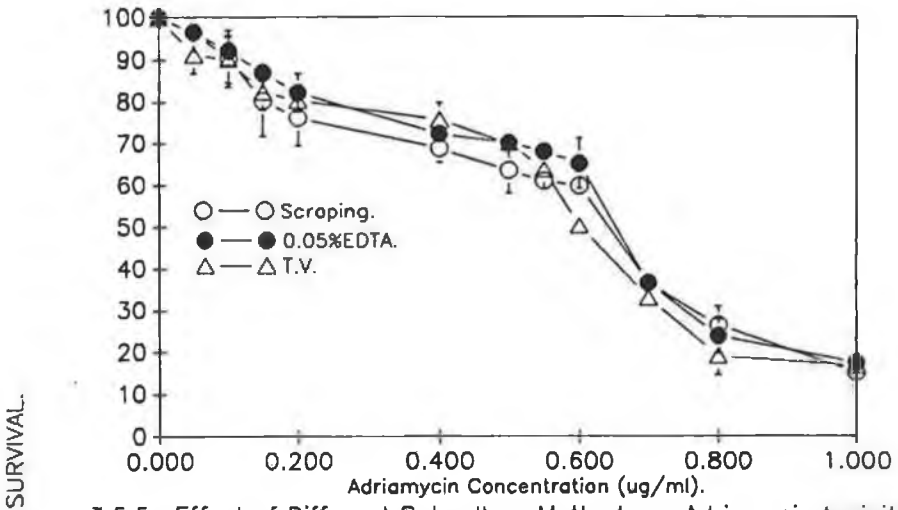
3.5.2. :Effect of Different Subculture Procedures on Adriamycin Toxicity to DLKP CEIls.



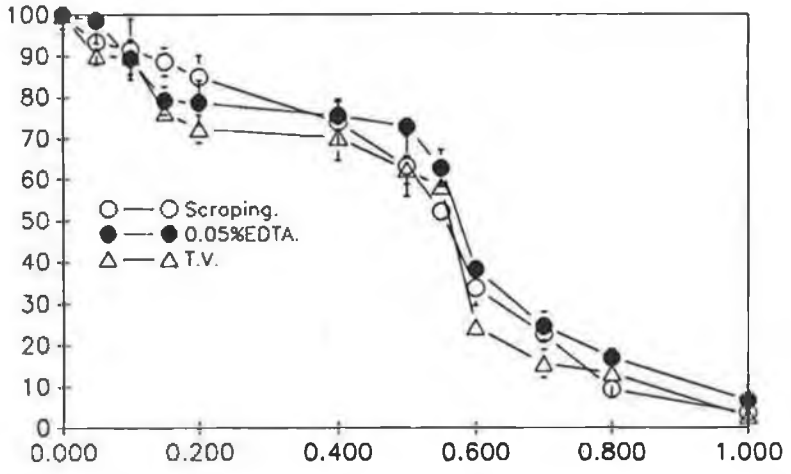
3.5.3. :Effect of Different Subculture Methods on Adriamycin Toxicity to OAW42-A cells.



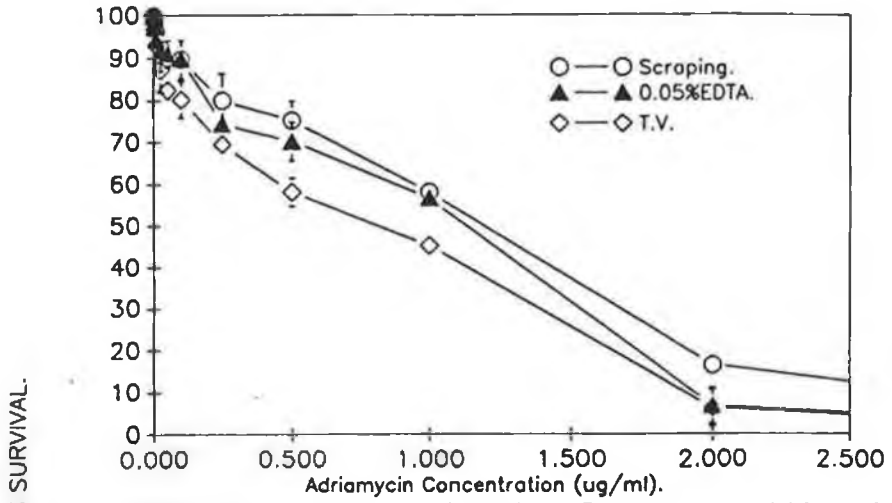
3.5.4.:Effect of Different Subculture Methods on OAW42 cells (P64).



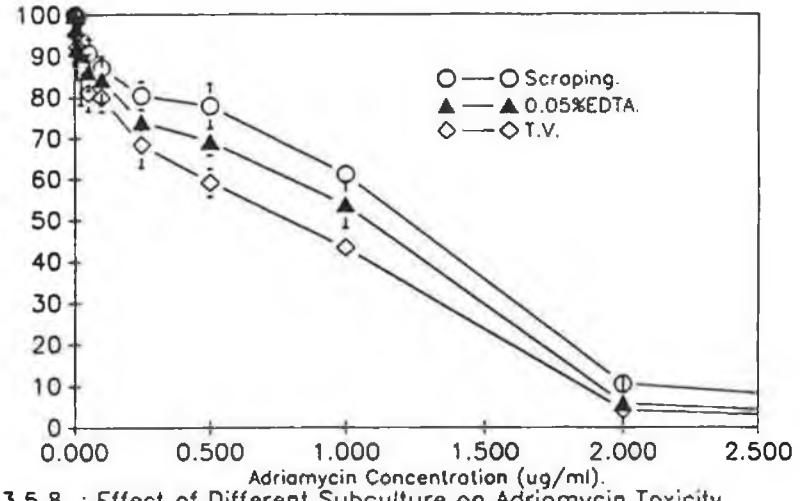
3.5.5. :Effect of Different Subculture Methods on Adriamycin toxicity To SKMES1–A Cells (0.55 ug/ml ADR).



3.5.6.:Effect of Different Subculture Methods on Adriamycin Toxicity to SLKU1–A (0.4ug/ml ADR).



3.5.7. : Effect of Different Subculture Procedures on Adriamycin Toxicity of HEP-2A (1.0ug/ml ADR).



3.5.8. : Effect of Different Subculture on Adriamycin Toxicity to HEP-2B(1.0ug/ml)

3.5.2. The Varying Sonication Required for MDR Variants and their Sensitive Counterparts.

During the preparation of the purified membrane fraction for Western blotting. It was noticed that the resistant variants were consistantly more resistant to sonication. During the sonication process microscopic examination was used to monitor cell lysis. The criteron for complete sonication was the lysis of majority of the cells, care must be taken not to over sonicate the cells as this can lead to membrane degradation. The time required for the resistant cells to lyse was definitely longer than there parental counterparts (See table 3.5.2 for details).

The increase in the sonication time and frequency required for all the MDR variants was noted in comparison to their respective parental cell lines. This observation is somewhat contradictory to our finding that MDR resistant cells are generally more sensitive to freezing and standard subculture procedures. The increase in sonication required for lysis of the resistant cells could be indicative of altered lipid composition of the resistant cells rendering them more mechanically sturdy rather than a P-170 related property.

Table 3.5.2. The Varying Sonication Required for MDR variants and their Sensitive Counterparts.

Cell Line	Sonication no.of pulses Hz
CHOK1	23 X -61
CHrC5	24 X -81
DLKP	10 X -81
DLKP-A	27 X -72
SKMES1	17 X -80
SKMES1-A	29 X -80
OAW42	13 X -70
OAW42-A	23 X -77
SKLU-1	13 X -67
SKLU1-A	25 X -71
HEP-2	18 X -85
HEP-2A	25 X -81
HEP-2B	26 X -82

3.6. Protein Analysis of P-170 by Western Blotting.

The presence of immunoreactive P-170 was investigated by Western blotting of purified membrane fractions of the MDR variants. The extraction procedure involved initial sonication to disrupt the cell membrane followed by differential centrifugation in order to obtain a purified plasma membrane fraction (see Section 2.12.1, figure 3.6.1.) One interesting observation was that the MDR variants required more sonication before cell lysis was obtained (See Table 3.5.2. for details). Immunoreactive P-170 expression was highest in CHrC5, DLKP-A (Figure 3.6.2) and SKMES1-A (Figure 3.6.3) with lower level expression in OAW42-A, HEP-2A and HEP-2B (Figure 3.6.4.). No expression of P-170 was noted in SKLU1-A (Figure 3.6.5). The presence of a second band was evident at 70KD M.W. in CHrC5. The significance of this band is unknown, it could signify the expression a related membrane glycoprotein or a degradation product. DLRP-A was not investigated.

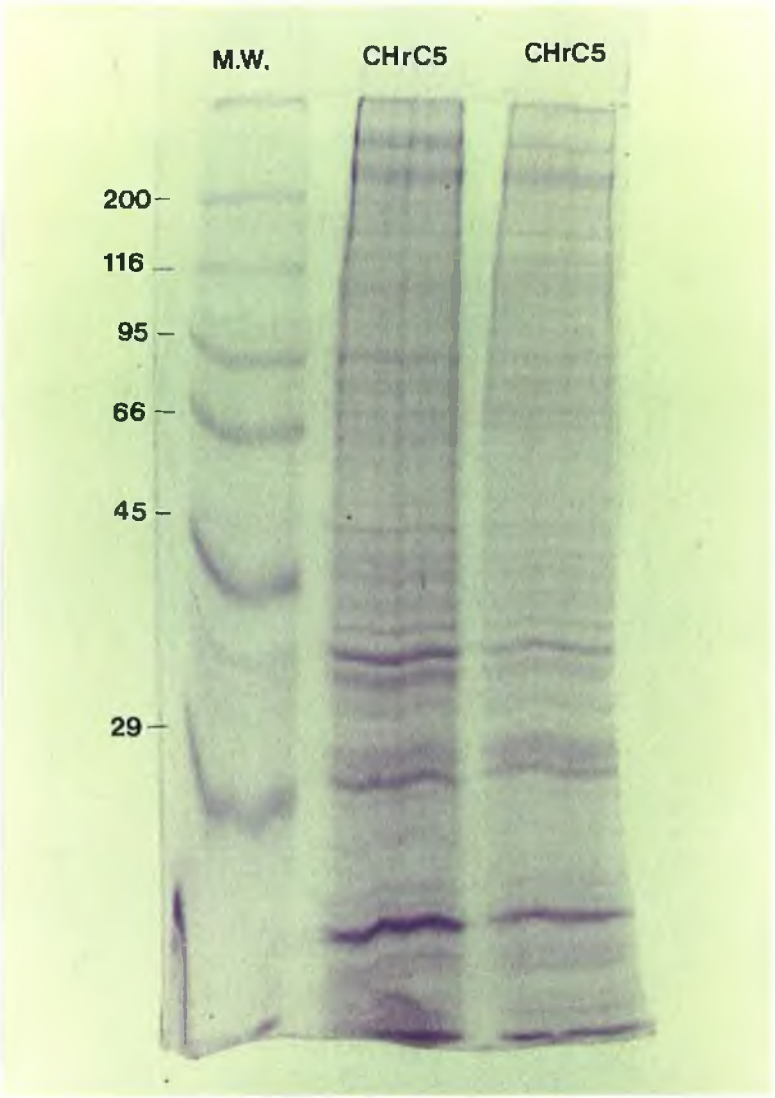


Figure 3.6.1. CHrC5 Membrane Proteins.

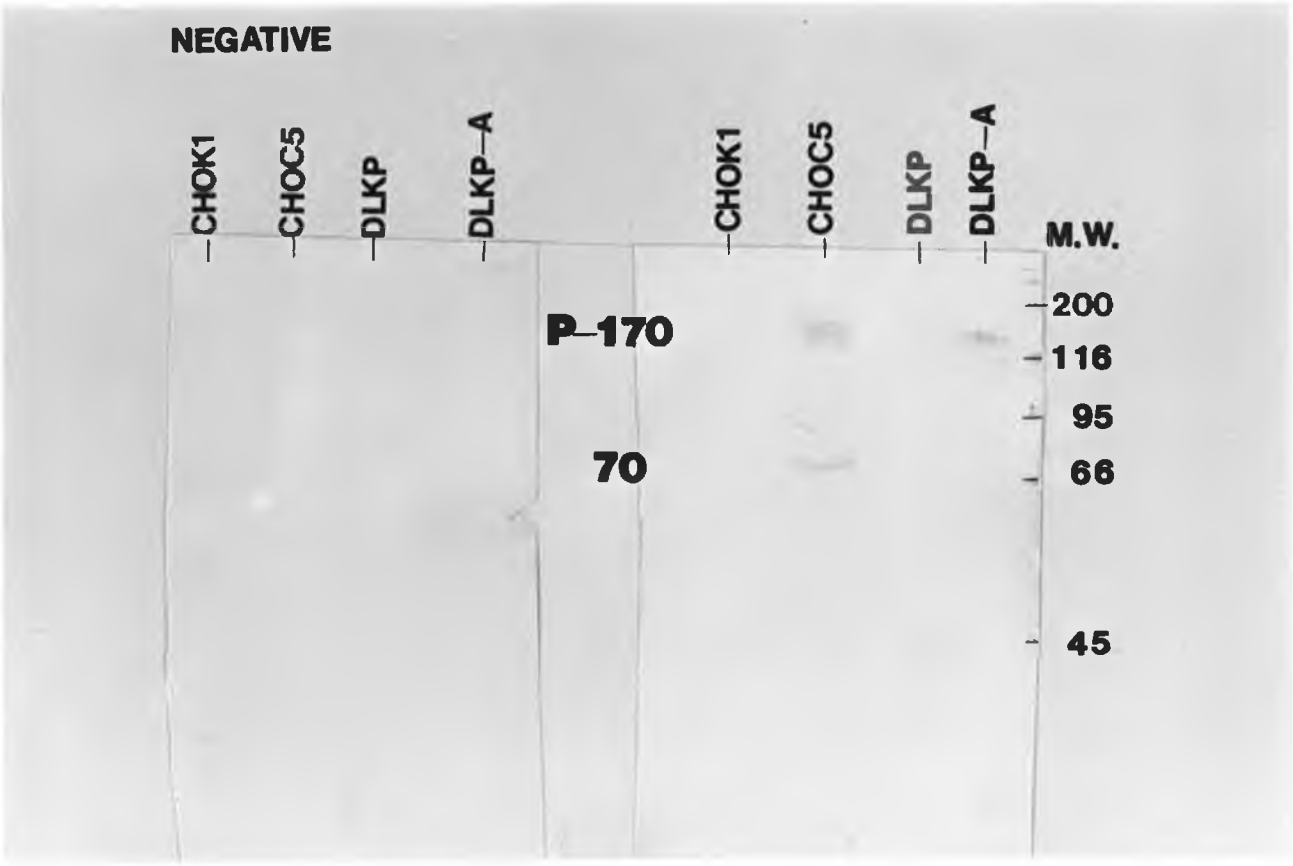


Figure 3.6.2. Western Blot CHOC5 (*), CHOK1, DLKP-A and DLKP.

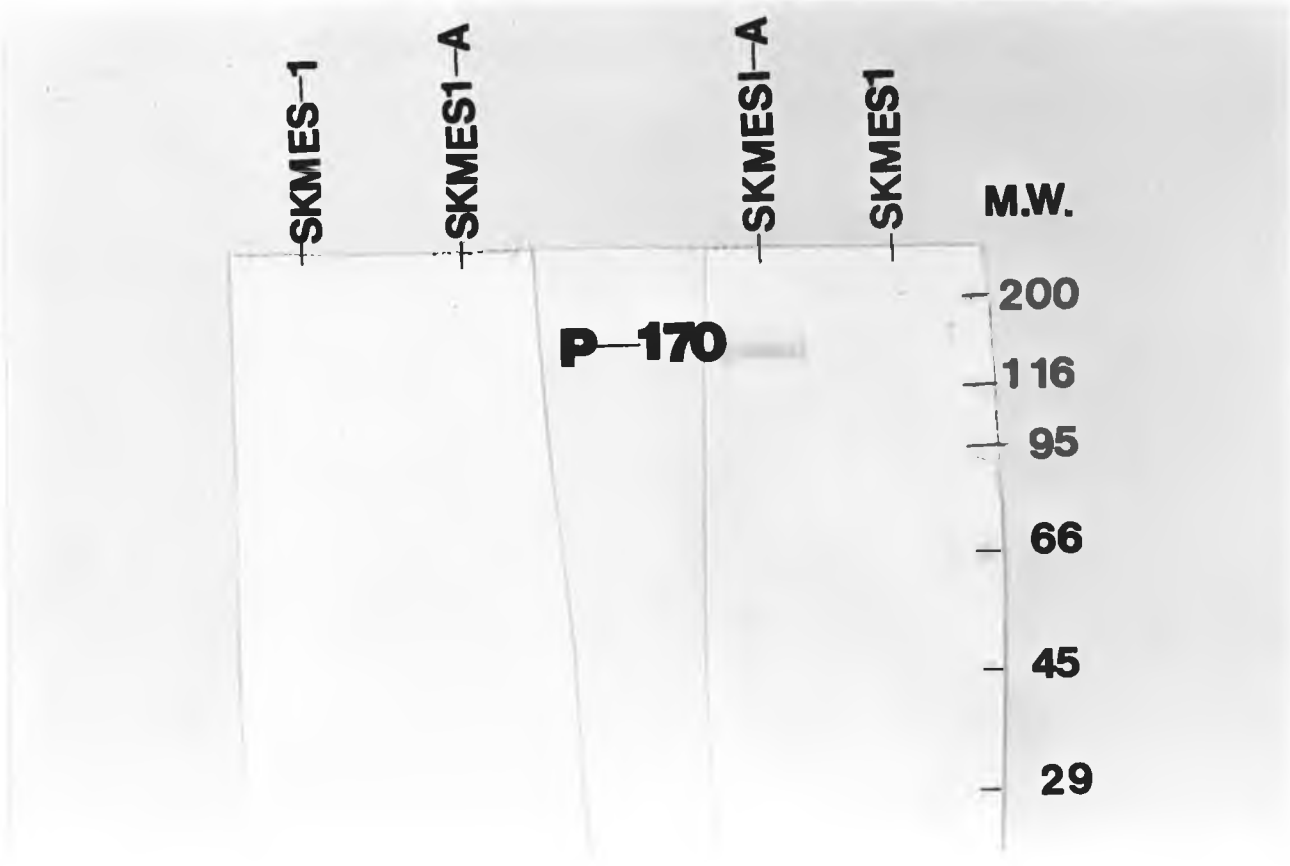


Figure 3.6.3. Western Blot of SKMES-1 and SKMES1-A.

(*) Correction : CHOC5 in Figure 3.6.2. should read CHrC5

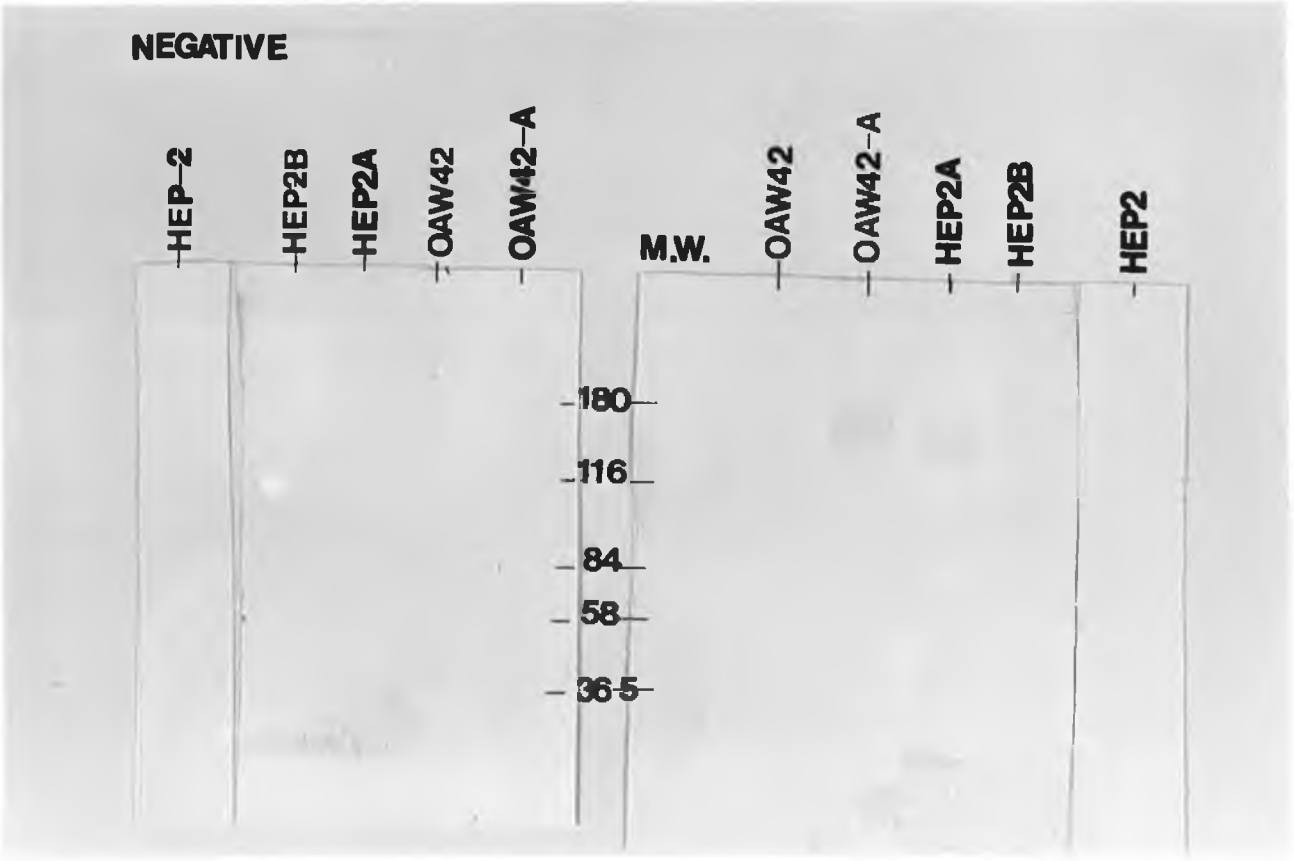


Figure 3.6.4. Western Blot of HEP-2, HEP-2A, HEP-2B,

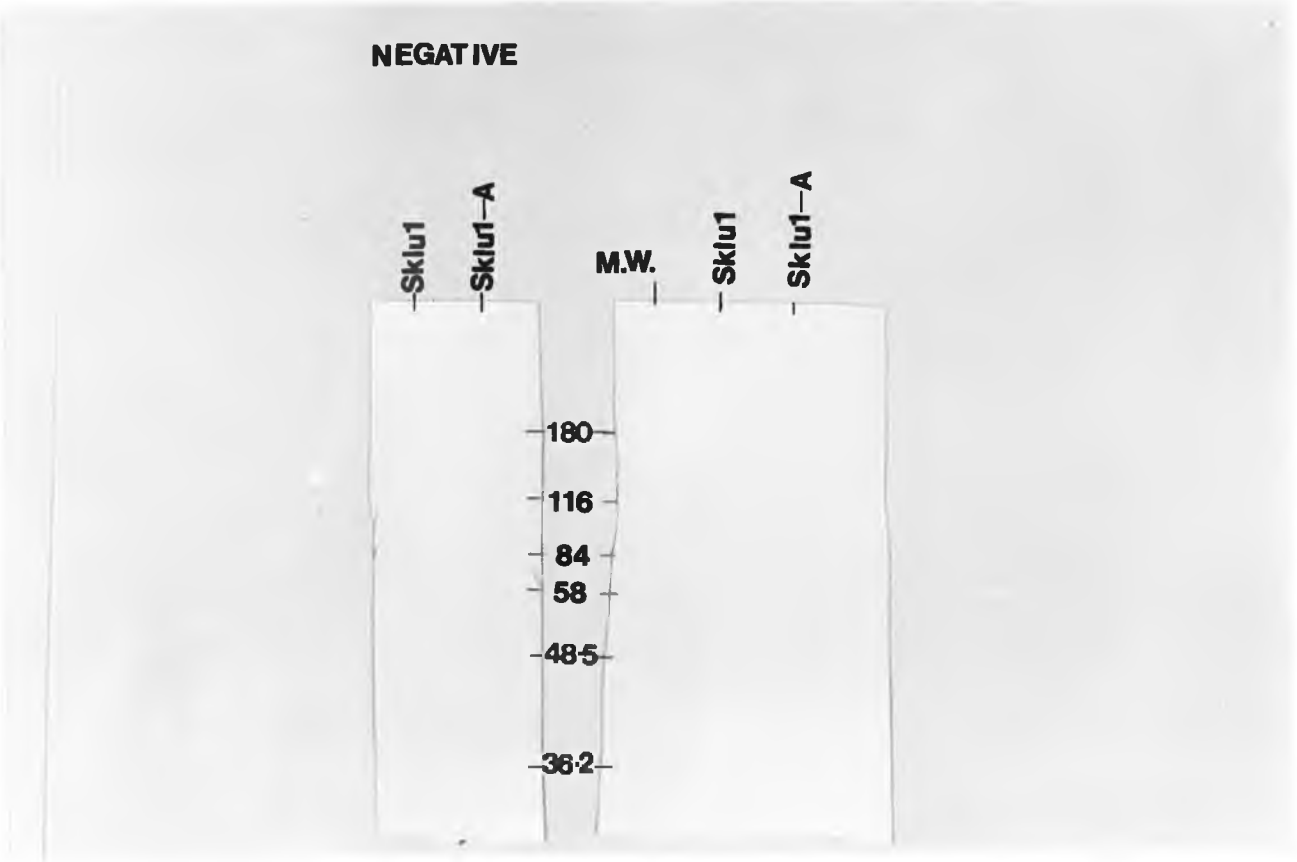


Figure 3.6.5. Western Blot of SKLU1 and SKLU1-A.

3.7. Immunohistochemical Detection of P-170.

The monoclonal antibody used for P-glycoprotein detection was C219 antibody (see tables 2.5 and 2.6 for details), which recognises an epitope lying in a cytoplasmic domain, 200 amino acids long, of the C-terminal region of the P-glycoprotein polypeptide. The positive control cell line used was CHrC5 (Figure 3.7.1). Three different patterns of staining was noted. Firstly, a pattern with numerous positively stained cells with fluorescence that appeared to be very even, was seen in CHrC5 and DLKP-A (Figure 3.7.2). Secondly, scattered positively staining cells indicating the presence of a very heterogeneous population with varied expression of P-170 was seen for SKMES1-A (Figure 3.7.4), HEP-2A (Figure 3.7.6), HEP-2B (Figure 3.7.7) and OAW42-A (Figure 3.7.8). Finally, no positive cells at all were seen in SKLU1-A. In initial immunohistochemical studies, photo-bleaching of the FITC fluorescence was a problem, this was overcome by the use of DABCO (1,4-diazobicylo-(2,2,2)-Octane) incorporated into the mounting buffer (Johnson et al.,1982). In the negative control slides of the parental cell lines, it was only possible to obtain photographs of DLKP (Figure 3.7.3.) and SKMES-1 (Figure 3.7.5.), the backround fluorescence was judged to negligible on the remaining parental cell lines.

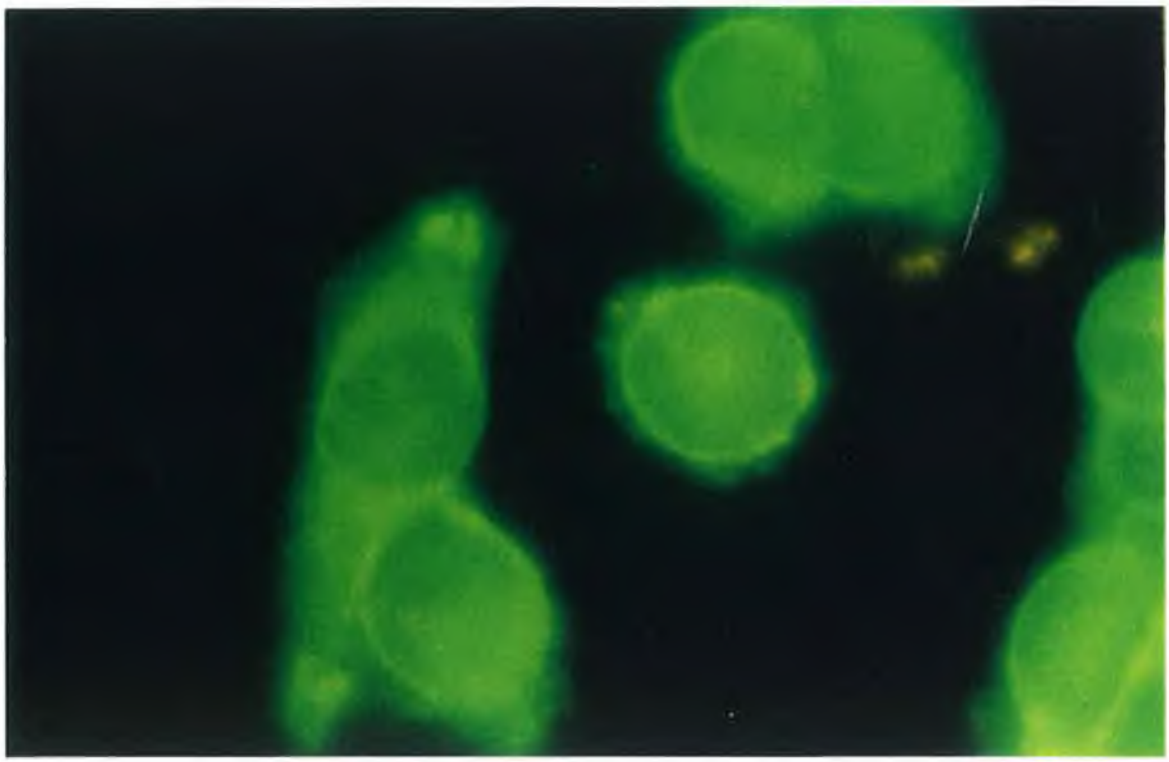


Figure 3.7.1. Immunofluorescence of CHrC5 Cell Line (X250)

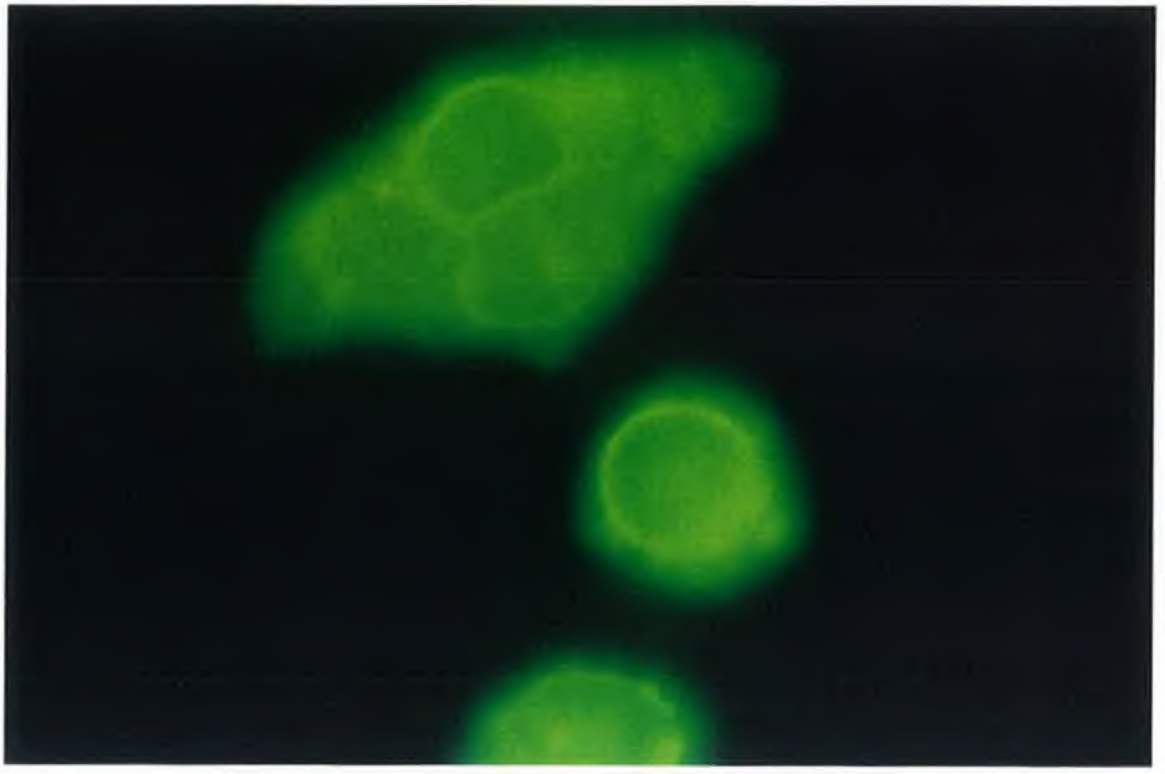


Figure 3.7.2. Immunofluorescence of DLKP-A Cell Line (X250)

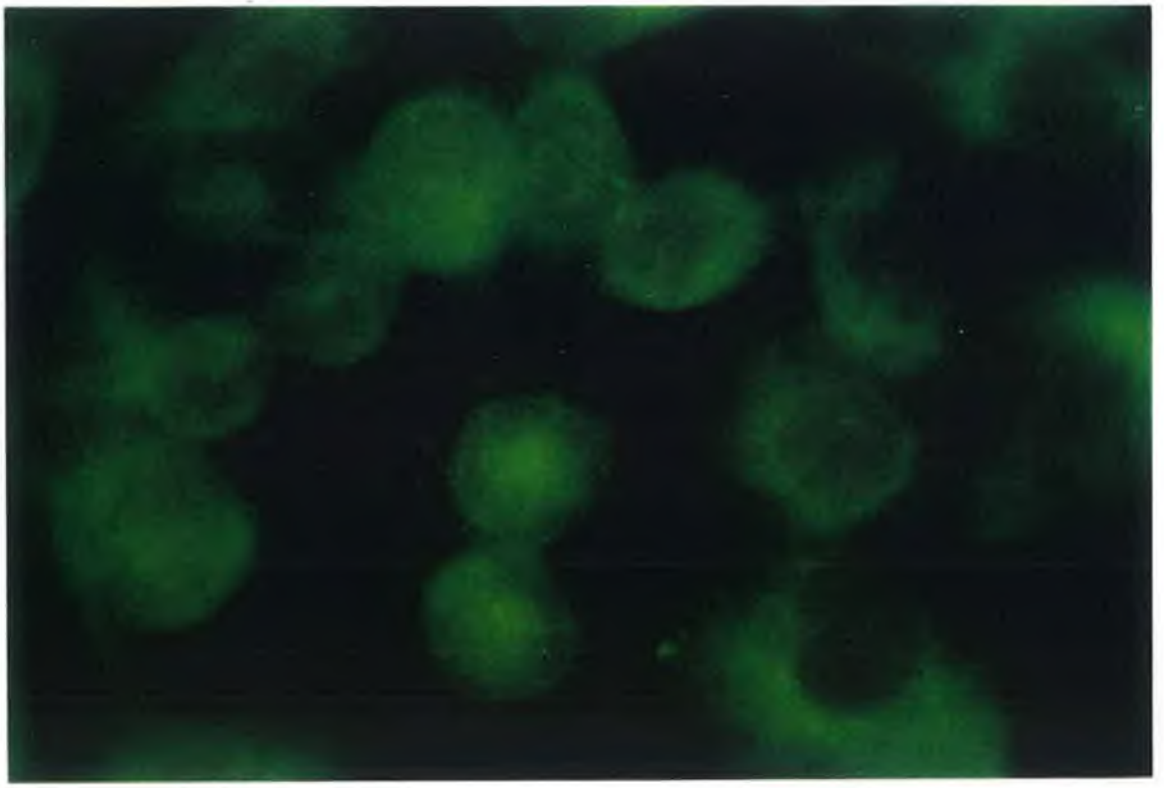


Figure 3.7.3. Immunofluorescence of DLKP Cell line (X250).

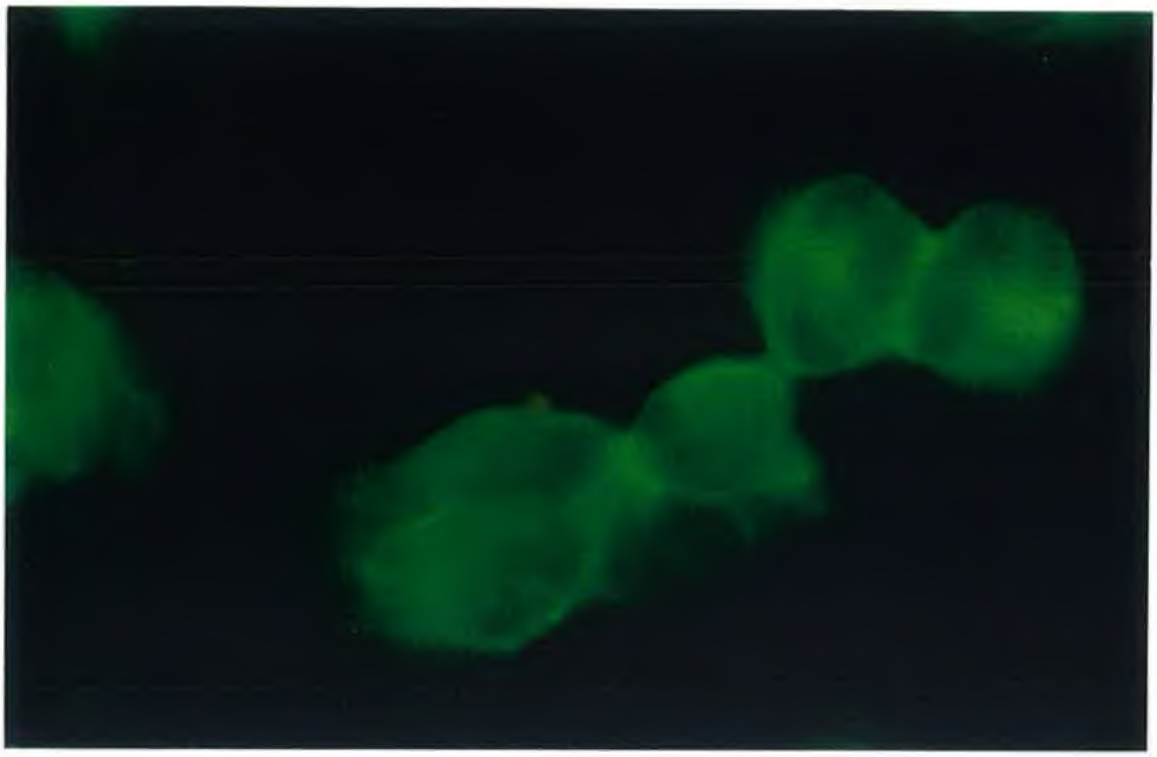


Figure 3.7.4. Immunofluorescence of SKMES1-A Cell Line (X250)

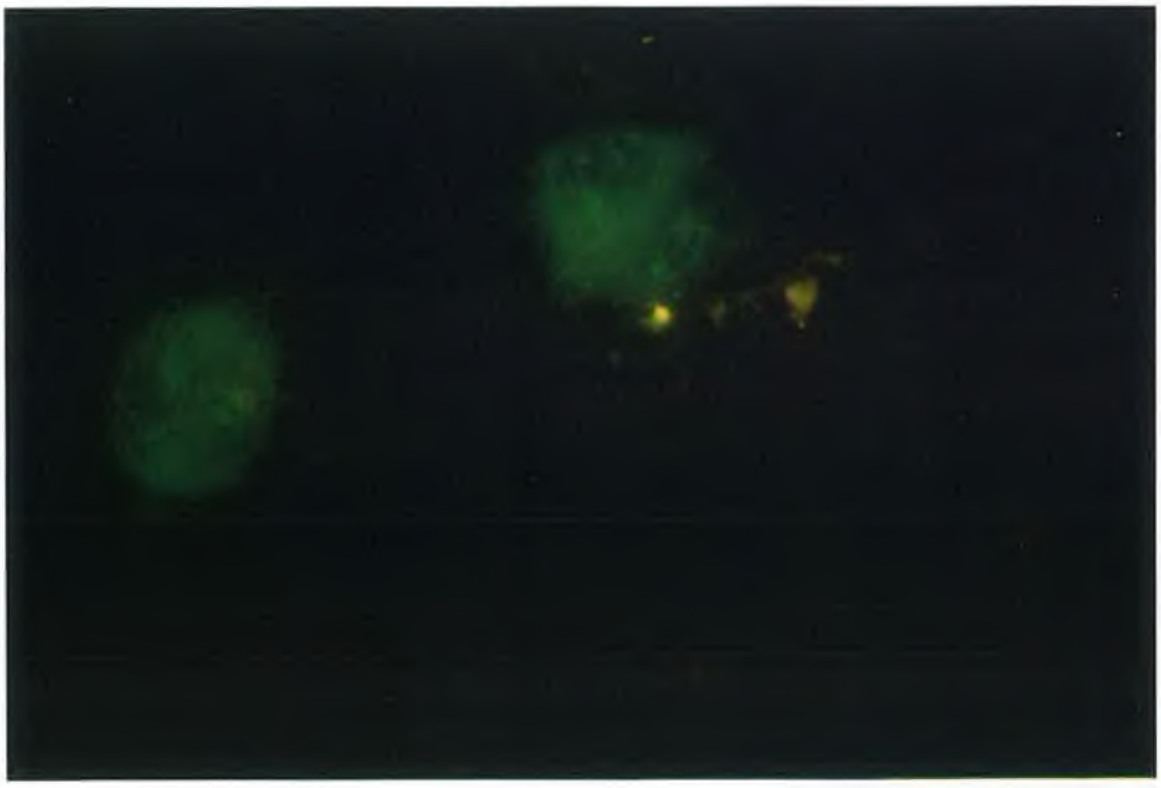


Figure 3.7.5. Immunofluorescence of SKMES1 Cell Line (X250)

Figure 3.7.6. Immunofluorescence of HEP-2A Cell line (X100)

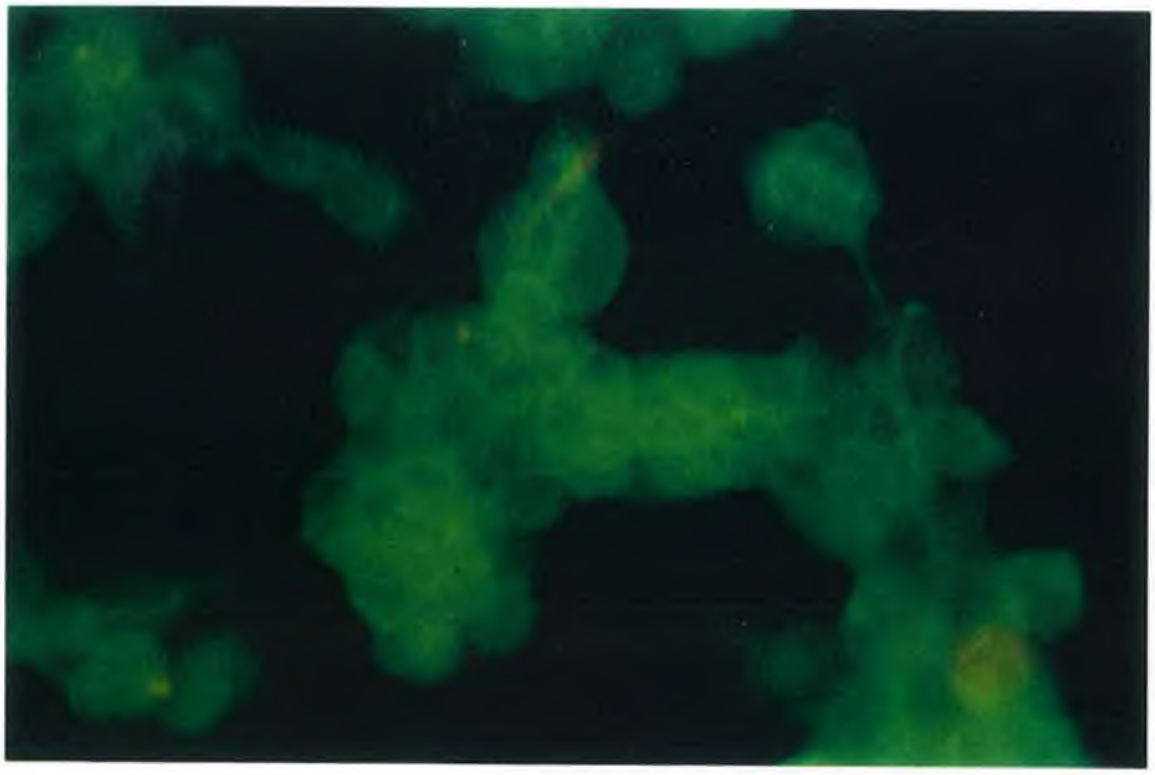


Figure 3.7.7. Immunofluorescence of HEP-2B Cell line (X100)

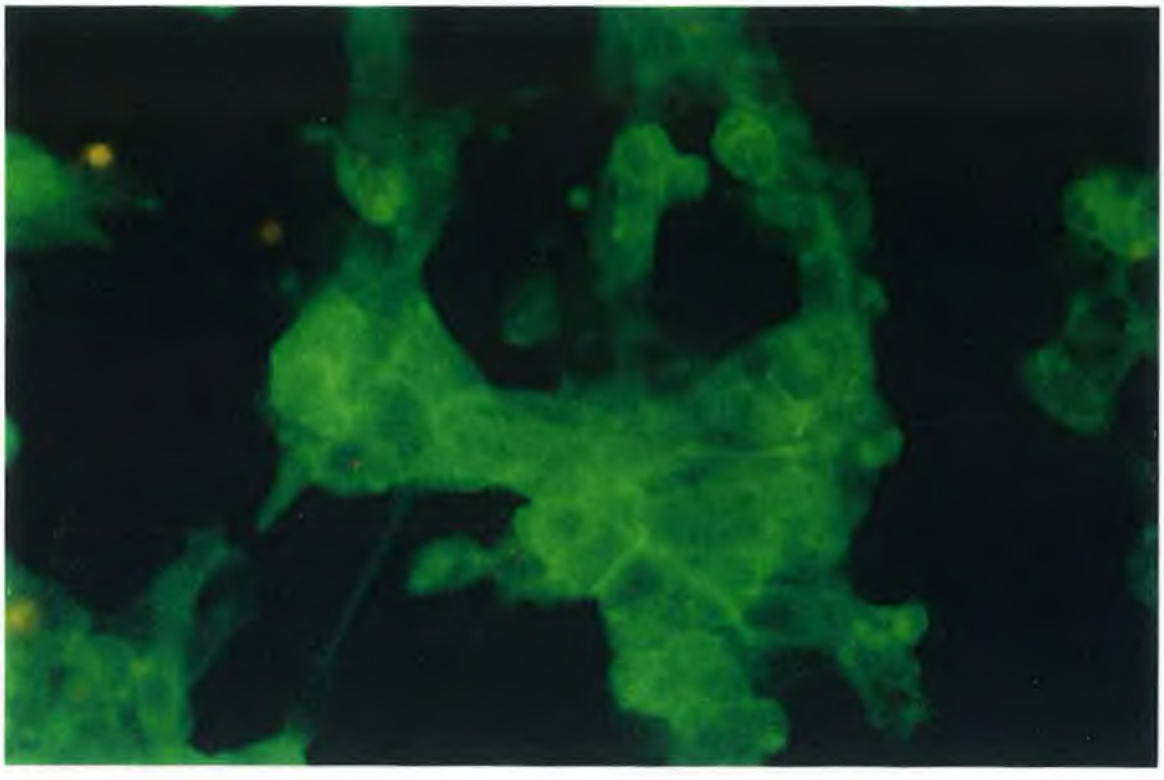


Figure 3.7.8. : Immunofluorescence of OAW42-A cell line (X100).

3.8. Cytogenetic Alterations in DLKP-A, HEP-2A and HEP-2B.

The presence of double minute (DM) chromosomes and homogeneously staining regions (HSRs) both indicative of gene amplification, are the most common cytogenetic mainfestation of MDR. Cytogenetic analysis was performed on three of the resistant cell lines and their parental counterparts, looking for the presence of double minute chromosomes. 100 cells were scored for each of the variants analysed type and the number of double minute chromosomes reported per metaphase spread analysed.

Additionally, on analysis of the slides a number of hugely abnormal spreads were encountered, with vast cytogenetic damage evident. The number of abnormal spreads noted per 100 metaphases is reported. (Note : No abnormal spreads were counted in the DM chromosome analysis)

Table 3.8. : Cytogenetic Evidence of MDR.

Cell Line	% of Cells With DMs					% of Cells with High degree of chromosome damage
	0	1	2	3	>4	
DLKP	97	3				2
DLKP-A	66	8	6	7	3	18
HEP-2	98	1	1			1
HEP-2A	53	34	8	1	4	23
HEP-2B	61	28	7	1	3	29

Double Minute chromosomes are a definite cytogenetic feature of DLKP-A (Figure 3.8.2), HEP-2A (Figure 3.8.5) and HEP-2B (Figure 3.8.7.), with little evidence of DM present in either of the parental cell lines DLKP (Figure 3.8.1) and HEP-2 (Figure 3.8.4). A ring chromosome was noted in DLKP-A (Figure 3.8.2) in addition to double minute chromosomes, it has been reported that ring chromosomes signify a transition stage between DM chromosomes and

HSR's (Barker et al.,1982). In HEP2-A (figure 3.8.5) a large number of DMs are evident in addition to a long chromosome, it is thought that this could be a HSR, further work with G-banded preparations is required to ascertain if HSRs are a definite karyotypic feature of HEP-2A.

A large number of abnormal chromosomal spreads were noted for all the resistant variants analysed (Table 3.8.1), 18 for DLKP-A, and 23 and 29 respectively for HEP-2A and HEP-2B. An extended period of time in hypotonic solution in order to achieve nuclear lysis, 45 minutes for DLKP-A, 35 minutes for HEP-2A and HEP-2B as opposed to 25 minutes for the parental cell lines. This indicates an alteration in the composition of the nuclear membrane.

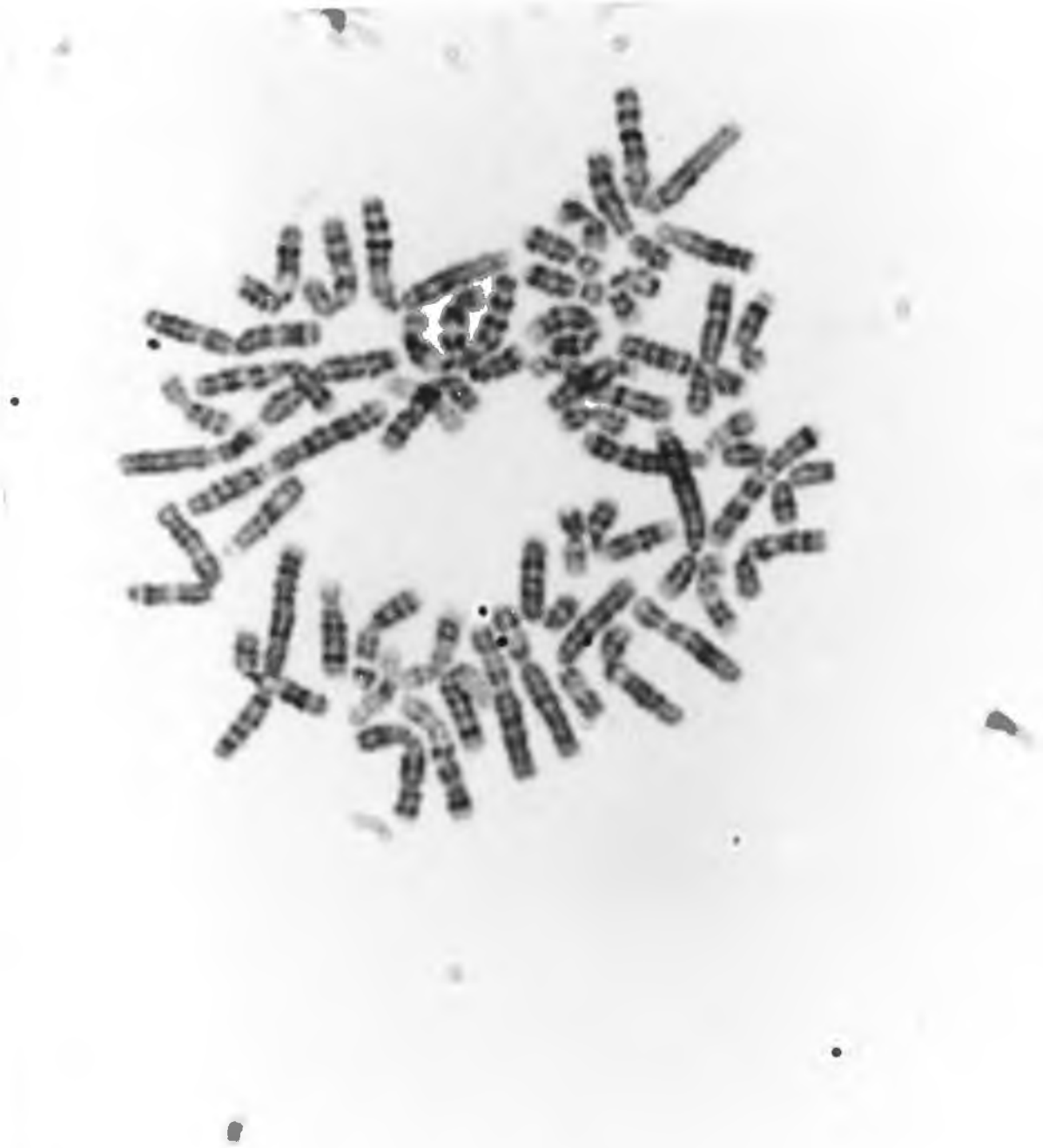


Figure 3.8.1. G-Banded DLKP Metaphase Spread.

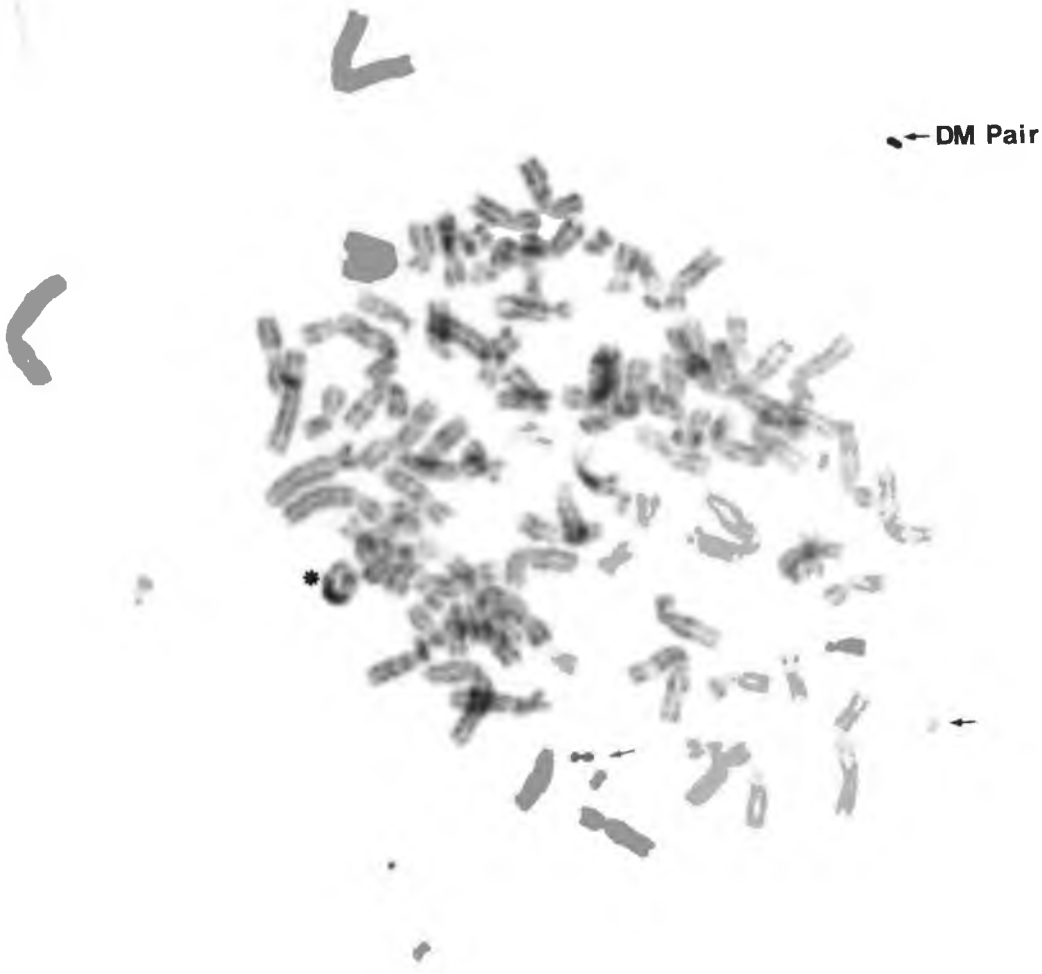


Figure 3.8.2. DLKP-A Metaphase Showing the Presence of DM Chromosomes and a Ring Chromomsome(*).

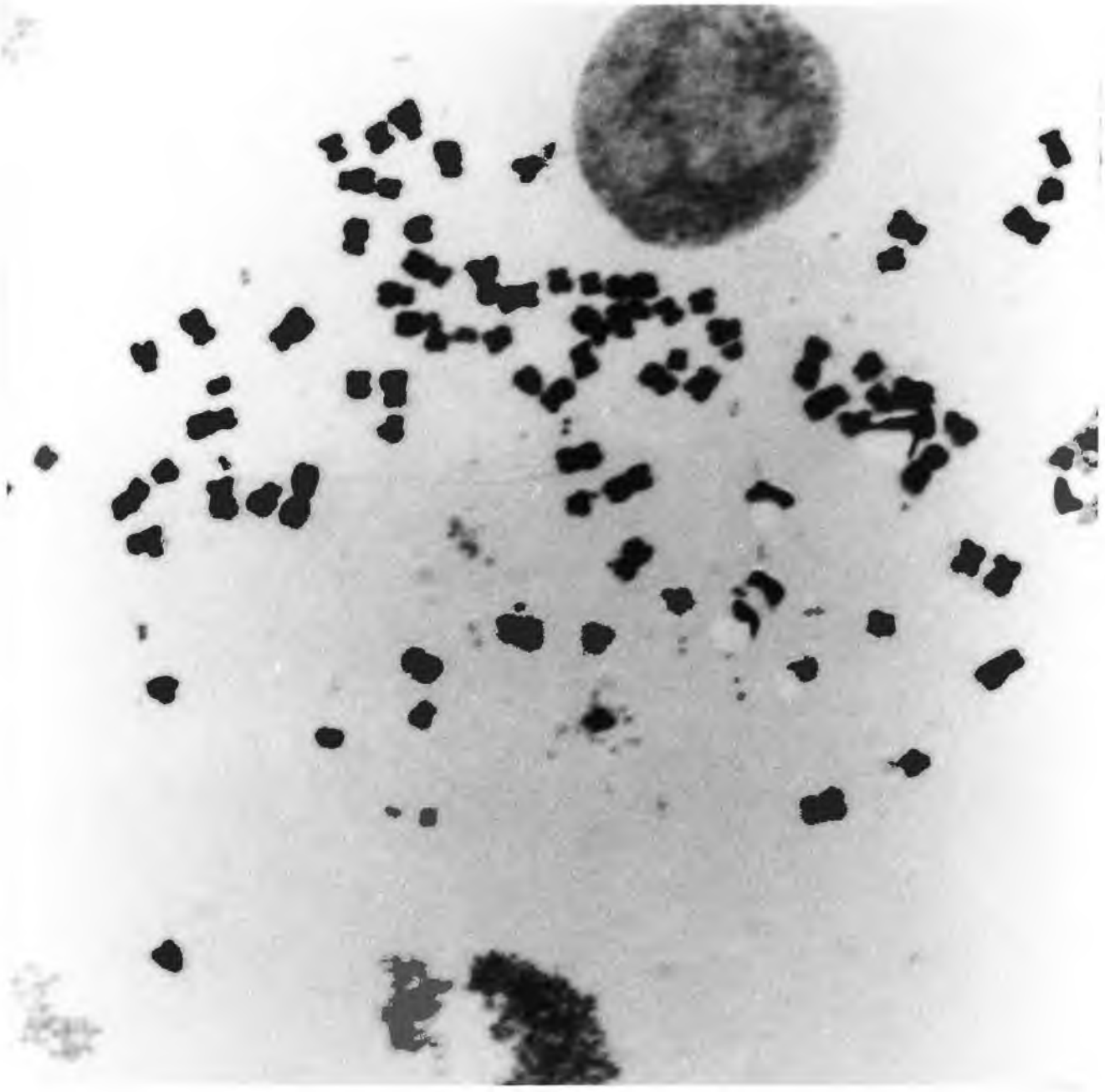


Figure 3.8.3. DLKP-A Abnormal Metaphase, Spread Showing Numerous DMs, Abnormal Breakages and Chromosome Condensation.

Figure 3.8.4. HEP-2 Metaphase spread.

Figure 3.8.5. HEP-2A Metaphase Showing Numerous DMs Ascentric Fragments and HSR.

Figure 3.8.6. HEP-2A Abnormal Metaphase Spread Showing Chromosome Condensation and Chromosome Breakage.

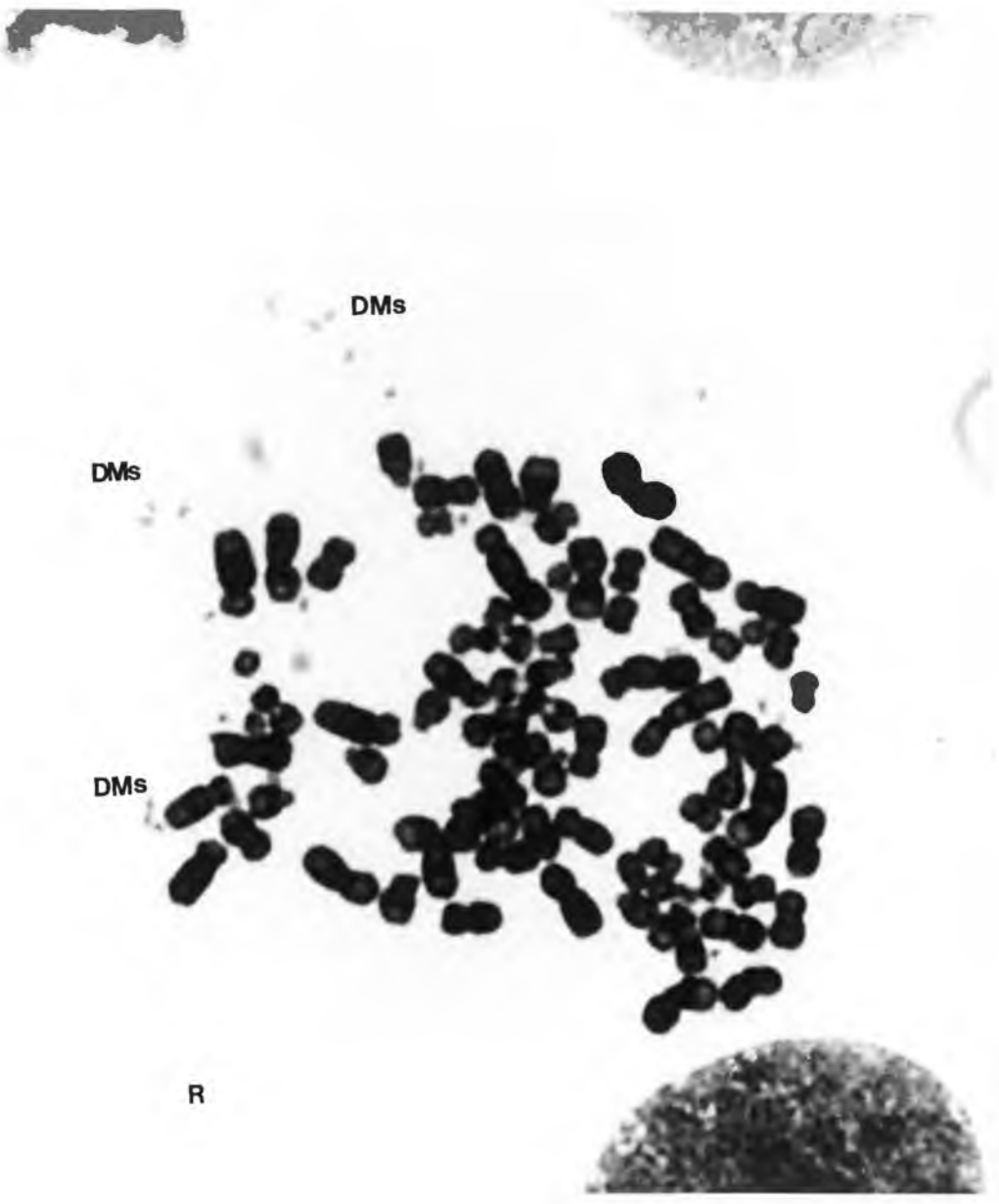


Figure 3.8.7. HEP-2B Metaphase Spread Showing Numerous Faintly Stained Double Minute Chromosomes.

Figure 3.8.8. HEP-2B Abnormal Metaphase Spread With Ascentric Fragments Chromosome Condensation and Chromosome Breakage.

3.9. Optimization of Transfection Techniques.

A number of transfection techniques were optimised with a view to transfection of the MDR phenotype, with the pHAMDR1a vector which encodes for the complete cDNA for the mdr1 gene and also with genomic DNA from a number of resistant cell lines. The techniques studied were that of $CaPO_4$ alone and in the presence of a number of facilatators which included chloroquinine, DMSO and PEG 6000. Also the novel transfection procedure involving polybrene/DMSO treatment was investigated in addition to electroporation. Other parameters included the investigation of the required concentration of DNA necessary for transfection.

3.9.1a. Investigation of the Effect of DNA Concentration on Transfection Frequency into Different Recipient Cell Lines.

Varying DNA concentration in the $CaPO_4$ method of transfection (Table 3.9.1a) shows that with an increase in DNA concentration a subsequent increase in transfection frequency occurs. The ease at which the mouse cell lines (LTK^- and A9) are transfected in comparison to human tumor cell lines is clear. On repeating of this experiment higher concentrations of DNA were included to ascertain if higher transfection frequencies were achievable,the results are shown in table 3.9.1b.

A very large, dense $CaPO_4$ precipitate was noticable at the higher concentrations of DNA (40 and 50μg), thus use of concentrations of DNA this high is not suitable for transfection purposes. It was decided that a total concentration of 20μg of DNA was suitable for further transfection experiments as firstly the precipitate was the correct texture and the transfection frequencies were quite high. A general requirement for the calcium phosphate procedure to be efficient, is the precipitate must be fine and opaque, this was an observation by Debenham et al.(1982). Two factors are thought to determine the quality of the precipitate, the quantity of the DNA present and the pH of the TE buffer used in the preparation of the $CaPO_4$ precipitate. Table 3.9.1c. summarises an experiment to investigate the optimum pH of the TE buffer. The pH of the TE was varied from 6.9-7.5 then a $CaPO_4$ precipitate was formed as described in section 2.11.1. and the precipitate examined microscopically. The optimum pH of the TE buffer pH 7.0-7.1. The number of transfectants was scored by the staining of the plates with 0.25% crystal violet after the relevant selection time (See section 2.11.6), and then manually counting the number of stained colonies.

Table 3.9.1a. Investigation of the effect of DNA concentration of PLW4 plasmid, on transfection efficiency into different recipent cell lines.

Cell line DNA Conc.μg	HEP-2	SW1088	LTK$^-$	A549	A9
1	7.5±2.1	4.1±0.9	14.2±0.9	0	15.6±0.6
5	15.6±0.6	9.7±0.8	17.8±1.6	4.9±0.2	17.2±2.3
10	17.4±1.8	13.2±2.6	24.1±3.7	9.3±1.6	——
15	18.7±2.3	13.9±0.9	45.2±4.5	9.9±2.6	19.4±1.3
20	21.3±3.3	12.4±1.7	64.6±2.1	11.5±2.3	——

n=5 ; Data =no.of tranfectants per 10^6 cells/100mm^2 culture dish ± S.D.

Table 3.9.1b. Effect of the concentration of PLW4 DNA on transfection frequency.

Cell line DNA conc.μg	HEP-2	SW1088	LTK$^-$	A549	A9
5	5.7±1.2	0	14.6±1.4	1.2 ± 1.6	——
10	14.3±2.4	3.3 ±1.6	23.5±2.3	8.9 ± 1.2	3.4 ± 1.3
20	23.4±0.9	6.3 ±0.9	46.2±0.8	9.7 ± 0.9	9.8 ± 2.6
40	15.6±0.6	5.6 ±0.5	42.3±0.7	6.3 ± 0.7	——
50	13.3±1.4	2.4 ±1.2	15.6±1.6	3.12± 1.9	1.2 ± 1.6

n=5 ; Data =No.of transfectants 10^6cells/100mm^2 culture dish ± S.D.

Table 3.9.1c. Investigation of the effect of the pH of TE buffer on the formation of the $CaPO_4$ precipitate.

pH of TE	Microscopic observations
6.9	Very fine crystals, small clusters
7.0	Crystals slightly larger, opaque
7.1	Crystals still fine, opaque
7.2	Crystal size increasing, opaque
7.3	Medium sized crystals, opaque
7.4	Large crystal visible with naked eye
7.5	coarse large crystals, uneven lumps

3.9.2. Transfection using $CaPO_4$ Plus Chloroquinine.

Chloroquinine (a lysosomotropic agent) + $CaPO_4$ has been proven to enhance the transfection frequency above $CaPO_4$ alone. It has been found that 30% kill due to chloroquinine is necessary to see enhanced efficiency of transfection. A toxicity assay was performed on a number of cell lines to acertain the concentration of chloroquinine to cause 30% kill.

Table 3.9.2a Experiment to establish concentration of chloroquinine required for transfection.

Cell Line	Chloroquinine Concentration IC30
RPMI-2650	180μg/ml
HEP-2	124μg/ml
LTK-	173μg/ml
SW1088	163μg/ml

n=3

3.9.2b. Transfection of pSV2NEO by $CaPO_4$ plus chloroquinine.

Cell Line / Transfection	RPMI-2650	HEP-2	LTK^-	SW1088
$CaPO_4$	17.6±2.3	19.2±0.7	47.2±3.2	12.4± 2.3
$CaPO_4$ +CHQ	28.4±1.73	25.6±2.5	U.C.>100	13.6±2.5

n=3 ; Data=No.of transfectants 10^6cells/100mm^2 ± S.D.
U.C.= uncountable.

Chloroquinine enhanced the transfection frequency substantially particularly in the case of LTK^-.

3.9.3. Transfection of pSV2NEO and PLW4 by $CaPO_4$ Plus DMSO.

DMSO has been found to increase transfection frequency. As with chloroquinine a certain degree of cell kill is reported to be necessary before DNA is transfected efficiently, 10% in the case of DMSO. A preliminary experiment showed that treatment of any of the cell lines with 30% DMSO solution for 4 minutes produced approximately 10% kill. In the literature DMSO has been used both before and after the $CaPO_4$ precipitate has been added. Both these parameters were investigated.

Pre-treatment of the cells before transfection with 30% DMSO proved to be a very effective transfection procedure for all the cell lines and the two plasmids tested. It was a much more effective strategy than treatment with DMSO after transfection.

Table 3.9.3. Transfection of pSV2NEO and PLW4 by $CaPO_4$ plus DMSO.

Cell line / Transfection	RPMI	LTK-	HEP2	A549
pSV2NEO $CaPO_4$	14.3±2.3	45.6±3.6	21.1±2.1	16.7±2.9
pSV2NEO B.T. $CaPO_4$+30%DMSO	41.2±4.7	U.C. >100	29.2±2.9	29.2±3.5
pSV2NEO A.T. $CaPO_4$+30%DMSO	33.2±1.2	77.5±4.2	33.1±3.7	25.2±3.9
PLW4 $CaPO_4$	32.1±3.4	49.8±5.6	27.5±4.5~~	18.22±2.9
PLW4 B.T. $CaPO_4$+30%DMSO	45.2±2.9	94.2±2.3	36.7±3.9	23.2±6.7
PLW4 A.T. $CaPO_4$+30%DMSO	29.2±3.4	65.2±2.8	29.4±2.1	24.1±3.9

n = 3 for all experimental data, but for ~~ n =2.
Data = No. of transfected colonies 10^6 cells/100mm^2 dish ± S.D.
B.T. = Before Transfection
A.T. = After Transfection

3.9.4. Transfection of pSV2NEO with the PEG-6000 and $CaPO_4$ Procedure.

The mechanism of action of PEG 6000 is unknown but it is thought that it perturbs the cell membrane on several levels. It has been suggested that PEG may induce various structural changes in the bulk liquid phase adjacent to membrane surfaces. It has found been to be an extremely effective method of transfection (Sutherland et al.,1984). A preliminary experiment showed that PEG 6000 at a concentration of 0.5g/ml is suitable for transfection purposes. As with DMSO a concentration which yielded about 10% kill is necessary for transfection.

Peg 6000 was found to substantially increase the tranfection frequency of the human cell lines (RPMI, HEP-2 and A549) and for the mouse cell line LTK^-, over $CaPO_4$ alone. The results obtained for each of the plasmids PLW4 and pSV2NEO were found to be very similar.

Table 3.9.4. Investigation of the effect of Peg 6000 on $CaPO_4$ transfection frequency.

Cell Line / Transfection	RPMI-2650	LTK-	HEP-2	A549
$CaPO_4$ pSV2NEO	18.2±2.1	45.1±2.7	22.2±1.4	18.2±1.9
$CaPO_4$ + PEG 6000	59.3±2.6	U.C.	63.4±0.7	35.1±2.3
$CaPO_4$ PLW4	22.1±4.6	49.1±2.9	25.8±1.8	27.2±4.7
$CaPO_4$ + PEG 6000	53.1±6.1	U.C.>150	65.1±4.5	41.2±3.4

n=3 ; data = no.of transfectants per 10^6 cells/100mm^2 dish ± S.D.

3.9.5. Transfection with Polybrene/DMSO.

Transfection with polybrene/DMSO has been found an effective procedure for the transfection of a number of cell lines. The optimal concentration of polybrene and DMSO in combination needed to be ascertained, 10% kill was required to achieve an enhanced transfection effect. The results of a preliminary experiment to optimize concentration of polybrene/DMSO are summarised in table 3.9.5a.

The efficiency of transfection with the Polybrene/DMSO procedure appears to be less than with $CaPO_4$ + Peg 6000, however its efficiency is higher than just $CaPO_4$ alone.

Table 3.9.5a Optimisation of Polybrene/DMSO concentration required for transfection.

Polybrene: DMSO μg/ml : %	Cell line IC10
10 : 10	A549
20 : 30	LTK^-
30 : 10	RPMI
20 : 30	HEP-2

These conditions were then used in the transfection of pSV2NEO.

Table 3.9.5b Investigation of the frequency of Transfection with Polybrene/DMSO compared to $CaPO_4$.

Cell line / Transfection	RPMI-2650	LTK-	HEP-2	A549
$CaPO_4$	18.6±0.9	37.9±2.6	19.4±1.2	12.7±2.7
Polybrene/ DMSO	24.1±2.6	97.4±9.1	38.4±3.4	14.2±1.8

n=3; Data=No. of transfectants per 10^6cells/100mm^2 dish ± S.D.

3.9.6a. Transfection of pSV2NEO by Electroporation.

The technique of electroporation has been developed to a large extent in the past three years (Potter et al.,1984). This technique is a lot less cumbersome than any of the transfection technique highlighted so far in this study. It involves the perturbation of the cell membrane by an electric current, allowing for the uptake of DNA. In the initial experiments a range of voltages and capacitances were studied. These experiments were performed in 25cm^2 flasks seeded at 5X10^5 cells per flask.

Table 3.9.6a Effect of varied voltage on transfection frequency of pSV2NEO at fixed capacitance of 25μF.

Cell Line / Voltage	HEP-2	DLKP	CHOK1	SKMES1	SKLU1
500	25±1.2	17±2.3	33±4.6	18±3.2	9±3.4
750	37±2.5	35±3.4	72±3.9	39±3.4	14±2.5
1000	52±3.5	61±7.1	U.C.>100	41±4.5	21±3.7
2000	48±3.9	69±6.1	U.C.>100	49±2.7	16±2.4

n=3; Data=Number of transfectants per 5X10^5 cells/25cm^2 flask ±S.D.

For all the cell lines increased voltage up to 1000V resulted in increased number of transfectants.

3.9.6b. Effect of Capacitance on the Number of Transfectants.

It has been found that voltage affects the levels of transfection; this experiment was performed to investigate if increase in capacitance either affects cell survival or transfection efficiency. A preliminary experiment showed that varying of capacitance from 0 to 25μF did not cause any alteration in cell survival for CHOK1, NIH3T3, DLKP, HEP2 and LTK^- (data not shown). The next experiment was performed to ascertain if an affect on transfection efficiency was noted. This experiment was performed using 10^6 cells in $100mm^2$ culture dishes with pSV2NEO the transfected plasmid at 1500 voltage, 25μF capacitance.

Table 3.9.6b Effect of capacitance on transfection frequency.

Cell Line Capacitance μF	CHOK1	NIH3T3	DLKP	HEP-2
1	36±2.8	39±2.5	16±2.9	21±2.6
5	37±3.9	43±4.1	19±2.4	19±3.5
25	39±2.7	40±2.7	19±3.1	22±1.8

n=3 ;Data=Number of transfectants per 10^6 cell/$100mm^2$ dish ± S.D.

Capacitance does not affect the efficiency of transfection for any of the cell lines tested.

3.9.7. Transfection of Genomic DNA.

A number of experiments were performed to optimise the transfection of genomic DNA with a view to transfection of genomic DNA from resistant cell lines. Co-transfection was the procedure adopted whereby the genomic DNA was transfected in the presence of plasmid DNA. The procedure of co-transfection allows for an internal standard within each experiment so that if plasmid DNA is taken up it is an indication that the genomic DNA has been also. Transfection of genomic DNA is much more difficult because the DNA must be integrated into the chromosome into a suitable position before expression can occur.

It can be seen from table 3.9.7 that it is possible to get stable expression of genomic DNA from mouse to a human recipient looking for expression of ouabain resistance. However, regardless of which procedure was attempted it was not possible to transfect genomic DNA encoding MDR, (resistance to adriamycin). The reasons for this are unknown, possibly an incorrect selection time was being used in these experiments. Judging by the transfection results from the plasmid DNA for those experiments, it seems as if genomic DNA was taken up but not expressed, probably due to insertion of the genomic DNA in a unsuitable chromosomal position, or DNA degradation before insertion.

Table 3.9.7. Summary of Genomic Transfections

DNA	Recipient Cell Line	Transfection Procedure	Selection Results per 10^6 cells.
1.RPMI+pSV2NEO	RPMI	$CaPO_4$	Geneticin $23/10^6$
2.RPMI+pSV2NEO	A9	$CaPO_4$+PEG 6000	Geneticin $0/10^6$ HAT $0/10^6$
3.RPMI+pSV2NEO	A9	Polybrene/ DMSO	Geneticin $0/10^6$ HAT $0/10^6$
4.A9 +pSV2NEO	RPMI	$CaPO_4$	Geneticin $10/10^6$ Geneticin+ Ouabain $6/10^6$
5.A9 +pSV2NEO	RPMI	$CaPO_4$+PEG 6000	Geneticin $30/10^6$ Geneticin+ Ouabain $11/10^6$
6.LTK$^-$+pSV2NEO	RPMI	$CaPO_4$	Geneticin $35/10^6$ Geneticin+ Ouabain $12/10^6$
7.LTK$^-$+pSV2NEO	RPMI	$CaPO_4$+PEG 6000	Geneticin $45/10^6$ Geneticin + Ouabain $30/10^6$
8.LTK$^-$+pSV2NEO	RPMI	Polybrene/ DMSO	Geneticin $29/10^6$ Geneticin+ Ouabain $21/10^6$
9.CHrC5+pSV2NEO	LTK$^-$	$CaPO_4$+PEG 6000	Geneticin $44/10^6$ Geneticin+ADR (0.1μg/ml) $0/10^6$
10.CHrC5+pSV2NEO	LTK$^-$	Polybrene/ DMSO	Geneticin $41/10^6$ Geneticin+ADR 0.075μg/ml $0/10^6$
11.CHrC5+pSV2NEO	LTK$^-$	Electroporation 1000 V 25 μF	Geneticin $61/10^6$ Geneticin+ADR 0.075μ/ml $0/10^6$
12.CHrC5+pSV2NEO	NIH3T3	$CaPO_4$+Peg 6000	Geneticin $56/10^6$ ADR(0.075 μg/ml) $0/10^6$
13.CHrC5+pSV2NEO	NIH3T3	Electroporation 2000 V 25 μF	Geneticin $58/10^6$ ADR(0.075μg/ml) $0/10^6$
14.CHrC5+pSV2NEO	NIH3T3	Electroporation 1500 V 25 μF	Geneticin $42/10^6$ ADR (0.075μg/ml) $0/10^6$

3.9.8. Transfection of pHAMDR1a Plasmid Encoding the full cDNA for the mdr1 Gene.

pHAMDR1a encodes the full cDNA for the mouse mdr1 gene (Mc Lachin et al.,1990). This was transfected into NIH3T3, HEP-2 and DLKP with a view to comparing the multidrug resistance profiles of a transfected gene and a selected cell line. The initial experiments were done on NIH3T3 cell line to ascertain the concentration of colchicine required to start the selection, the preselection period required and the optimal transfection technique.

Table 3.9.8a. Experiment to estimate optimal voltage for transfection of NIH3T3 at constant capacitance of 25μF.

VOLTAGE (V)	NIH3T3 % SURVIVAL
0	100 ±1.3
0 (ice)	98.2 ±0.7
50	96.2 ±1.9
100	96.5 ±2.4
500	94.3 ±2.4
750	81.2 ±1.7
1000	69.2 ±1.6
1500	68.4 ±2.3
2000	49.2 ±1.9
2500	36.2 ±2.7

Voltage to be used in subsequent experiments 1000 V or 1500 V.

3.9.8b. Experiment to Estimate Optimal Transfection of pHAMDR1A into NIH3T3.

The aim of this experiment was to establish the correct concentration of colchicine to use for selection of transfectants (to eliminate backround), and to ascertain the preselection time required for pHAMDR1A (Table 3.9.7).

At a concentration of colchicine of 5-15ng/ml the backround number of colonies was too high, 20 ng/ml colchicine was chosen as a suitable concentration for initial selection of transfectants. Transfection frequency very found to be very low . Three different transfection procedures were investigated to try and increase transfection frequency, $CaPO_4$ + PEG 6000, $CaPO_4$ + Chloroquinine and electroporation at 2000V, 25μF Capacitance.

Table 3.9.8b. Investigation of optimal transfection of pHAMDR1a with NIH3T3 as the recipent cell line.

Colchicine Conc. (ng/ml)	Control no Plasmid	Preselection Time (Hours)				
		0	24	48	72	96
5	63 ±5.2	61±2.8	72±2.7	69±2.1	--	--
10	60 ±4.8	65±4.9	75±7.5	68±2.1	79±2.1	72±2.1
15	16 ±2.1	19±3.8	25±2.4	21±2.4	20±2.1	24±3.4
20	0	3±2.6	11±1.2	9±1.2	12±0.5	11±2.1
30	0	0	0	0	0	0

n=3; Data=Number of transfected colonies depending on preselection time and colchicine concentration, ± S.D.

3.9.9. Investigation to Increase Transfection Frequency, and Obtain Transfectants for Toxicity Assays.

Transfection frequency was found to be very low for experiment 3.9.8b. A number of different transfection procedures were investigated to try to increase transfection frequency, $CaPO_4$ + PEG 6000, $CaPO_4$ + Chloroquinine and electroporation at 2000V, 25μF Capacitance. This experiment was in triplicate in 75 cm^2 flasks;one was stained and counted (the results are presented in Table 3.9.8) the other two were grown up further for use in toxicity assays.

Table 3.9.9. Investigation to Increase Transfection Frequency, and to Obtain Transfectants for Toxicity Assays.

Transfection Method	Transfection frequency NIH3T3 /10^6 cells.
CaPO4 +PEG 6000 2g/ml	35
CaPO4 + Chloroquine 150μg/ml	41
Electroporation 2000V, 25μF	62

n=1; Data=Number of transfectants per 10^6cells/75cm^2 flask.

Electroporation at 2000V proved to be the most efficient, even though initial cell death was 50%. Electroporation at 2000V, 25μF was the transfection procedure used for the transfection of HEP-2 and DLKP. The transfection frequencies were 26/10^6 cells for HEP-2 and 31/10^6 cells for DLKP. Cells were grown to varying concentration of colchicine resistance (For details of selection procedure see section 2.11.6.) and their drug resistance profiles analysed. HEP-2 transfectants were analysed at 40ng/ml colchicine

resistance, DLKP tranfectants at 50ng/ml (Tables 3.9.12. and 3.9.13.), and NIH3T3 at 30ng/ml and 50ng/ml resistance (Tables 3.9.10. and 3.9.11.). Results are presented in the form of IC50 nM. It is noted that with a increase in resistance to colchicine that a concomitant increase in resistance to each of the test compounds is observed. The highest fold resistance was noted for adriamycin, vincristine, colchicine and VP16, with low level resistance to 5-FU and cis-platin. Both HEP-2pHa40 and DLKPpHA50 showed diverse patterns of resistance, with DLKPpHA50 showing marked resistance to adriamycin, 39.62 fold resistance as compared to 4.04 fold resistance for HEP-2pHA40. In addition DLKP-A was much more resistant to vincristine 33.58 fold compared to 3.5 fold for HEP-2pHA50. Resistance to the other test compounds was quite similar with substantial resistance to VP16 noted for both the cell lines. The cross resistant profiles of the three transfected cell lines are not at all comparable. This is possibly indicative of the fate of the pHAMDR1A DNA on entering the cells and the number of copies of pHAMDR1a integrated into the genome. In addition point mutations in the P-170, caused by colchicine exposure may give rise to altered patterns of resistance.

Table 3.9.10. Cross resistance patterns of NIH3T3 NIH3T3pHA30 and NIH3T3pHA50.

Cell line / Drug	NIH3T3	NIH3T3 pHA30	NIH3T3 pHA50
COLH	10.82±1.034	223.8±41.9	317±41.14
ADR	95.05±12.77	2047.2±214	4047±367.8
VCR	2.75±0.36	27.1±4	33.9±0.39
5-FU	949.5 ±476	1633.1±135	3163±328.4
C-PT	899.2 ±33.35	2216.6±83.9	6228±299.4
VP16	67.97±8.49	1563.1±50.9	2208±169.9

n=2 ; Data= IC50nM, mean ± S.D.

Table 3.9.11. Fold resistance of the NIH3T3pHA30 and NIH3T3pHA50 relative to the parental control.

Cell Line / Drug	NIH3T3pHA30	NIH3T3pHA50
COLH	20.7	29.29
ADR	21.53	42.59
VCR	9.85	12.3
5-FU	1.72	3.332
C-PT	2.47	6.93
VP16	22.99	32.48

Table 3.9.12. Cross Resistance Patterns of HEP-2pHA40 and DLKPpHA50.

Cell line / Drug	HEP-2	HEP-2pHA40	DLKP	DLKPpHA50
COLH	24±1.55	190±7.2	26±3.34	276±10.9
ADR	22±1.86	89±11.4	27±0.52	1070±103
VCR	1.13±0.17	4±0.73	1.31±0.14	44±4.36
5-FU	4022±569	15779±5019	8032±557	22819±3728
C-PT	1233±133	2433±300	1399±333.5	4000±300
VP16	144±3.6	3150±286.4	119±8.1	2693±438

n=2 ;Data =IC50nM, Mean ± S.D.

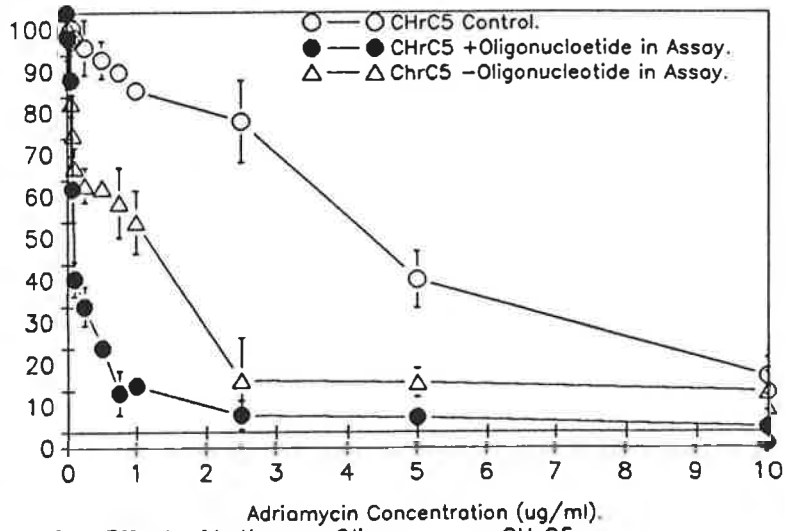
Table 3.9.13. Fold Resistance of HEP-2pHA40 and DLKPpHA50 Relative to the parental Controls.

Cell line / Drug	HEP-2pHA40	DLKPpHA50
COLH	7.9	10.6
ADR	4.04	39.62
VINC	3.5	33.58
5-FU	3.9	2.85
C-PT	1.97	2.8
VP16	21.87	22.6

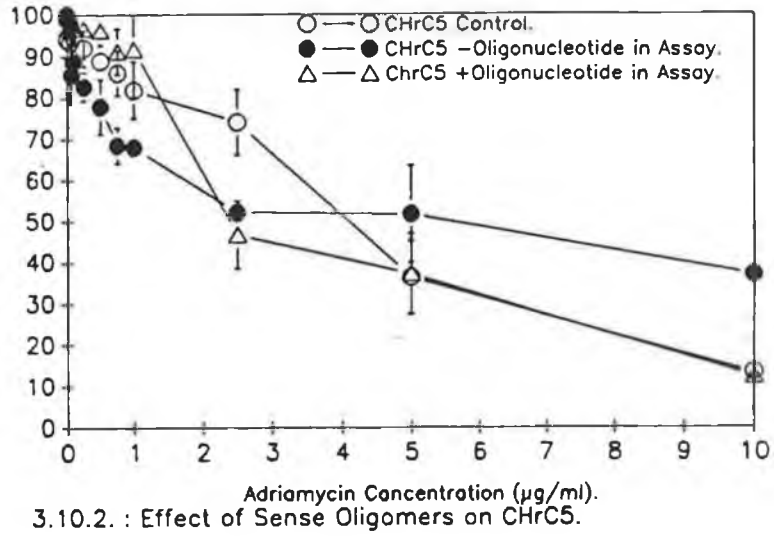
3.10. Transfection of Antisense and Sense Oligonucleotides.

A direct role for the evaluation of the role of P-170 overexpression as a mechanism of resistance was studied by the transfection of antisense and sense oligonucleotides, using an 18mer (for details see section 2.11.7.) which codes for 18 bases down stream of the mdr1 promoter. After culturing for three days in the presence of oligomers, the expression of P-170 was assessed by an adriamycin toxicity assay (4 Day); the stability of the altered sensitivity was ascertained by the assaying in the presence and absence of oligomer.

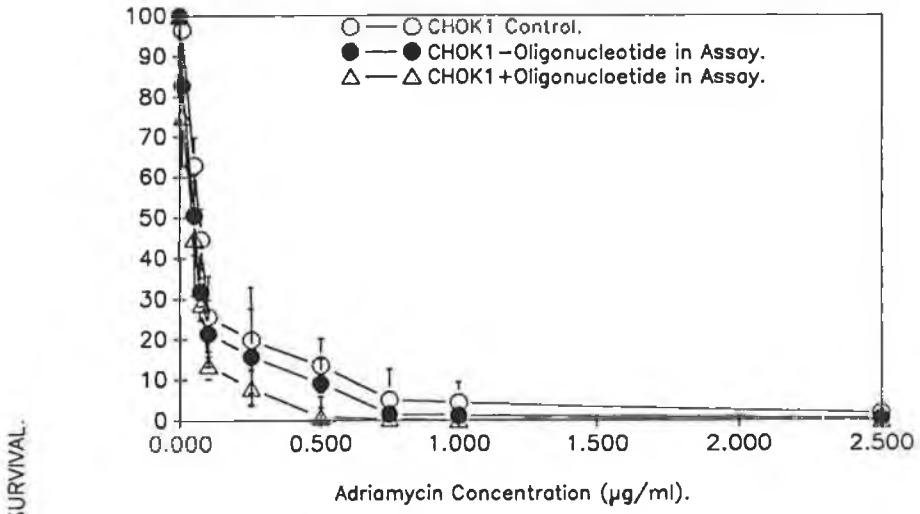
No change in adriamycin resistance was seen after culture with sense oligomers for any of the resistant variants , for example figure 3.10.8 DLKP-A. Only one of the parental cell lines cultured in the presence of antisense oligomer showed a decrease in IC50 SKMES1-A (Figure 3.10.9) possibly indicating the presence of low level P-170 expression in the parental cell line. In all the MDR variants tested putative reduced expression of P-170 protein (measured as increased adriamycin sensitivity) with antisense oligomers was noted, but to markedly varying degrees. The greatest degree of altered resistance was noted for CHrC5 (Figure 3.10.1.), the resistant control cell line. A reduction in IC50 by 3-4 fold was noted for DLKP-A (Figure 3.10.7), SKMES1-A (Figure 3.10.11), HEP-2A (Figure 3.10.17) and HEP-2B (Figure 3.10.19). Reduced resistance but to a lesser extent, was noted in OAW42-A (Figure 3.10.13), with a reduction in IC50 of approximately 1.6 fold. Only a very slight alteration in the IC50 of SKLU1-A (Figure 3.10.15) was noted.



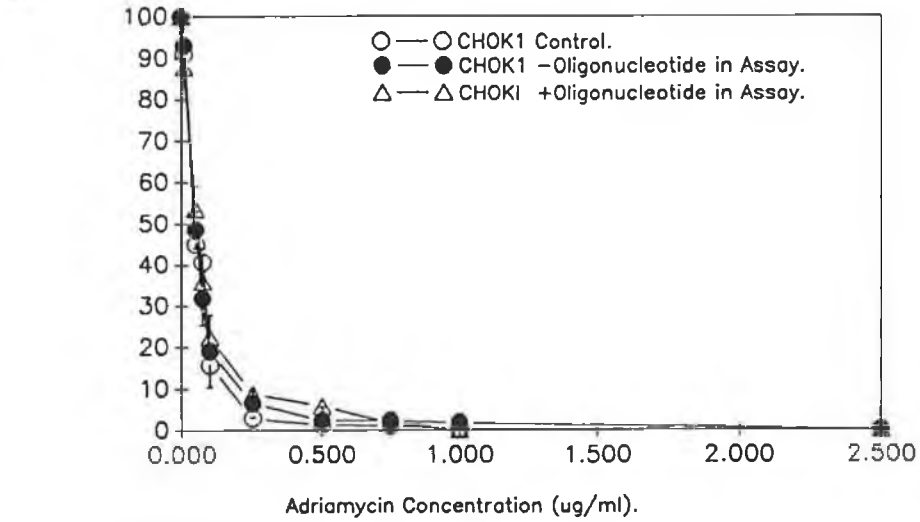
3.10.1.:Effect of Antisense Oligomers on CHrC5.



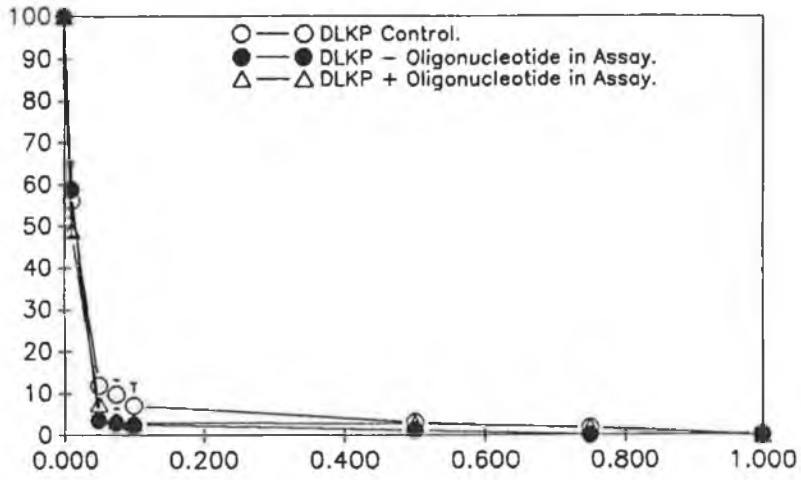
3.10.2. : Effect of Sense Oligomers on CHrC5.



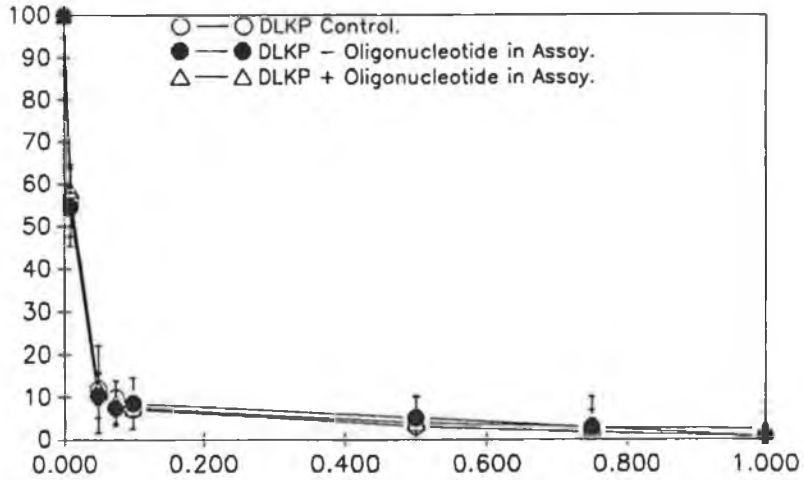
3.10.3. :Effect of Antisense Oligonucleotides on CHOK1.



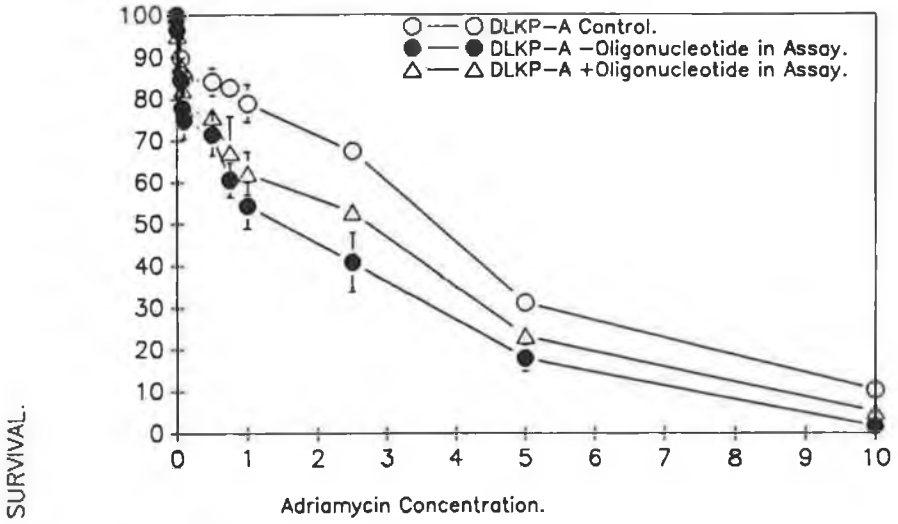
3.10.4. :Effect of Sense OLigonucleotides on CHOK1.



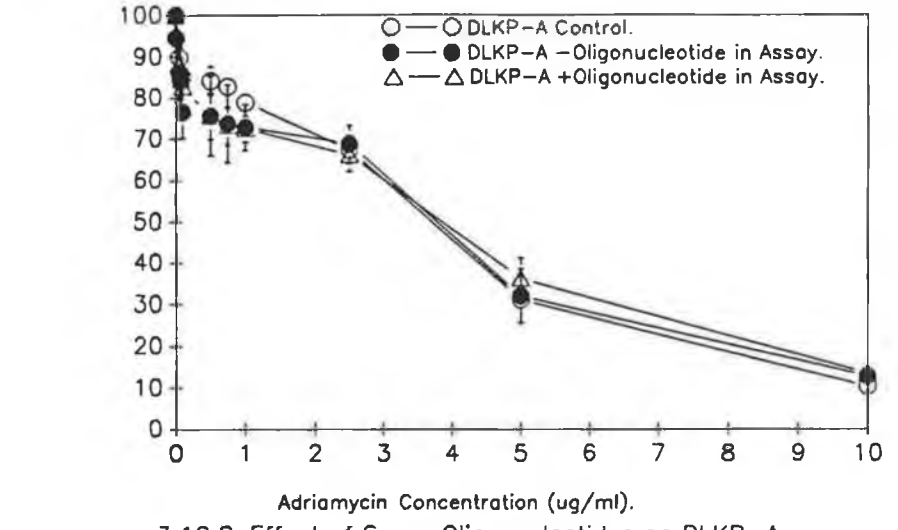
3.10.5.:Effect of Antisense Oligonucleotides on DLKP.



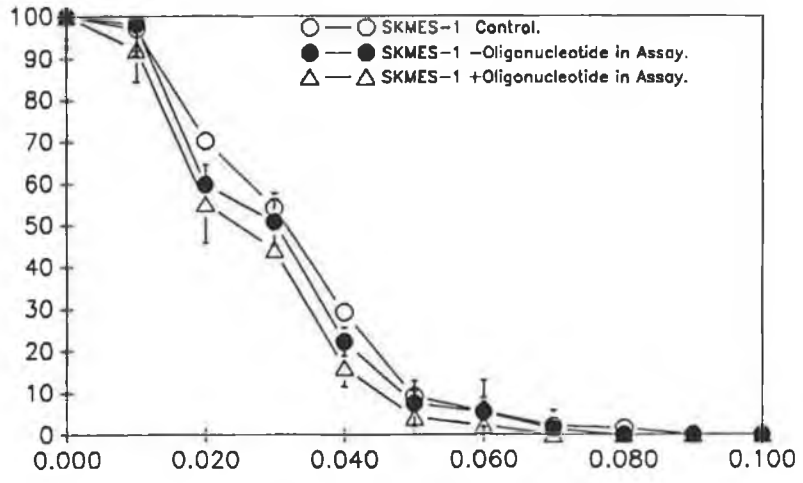
3.10.6. :Effect of Sense Oligonucleotides on DLKP.



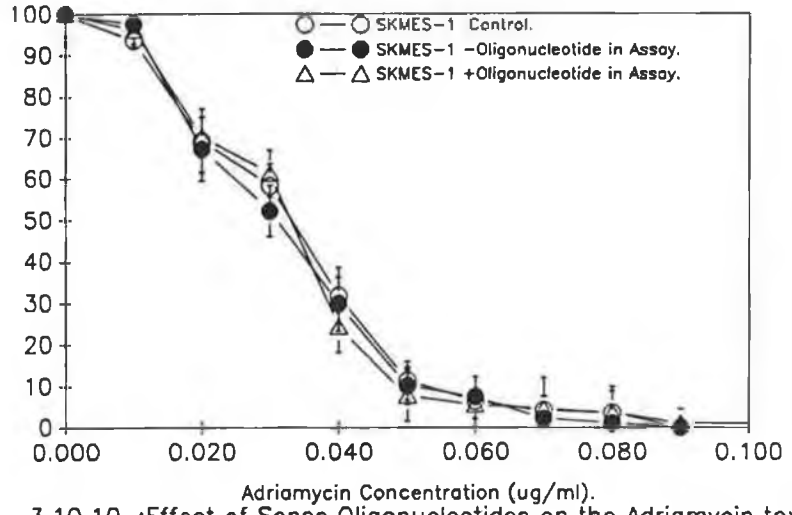
3.10.7.:Effect of Antisense Oligonucleotides on DLKP–A.



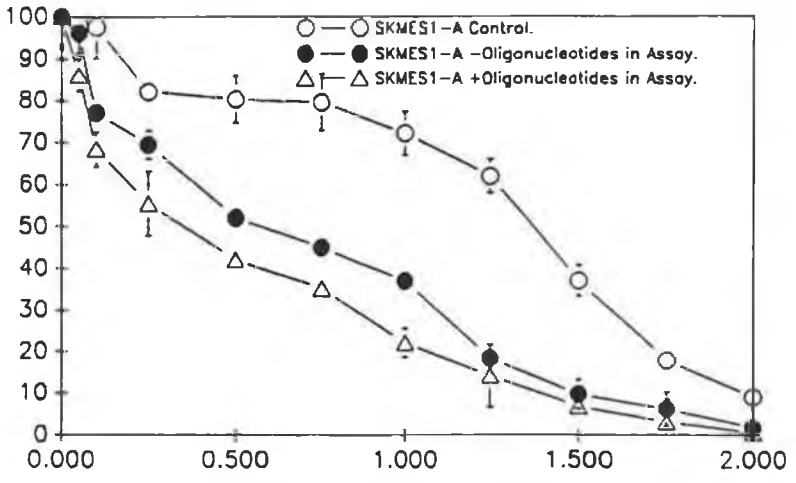
3.10.8.:Effect of Sense Oligonucleotides on DLKP–A.



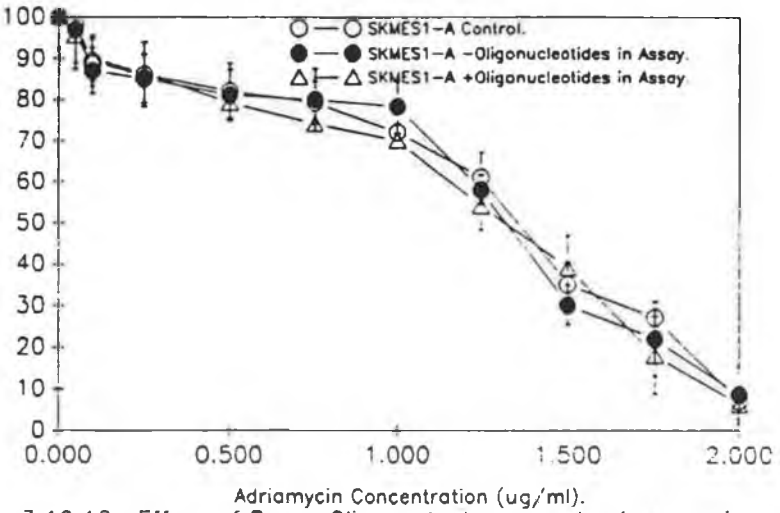
3.10.9.:Effect of Antisense oligonucleotides on the Adriamycin toxicity of SKMES-1(p56).



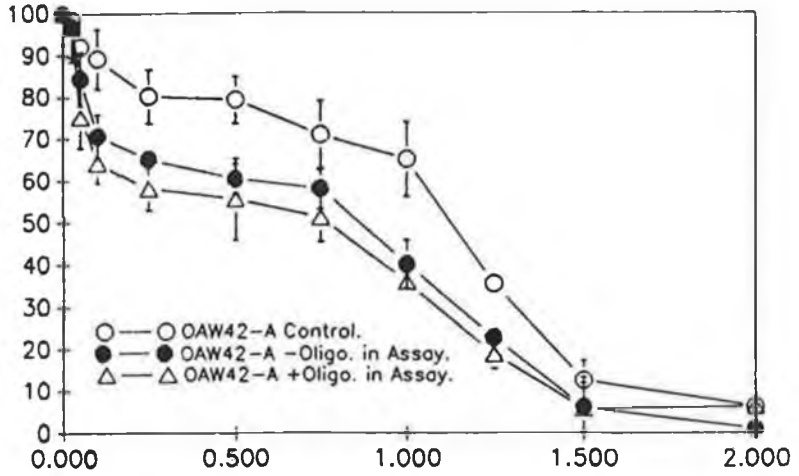
3.10.10. :Effect of Sense Oligonucleotides on the Adriamycin toxicity of SKMES-1(p56).



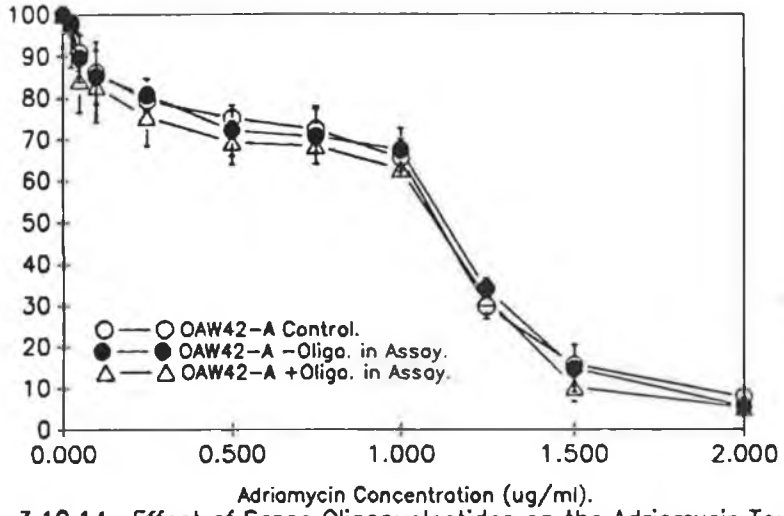
3.10.11. :Effect of Antisense Oligonucleotides of the Adriamycin toxicity of SKMES1-A (0.95ug/ml ADR)



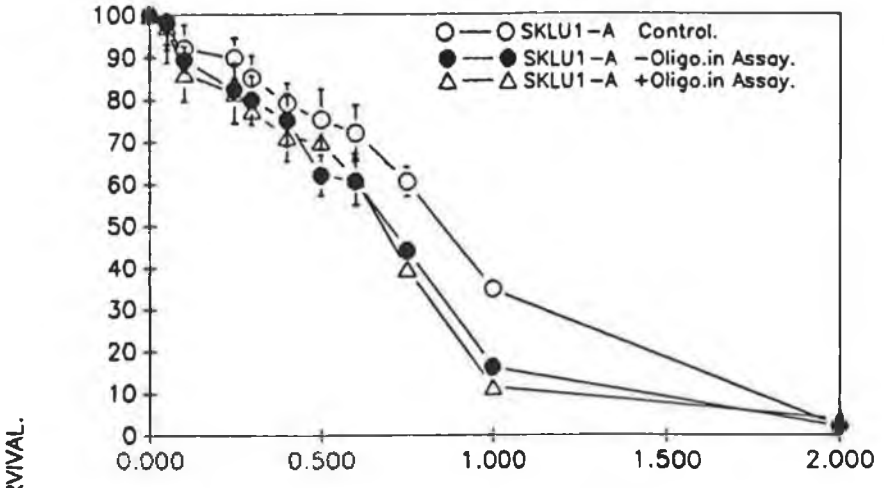
3 10.12 :Effect of Sense Oligonucleotides on the Adriamycin toxicity of SKMES1-A (0.95ug/ml ADR)



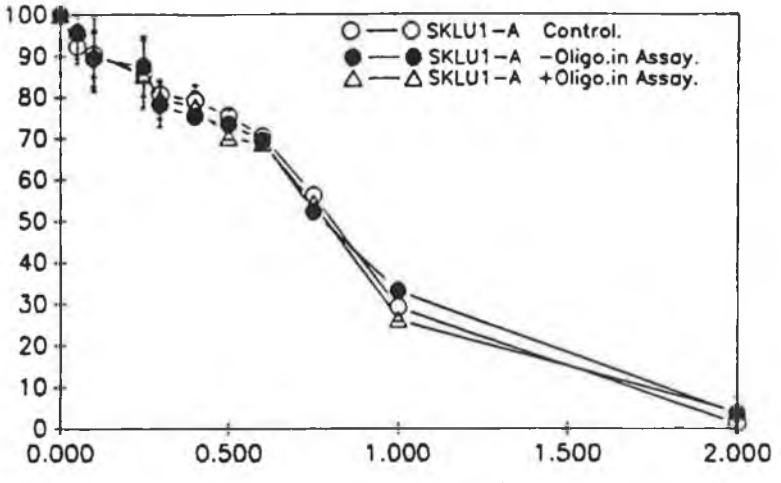
3.10.13.:Effect of Antisense Oligonucleotides on the Adriamycin toxicity of OAW42-A (0.85 ug/ml ADR).



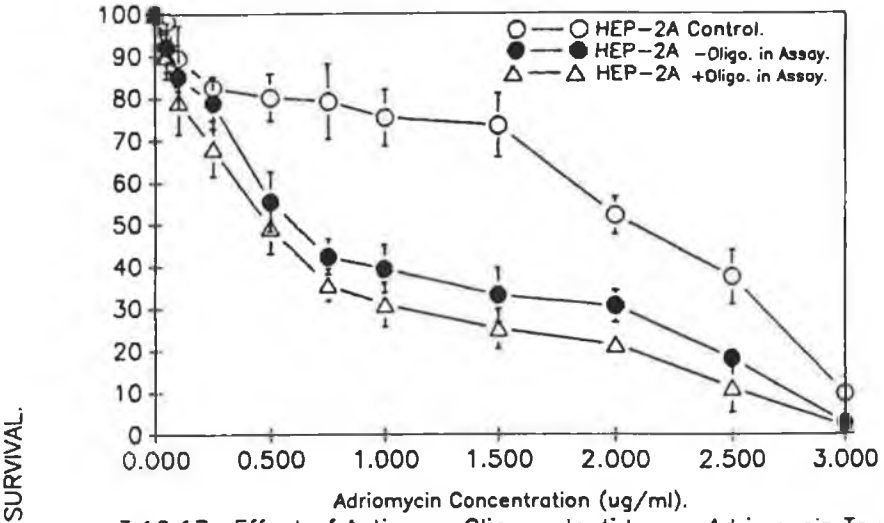
3.10.14.: Effect of Sense Oligonucleotides on the Adriamycin Toxicity of OAW42-A (0.85 ug/ml ADR).



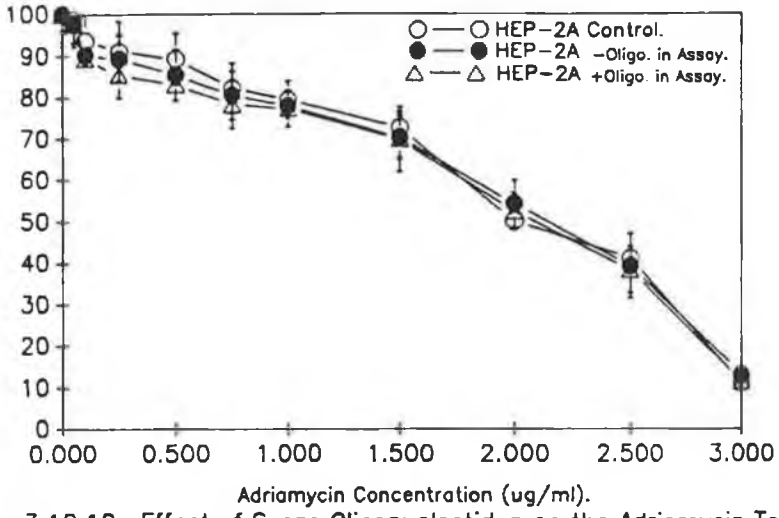
3.10.15. : Effect of Antisense Oligonucleotides on the Adriamycin Toxicity of SKLU1-A (0.55ug/ml ADR)



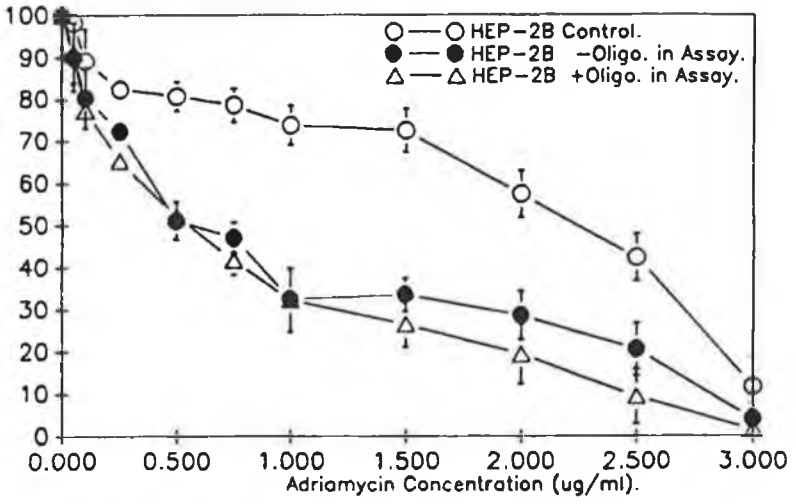
3.10.16.:Effect of Sense Oligonucleotides on the Adriamycin toxicity of SKLU1-A (0.55ug/ml ADR)



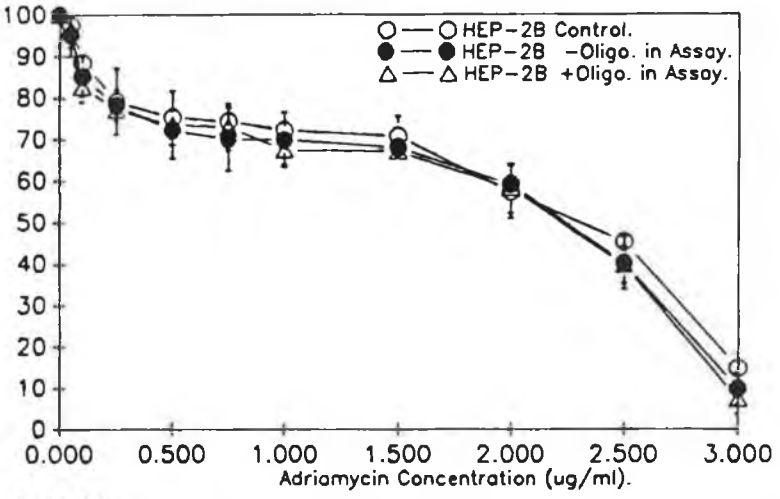
3.10.17.: Effect of Antisense Oligonucleotides on Adriamycin Toxicity of HEP-2A (1.5 ug/ml ADR).



3.10.18. :Effect of Sense Oligonucleotides on the Adriamycin Toxicity of HEP-2A (1.5ug/ml ADR).



3.10.19. ;Effect of Antisense Oligonucleotides on the Adriamycin toxicity of HEP-2B Cells (1.5 ug/ml ADR).



3.10.20. : Effect of Sense Oligonucleotides on the Adriamycin Toxicity of HEP-2B Cells (1.5 ug/ml ADR).

3.11. Circumvention of MDR.

Novel chemotherapeutic agents and combination chemotherapies have resulted in promising new protocols for the treatment of recurrent and drug-resistant malignancies. These regimes allow for increased therapeutic efficacy but are often limited to a specific tumor type or to specific cytotoxic agents.

Verapamil, quinine, quinidine, chloroquinine, nifedipine, aspirin and caffeine were studied for their efficacy in the reversal of resistance. The emphasis of this study was on compounds and concentrations that can be used *in vivo* situations, with little or no toxicity noted to normal tissue. Initially toxicity assays (See tables 3.11.1a. and 3.11.1b.) were performed with each of the agents to ascertain non toxic dose levels to use in circumvention assays (see table 3.11.1c.). This concentration of agent was then incorporated into an adriamycin toxicity assay to look at the fold reversal in IC50 due to the presence of circumvention agent. Results presented are the mean of two experiments, S.D. were ± 10% (data not presented), the results are presented in the form of IC50 of adriamycin in the presence and absence of circumvention agent (C.A.), RMR signifies Resistance Modifying Ratio.

$$RMR = \frac{\text{IC50nM ADR}}{\text{IC50 nM ADR + C.A.}}$$

Table 3.11.1a. IC50nM of the Circumventions Agents.

Cell Line IC50nM	HEP-2	HEP-2A	HEP2B	DLKP	DLKP-A	SKMES1	SKMES1A
Verapamil	27,034	31,789	29,000	32,032	36,670	25,431	26,254
Nifedipine	80,854	82,006	81,946	74,123	69,623	58,217	62,163
Chloroquine	44,482	39,162	44,217	62,188	60,943	52,165	51,179
Caffeine	200823	211122	187124	175129	162456	131129	146358
Quinine	382479	412345	398721	356783	367582	321456	335671
Quinidine	46789	47435	45983	51256	53198	53458	53987
Gen.Aspirin (mg/ml)*	3.6	3.94	4.52	7.15	6.98	4.95	5.23
Std.Aspirin (mg/ml)*	11.24	9.76	6.58	7.45	8.17	7.99	7.45

Table 3.11.1b. IC50nM of Circumvention Agents.

Cell Line IC50nM	OAW42	OAW42-A	SKLU-1	SKLU1-A
Verapamil	35,416	34,324	25,129	26,485
Nifedipine	45,176	39,326	53,764	51,129
Chloroquine	53,176	59,125	47,359	45,916
Caffeine	189746	173129	169723	154123
Quinine	299876	304678	334567	329876
Quinidine	51,673	49,897	35,347	367113
Gen.Aspirin (mg/ml)*	7.64	7.01	3.94	4.76
Std.Aspirin (mg/ml)*	14.12	13.38	6.75	6.24

*These figures are given in mg/ml rather than nM, since all the components of the generic aspirin preparation are not known, it was impossible to convert to a molar concentration (This can however be done for standard aspirin, using a molecular weight of 180.2).

It is noted from the above tables that there is no marked difference in IC50 for any of the circumvention agents comparing the sensitive parent to the resistant cell line.

Table 3.11.1c. Concentrations of agents used in circumvention assays.

Circumvention Agent	Concentration
Verapamil	1 μg/ml
Nifedipine	4.5 μg/ml
Quinine	50.0 μg/ml
Quinidine	2.0 μg/ml
Chloroquinine	2.0 μg/ml
Std.Aspirin	0.1 mg/ml
	0.25mg/ml
	0.5 mg/ml
Generic Aspirin	0.1 mg/ml
	0.25mg/ml
	0.5 mg/ml
Caffeine	25.0 μg/ml

Circumvention results are shown in tables 3.11.2. to 3.11.7.

CHrC5 and CHOK1:

Verapamil proved to be the most effective agent producing a RMR in CHrC5 of 14.9. It must be noted that this was the highest level of reversal for any of the agents tested on all the cell lines. This is consistent with findings from the literature that verapamil is extremely effective in the reversal of P-170 mediated mechanism of resistance. Quinidine, a stereoisomer of verapamil, plus generic and standard aspirin (at a concentration of 0.25mg/ml) proved effective in reversal of resistance with a RMRs of 4.39, 3.44 and 3.915 respectively. Generic aspirin at a concentration of 0.5mg/ml and quinidine both induced a reversal of resistance in parental cell line CHOK1.

DLKP and DLKP-A :

The circumvention results for DLKP-A were very surprising in that every compound tested appeared to cause a reversal effect, with the most prominent effect noted for standard (0.25 mg/ml) and generic aspirin at concentration of 0.25mg/ml. Generic aspirin was noted to cause a 2.375 RMR on the parental cell line DLKP.

SKMES-1 and SKMES1-A:

The most effective compound for the reversal of Adriamycin resistance in SKMES1-A was verapamil. High levels of P-170 have

been detected in SKMES1-A, this possibly explains the enhanced effect of verapamil. Quinine and generic aspirin both produced low level circumventing activity in SKMES1-A.

HEP-2, HEP-2A and HEP-2B:

Quinidine was found to be the most effective agent for the reversal of resistance to HEP-2A and HEP-2B with a 3.3 and 3.5 fold respective reversal of resistance. Verapamil proved to be an effective circumvention agent producing a RMR of 2.4 for HEP-2B, and 1.96 for HEP-2A. Nifedipine and verapamil both resulted in similar fold reversal of resistance of 2.85 and 2.82 respectively. Standard aspirin at a concentration of 0.25mg/ml proved to be the next most effective agent in these cell lines.

OAW42 and OAW42-A :

Both standard aspirin and verapamil were noted to affect the toxicity profile of the parental cell line OAW42. In Addition nifedipine had marked affect with a RMR of 2.85.

SKLU1 and SKLU1-A :

Quinidine was the most effective circumvention agent for SKLU1-A , with a 2.69 fold reversal of resistance. In addition quinine and generic aspirin in both produced clinically relevant RMRs.

Table 3.11.2 Effect on IC50 of Circumvention Agents on CHOK1 and CHrC5.

Cell line / Agent	CHOK1 -	CHOK1 +	(RMR)*	CHrC5 -	CHrC5 +	(RMR)
Verapamil	45.99	27.59	(1.66)	7579	507.7	(14.9)
Quinidine	55.2	21.03	(2.62)	8003	1819	(4.39)
Nifedipine	49.67	31.27	(1.58)	6273	4433	(1.4)
Chloroquinine	46.21	40.98	(1.127)	7594	7012	(1.08)
Std.Asp (0.1)	44.59	37.26	(1.19)	7322	3012	(2.4)
(0.25)	51.64	31.34	(1.64)	7216	2097	(3.44)
Gen.Asp.(0.1)	49.21	40.12	(1.22)	6776	3642	(1.86)
(0.25)	55.27	47.23	(1.17)	7521	1921	(3.915)
(0.5)	48.71	21.12	(2.306)	7921	4726	(1.676)

* RMR-Resistance Modulating Ratio.

Table 3.11.3 Effect on IC50 of Circumvention Agents on DLKP and DLKP-A.

Cell line / Agent	DLKP -	DLKP +	(RMR)	DLKP-A -	DLKP-A +	(RMR)
Verapamil	5.33	6.43	(0.828)	5749	1648	(3.48)
Quinidine	6.9	4.59	(1.5)	5748	1548	(3.71)
Quinine	4.04	4.49	(0.899)	5546	1703	(3.25)
Nifedipine	5.7	7.783	(0.73)	5751	1924	(2.98)
Caffeine	5.23	5.1	(1.025)	5654	2595	(2.17)
Std.Asp.(0.1)	4.599	3.863	(1.191)	5745	1769	(3.24)
(0.25)	3.86	1.287	(2.99)	5726	958.5	(5.97)
Gen.Asp.(0.1)	5.702	4.921	(1.158)	5699	2818	(2.02)
(0.25)	5.138	2.163	(2.375)	5546	1326	(4.18)

Table 3.11.4 Effect on IC50 of Circumvention Agents on SKMES-1 and SKMES1-A.

Cell line / Agent	SKMES-1 -	SKMES-1 +	(RMR)	SKMES1-A -	SKMES1-A +	(RMR)
Verapamil	8.279	10.67	(0.77)	2808	715.67	(3.9)
Quinidine	10.85	9.04	(1.2)	2802	1810	(1.54)
Quinine	9.014	6.99	(1.29)	2627	866.9	(3.03)
Nifedipine	8.83	8.4	(1.04)	2842	2012	(1.4)
Std.Asp.(0.25)	9.014	6.43	(1.39)	2911	1804	(1.6)
Gen.Asp.(0.25)	7.827	2.876	(2.72)	2678	1246	(2.2)

Table 3.11.5. Effect on IC50 of Circumvention agents on HEP-2,HEP2-A and HEP-2B.

Cell Line / Agent	HEP-2 -	HEP-2(RMR) +	HEP-2A -	HEP-2A(RMR) +	HEP-2B -	HEP-2B(RMR) +
Verapamil	12.7	8.3(1.5)	3975	2018(1.96)	4121	1701 (2.4)
Quinidine	11.9	9.2(1.3)	4021	1213(3.3)	3896	1124 (3.5)
Quinine	11.03	10.5(1.05)	3929	2844(1.38)	4314	3809 (1.5)
Nifedipine	12.75	9.9(1.28)	3819	2930(1.30)	3946	2466 (1.6)
Chloro-Quinine	13.06	13.0(1.06)	3655	1788(2.04)	3843	2231 (1.7)
Std.Asp (0.25)	12.51	6.63(1.88)	3707	2382(1.55)	4065	2621 (1.6)

Table 3.11.6. Effect on IC50 of Circumvention Agents on OAW42 and OAW42-A.

Cell line / Agent	OAW42 -	OAW42 +	(RMR)	OAW42-A -	OAW42-A +	(RMR)
Verapamil	12.5	6.439	(1.94)	2163	767	(2.82)
Quinidine	7.95	7.727	(1.028)	2071	1814	(1.14)
Quinine	8.28	4.78	(1.732)	2231	1446	(1.54)
Nifedipine	7.54	6.439	(1.17)	2235	784	(2.85)
Chloroquinine	8.79	8.34	(1.05)	2388	1457	(1.63)
Std.Asp.(0.1)	11.41	7.175	(1.59)	2126	1755	(1.21)
(0.25)	7.727	3.216	(2.4)	2698	1113	(2.42)
Gen.Asp.(0.25)	9.014	5.335	(1.68)	2132	1048	(2.03)

Table 3.11.7. Effect on IC50 of Circumvention Agents on SKLU-1 and SKLU1-A.

Cell line / Agent	SKLU-1 -	SKLU-1 +	(RMR)	SKLU1-A -	SKLU1-A +	(RMR
Verapamil	9.75	7.48	(1.3)	1338	1146	(1.17)
Quinine	8.23	8.01	(1.027)	1568	690	(2.27)
Quinidine	7.73	6.62	(1.168)	1356	503	(2.69)
Nifedipine	7.23	6.59	(1.097)	1315	901.8	(1.46)
Chloroquinine	6.923	5.42	(1.227)	1501	1406	(1.13)
Gen.Asp.0.25	6.521	3.55	(1.836)	1547	832	(1.85)

CHAPTER 4

DISCUSSION

4.1. General Discussion.

Resistance of cancer cells to chemotherapeutic agents may be an inherent property of a tumor, or may develop during the course of treatment, perhaps due at least in part to the mutagenising effects of the drugs used in initial chemotherapy (Shoemaker et al.,1983; Kees, 1987). Two theories have been put forward to account for the development of cellular drug resistance. The first suggests that the tumor population is very heterogenous with populations of cells present that vary in biological properties and their drug sensitivity. It is thought that as treatment proceeds the sensitive cells will be preferentially destroyed but the primarily resistant population will not be affected and will expand to repopulate the tumor ; so after an initial period of regression tumor growth will continue as before. The second theory suggests that the resistance is acquired as a result of the appearance, during treatment, of mutant tumor cells which are unaffected by the administering cytotoxic drug. By a process of natural selection these resistant cells will then proliferate and come to dominate the tumor population. In the past few years, analysis of mechanisms MDR and the biochemical properties of resistant cells has much improved our understanding of drug resistance in human cells, particularly in relation to development of *in vitro* resistance. Yet little of this knowledge has been transformed into clinical gains in the form of alterations in standard chemotherapeutic regimes.

The major focus of this thesis was to develop a number of multiply drug - resistant cell lines of different tumor origin and to characterise these using pharmacological, biochemical, biophysical and genetic criteria. A number of lung carcinomas were chosen for this study due to the lack of substantial data accumulated on mechanisms of resistance in lung tumor cell lines compared to other tumor types. Much emphasis was focused on the area of circumvention of MDR with the use of compounds like nifedipine, aspirin, caffeine and chloroquinine, that could be used in the clinical situation without marked toxicity to normal tissue.

The drug adriamycin was chosen as the selection agent due to its well documented involvement in the MDR phenotype both in vitro and in vivo. It has been used by other researchers for the selection of a number of mulitple drug resistance variants of varied tumor type, for example; SW-1573, a human lung tumor cell line (Keizer et 1989), Colon carcinomas LOVO, DLD1 and SW948 (Toffoli et al.,1991); and leukemic T cells B1 (Snow et al.,1991). This drug shows marked activity against leukemias and has subsequently proved to be one of the most effective of all cytotoxic drugs with activity against a wide range of solid tumors including carcinoma of the breast, bronchogenic carcinoma, gastric cancers and soft tissue sarcomas. The mode of action of adriamycin is still not completely understood but at least five potentially cytotoxic effects of this agent have been identified :

1.**Intercalation** : This means that the molecular structure of the drug is such that it can insert itself between duplex DNA strands, and bind to specific components of the DNA. This results in disturbance of various DNA functions and in the case of the

anthracyclines it seems that DNA and RNA synthesis are inhibited.

2.Membrane Binding : Anthracyclines have been shown to bind to various components of cell membranes, leading to altered membrane fluidity and changes in permeability to various ions.

3.Free Radical Formation : Free radicals possess an unpaired electron and they are often highly reactive towards DNA and other biologically important macromolecules. Anthracyclines undergo enzymatic transformations which release free radicals within the cell and these can cause breaks in the DNA chain, thereby preventing mitosis.

4.Metal Ion Chelation : Anthracyclines have the ability to chelate, or bind, various metals including copper, zinc and iron. Some of the resultant chelates are thought to be cytotoxic.

5.Alkylation : It has been shown that metabolites of the anthracyclines act as alkylating agents.

This summary demonstrates the multiple possibilities for cytotoxic activity that the anthracyclines possess, though the relative importance of these various properties in relation to their antitumor effect is unclear.

The selection process differed markedly for each of the selected cell lines. DLKP-A was the quickest to develop to resistance. This is possibly attributable to a resistant population present in the parental cell line that was subsequently selected out. At each increase in concentration of adriamycin the DLKP-A cells adapted with ease ; an initial slowing in growth rate was evident for the first three weeks in the presence of higher concentration of drug but generally after that time period the proliferation rate increased again. In the case of all the selections a period of at

least six weeks at each new drug concentration was found to be necessary to allow the cells to fully adapt to that level of resistance. All the other selections (SKMES1-A, SKLU1-A, HEP-2A, HEP-2B, OAW42-A and DLRP-A) proceeded at a much slower rate. The DLRP-A and SLKU1-A selections proceeded at an extremely slow pace. It was necessary to restart both these selections a number of times; this is possibly indicative of a relatively homogenous population as regards drug resistance of the parental cell line. It is evident from looking at the clonal variation of the SKMES-1 parental cell line that a slightly resistant population (approximately 1%) was present. It was thought that this population would select out easily as in the case of DLKP-A; however this was not the case, the SKMES1-A selection proceeded very slowly at the initial stages. The cells appeared dormant in the presence of drug with the pH of the medium altering indicating metabolic activity but no cell doubling was noticed. At the later stages of selection the cells adapted very well to increases in drug concentration. The OAW42-A selection evolved very similarly to that of SKMES1-A but no drug resistant parental population was evident in OAW42-A. In the cases of the HEP-2A and HEP-2B both selections adapted initially quite quickly from 0.05μg/ml to 1μg/ml adriamycin resistance, but then slowed down markedly so that the cells had to be grown in drug free medium for two months. On reaching a fold resistance of 189 and 200 (respectively for HEP-2A and HEP-2B), the cells reacted very badly to any further increase in adriamycin concentration, with a large proportion of the cells dying with an increase of 0.2 μg/ml of adriamycin. It is assumed that HEP-2A and HEP-2B reached a threshold as regards drug resistance. Additionally it could indicate that the DNA damage

resulting from prolonged periods of growth in adriamycin have affected basic cellular survival functions to such an extent that any further drug induced DNA damage render the cells non-functional as regards basic cellular processes.

The method by which MDR variants are selected is a very important factor to consider in the study of MDR *in vitro* (Yang et al.,1990b). To date most investigators have used the prolonged exposure of one single agent usually the anthracycline adriamycin or certain antimitotic agents (Vinca alkaloids), as the method of obtaining MDR variants.How representative this is of the *in vivo* situation is unknown. It must however be noted that some results of studies using cell lines in which resistance was induced/selected using the conventional method have contradicted those from studies using fresh tissue. This factor was particullarly noted (Carulli et al.,1990) in the study of haematological maligancies; *mdr1* expression was noted in chronic lymphoblastic leukemia in the *in vivo* situation however this was not noted in the *in vitro* selected cell lines. Future *in vitro* MDR studies will have to include multidrug selections in which drug combinations representative of those used in *in vivo* treatment regimes are used for the *in vitro* selection process. Secondly, the relevance of selection by prolonged exposure to drug needs to be addressed. In the clinic patients may develop resistance at relapse after only several courses of chemotherapy. Therefore in terms of the method of induction of resistance, the drug resistance induced by a short term exposure to antitumor agents may have more clinical relevance than that produced by long term exposure.

4.2. Analysis of the cross resistance profiles of the MDR adapted variants and transfected variants.

The phenomenon of multidrug resistance suggests that a wide spectrum of structurally and functionally unrelated lipophilic drugs are recognised and processed by the molecular system, presumably the P-170, which protects MDR cells against toxicity of lipophilic substances. On analysis of the toxicity profiles from the MDR variants developed in this thesis, there is a consistent rank order correlation between resistance to the selecting drug and cross resistance to other drugs. However the detailed cross resistance profile varies among cell lines selected with the same drug. This is a consistent finding from the literature whereby cell lines are usually cross resistant after selection but the order of magnitude of resistance to the different agents is varied. Table 4.1. summarises the cross resistance profiles for a number of MDR variants, reported in the literature.

Table 4.1. Diversity of collateral resistance in MDR cell lines.

Cell line/Ref.	ADR	C-PT	VLB	VCR	VP16	COLH
SW-1573/50	10	0.6	N.D.*	5.5	23	2.3
SW-1573/500	250	0.25	N.D.	540	270	62
SW-1573/10,00 (Keizer,1989)	2000	0.7	N.D.	N.D.	750	220
SKVLB1	260	N.D.	2000	10,000	N.D.	N.D.
SKVCR2 (Bradley,1989	510	N.D.	4100	1,000	N.D.	N.D.
K562/ADM	527	N.D.	N.D.	841	14	N.D.
2780AD	96	N.D.	N.D.	357	25	N.D
KBC-4 (Sato,1991)	1	N.D.	N.D.	3	6	N.D
SW948-R-6	4.5	1.5	N.D.	2	N.D.	N.D
DLS1-R-8	11.1	1.0	N.D.	6.8	N.D.	N.D
LOVO-R-5 (Tollifi,1991)	19.3	0.98	N.D.	10.3	N.D.	N.D
K562-R (Yanovich,1989)	850	N.D.	4.0	5.0	N.D.	N.D

* N.D.-Not Done.

The data in table 4.1. illustrates the variation obtained in cross resistance patterns for a number of MDR selections. An interesting observation noted from the drug profiles of the ovarian resistant cell line SWVLB1 is that the level of cross resistance to vincristine greatly exceeds the level of resistance to the selecting drug vinblastine (Bradley et al.,1989). Similarly the lung carcinoma SW-1573/500 (which was selected with adriamycin), shows greater cross resistance to vincristine and VP16 (Keizer et al.,1989). None of the MDR variants characterised in our study exhibited this characteristic of a higher degree of cross resistance to the non selecting drug. It was however noted in the cases of the transfected cell lines, for example in the case NIH3T3pha50 higher levels of resistance to adriamycin and VP-16 were evident, HEP-2pha40 was more resistant to VP-16 and DLKP-pha50 was more resistant to adriamycin, vincristine, and VP16 compared to drug used for selection (Colchicine). DLKP-A was the most cross resistant of the MDR cell lines selected in the programme described in this thesis (Table 3.1.2), the magnitude of cross resistance noted in descending order ADR > VCR > VP16 > VM26 > COLH > C-PT > 5-FU. With progessively increased selective concentrations of adriamycin the cross resistance patterns increased for all drugs except for vincristine. An increase in the fold resistance from 67.46 for DLKP-A at 0.8μg/ml ADR to 106 for DLKP-A at 1.75μg/ml ADR was followed by a decrease in fold resistance to 39.75 for DLKP-A at 2.45μg/ml ADR. This could possibly signify a gene switching event in the predominant isoform of the P-170 produced at latter stages of resistance. A similar finding was highlighted by Lothstein et al., (1989) who found that a switch from the mdr1b to the mdr1a gene product was associated with an increase in resistance to vinblastine, taxol and adriamycin

with no detectable increase in the amount of immunoreactive P-glycoprotein. (Resistance to 5-FU and cisplatin will be discussed relative to all the resistant variants together). SKMES1-A also exhibited collateral resistance (Table 3.1.4) with highest levels of resistance to VP16, VM26 and vincristine and lower levels of resistance to C-PT, COLH and 5-FU. OAW42-A was most resistant to cis-platin (Table 3.1.4); cisplatin resistance is a very common finding in ovarian carcinoma in vivo. This could indicate that OAW42-A is a suitable model for the study of resistant ovarian carcinoma. OAW42-A is also resistant to VP16,VM26 and vincristine. HEP-2A and HEP-2B (Table 3.1.8.) exhibit identical spectra of cross resistance ,being most resistant to vincristine, with lower level resistance observed to colchcine, VP16, VM26 and cis-platin. The initial treatment of HEP-2B with EMS did not alter either the time required for selection or the cross resistance profiles. It was reported by Debenham et al.,(1982) that prior exposure to EMS accelerated the rate of selection ; this was not noted in the case of HEP-2B. HEP-2A and HEP-2B reacted very similarly in all the biochemical, pharmacological and genetic characterisation, the only exception being the lesser reactivity of the HEP-2B in the Western blotting procedure. Due to the very close similarities it is suggested that despite the differing selection process that the same population is selected out in both HEP-2A and HEP-2B. SKLU1-A (Table 3.1.6) exhibited low level resistance to vincristine, colchicine and VP16 with no other marked collateral sensitivity to any other agents. It was noted in DLKP-A, HEP-2A, HEP-2B and SKLU1-A that the highest level of resistance displayed after adriamycin was to vincristine. This was found to be the only correlation in cross resistance

patterns observed with all the cell lines selected. DLRP-A was found not be significantly cross resistant to other drugs, the low level of resistance to adriamycin probably accounts for this.

We have shown that a full length cDNA clone for the mouse mdr1 gene on transfection of pHAMDR1a into HEP-2, DLKP, and NIH3T3 can efficiently confer resistance to otherwise sensitive cells in transfection experiments. On analysis of the cross resistance patterns of the transfectants, cross resistance was evident in each of the transfectants, however the magnitude of resistance to different agents is varied. This observation was also noted by a number of investigators (Croop et al.,1987; Schurr et al.,1989, Lincke et al.,1990; Yang et al.,1991). Croop et al., (1987) examined nine different clones of a transfected cell line for collateral resistance to adriamycin, colchicine, daunomycin, vinblastine and actinomycin D. A very varied spectrum of resistance was noted for all the clones analysed. Diversity in the spectrum of cross resistance was observed in the transfected NIH3T3pha30, NIH3T3pha50, DLKPpha50 and HEP-2pha40. With NIH3T3pha30 and NIH3T3pha50 a concominant increase in resistance to each of the test compounds is observed. The highest fold resistance was noted to adriamycin, vincristine, colchicine and VP16, with low level resistance to 5-FU and cisplatin. Both HEP-2pha40 and DLKPpha50 show very diverse patterns of resistance, with DLKPpha50 showing marked resistance to adriamycin 39.62 fold resistance as compared to 4.04 fold resistance for HEP-2pha40. In addition DLKPpha40 was more resistant to vincristine (33.58 fold) compared to 3.5 fold for HEP2pha40. Resistance to other compounds was quite similar with marked resistance to VP16 for both these

cell lines. On comparison of the cross resistance patterns it is found that DLKP-A and DLKPpha50 are most resistant to the same drugs (Adriamycin, Vincristine and VP16) with DLKP-A exhibiting higher fold resistance to these drugs.

No similarities are noted in the MDR profile of HEP-2A and HEP-2pha50, with HEP-2A most resistant to adriamycin (189), vincristine (16.45) and colchicine (5.22) while HEP-2pha50 is most resistant to VP16 (21.87), colchicine (7.9) and adriamycin (4.04). This could be due to a number of factors. Firstly the mdr1 gene transfected is the full cDNA for the mouse mdr1 gene, and there are differences in the cross resistance profiles of the transfection of the mouse mdr1 gene and the human mdr1 gene (Croop et al.,1987). Secondly differences in the degree of amplification could be evident depending on the particular cell line or transfectant in question and this could result in an altered patterns of cross resistance. Thirdly, the differing patterns of cross resistance could be due to the presence of non P-170 mechanisms of resistance in HEP-2A. Further work on the mechanism of resistance in HEP-2A is required to answer this question fully. Additionally further work on the stability of the transfectants is essential to establish if the plasmid has been stably incorporated in the genome of the transfected cell lines.

Point mutations in the mdr1 gene induced by the action of the selecting drug could also account for the lack of homogeneity as regards cross resistance patterns in MDR variants and transfected variants selected with the same drug under similar selection conditions. Independent evidence indicates that the nucleotide binding sites in each half of the P-170 are important in drug

resistance, but the two sites apparently are not identical. Inactivation of both binding sites abolishes all activity ,while inactivation of either site alone leaves either no activity or only a small amount of residual resistance to colchicine, vinblastine and actinomycin D (Azzaria et al.,1989). The patterns of residual resistance are different depending on which nucleotide binding site is mutated. This suggests that both halves of P-170 and possibly the nucleotide binding sites themselves are somehow involved in the specificity of drug recognition or binding. Indeed the two halves might be closely associated with each other in the folded protein, such that the integrity of P-170 structure or conformation is important. The issue of drug binding and specificity is also intriguing in the light of very early observations that MDR cell lines are usually most resistant to the drug in which resistance was established (Croop et al.,1988; Moscow et al.,1989a ; Shen et al,1986). There are as stated above a number of exceptions to this general finding (Bradley et al.,1989 ; Keizer et al.,1989). These cells are resistant to a whole battery of drugs within the MDR phenotype, but the pattern of resistance can vary from cell line to cell line. Some information on drug specificity comes from the discovery that P-170 in colchicine-selected human cells differs from that in vinblastine-selected or in wild type, drug sensitive cells at only a single amino acid residue (Choi et al.,1988;Kioka et al.,1989). The 'mutant' protein encoding a preference for colchicine resistance has a valine at codon 185 in the P-170 sequence, while the 'wild type' sequence is a glycine at that residue. When the mutant gene is transfected into drug sensitive cells, the cells become relatively more resistant to colchicine than vinblastine or adriamycin. Thus residue 185 seems to be responsible for preferential resistance to colchicine, and might be

involved in the specificity of drug interaction with P-170. It is hypothesised that alterations of other amino acid residues by point mutation may cause preferential resistance to other MDR drugs and this could account for the variances noted in spectrum of cross resistance to different chemotherapeutic drugs.

The study of MDR by transfection of cloned genes has a number of advantages over the use of step wise selection of variants. The individual contribution of different members of the mdr1 gene and related family members to the multidrug resistant phenotype remains at present unknown. Cell clones stably transfected with full length cDNA for individual members of the MDR family provide an ideal system to study the structural and functional features of the corresponding MDR protein in an otherwise drug sensitive cellular backround. A number of reported alternative mechanism to P-170 have been reported, including alterations in a number of enzymes of the GST family (particullary π), Topoisomerase 11, DNA Polymerase α and ß. One such experiment has been reported (Fairchild et al.,1990) with the transfection of the full cDNA for the mdr1 gene and the GSTπ gene individually and comcomitantly. The possibility of interaction of the mdr1 gene and the GSTπ was investigated by transfection into the MCF-7 breast carcinoma cell line. It was reported that the transfection of the GSTπ vector alone is insufficient to confer resistance to adriamycin. Additionally on co-transfection of GSTπ and the mdr1 genes, no alterations in resistance patterns was noted above those resulting from transfection of the mdr1 gene alone. These findings leave a number of questions unanswered. A possible hypothesis it that the P-170 works in conjunction with cytosolic transport proteins to pump drugs out of the cells, although such accessory proteins have not

yet been identified. Furthermore because GSTπ amd mdr1 genes are both overexpressed in several models of resistance, it is apparent that there may be several mechanisms of resistance. There may be common factors to the regulation of these two genes. Finally, although co-transfection of GSTπ and mdr1 genes did not accentuate the resistance found on the transfection of the mdr1 gene alone, indicating that GSTπ may not have a direct role in producing MDR, evidence that this enzyme is found elevated in some tumors, compared to the normal tissue (Moscow et al.,1989a,b) suggests that it may be a clinical marker for carcinogen induced tumors or de novo drug resistance. Further transfection studies into different cell lines could further evaluate the role of GSTπ in MDR. An interesting study would involve the transfection of full cDNA of different combinations of the above stated genes and the pharmacological, biochemical and genetic alterations noted. Experiments of this style might lead to implications of synergistic actions of different genes causing specific alterations in resistant cells. Cells lines transfected would be very useful for the screening of potential circumvention agents. The transfectants would be particullary useful for this purpose as individual compounds could be tested on a range of transfectants each expressing different combinations of mechanisms of resistance. However, how representative transfected cell lines are of the in vivo situation is difficult to ascertain. They represent model systems that can be used to study the effect of different combinations of drugs and the screening of potential circumvention agents,but owing to the diversity of the MDR phenotype transfected cell lines could never be representative of the in vivo situation.

4.3 Transfection Techniques.

On analysis of the results of the different transfection techniques it can be concluded that it is possible to increase the efficiency of the $CaPO_4$ transfection procedure by the addition of facilitators PEG 6000, chloroquinine and DMSO. The most efficient procedure for the transfection of plasmid DNA was found to be electroporation. Generally human cell lines were much more difficult to transfect than mouse or hamster cell lines. The most effective procedure for the transfection of genomic DNA was found to be $CaPO_4$ + PEG 6000, for the transfection of ouabain resistance. Generally it is found that it is necessary to optimise transfection techniques for the particular cell line in use and DNA being transfected.

4.4. Resistance to Non-MDR drugs.

Resistance to non-MDR drugs has been noted in a number of the selected variants and transfected cell lines. This finding has been attributed to a number of factors. Cancers are thought to originate as a result of cellular mutations which allow an individual cell or group of cells to escape from normal control mechanisms. In such an unstable population further mutations are likely to occur with passage of time and some of these mutated cells may carry structural or biochemical abnormalities which render them resistant to a broad range of cytotoxic drugs; included in this category are non-MDR related drugs. This results in a subpopulation of inherently resistant cells. Many cytotoxic drugs themselves are mutagenic agents and when drug treatment begins the rate of mutation may be expected to increase with the appearance of resistant clones. The mutagenic affects of numerous chemotherapeutic agents has been proven and much documented. The mechanism of carcinogensis by antitumor drugs is still a subject to intensive research. One theory that has evolved relates to a direct action of certain drugs on cellular DNA. The suggestion is that agents which attack or bind intimately with DNA in the cell nucleus, may not always kill but might cause sublethal damage which can cause multiple mechanism of resistance. Support for this concept comes from the observation that cancer formation, following chemotherapy, in animals and man has been seen mainly with those agents that react directly with DNA (Daunorubicin) whereas agents which have no direct effect on DNA, such as methotrexate have not shown any carcinogenic potential. The second explanation for cytotoxic carcinogenesis is immunosuppression. It has been

suggested that the body is constantly producing small cancers which are recognised by the immune system and destroyed by host defences at a very early stage in their development: this is the theory of immune surveillance. If this theory is correct then prolonged immunosuppression would inhibit host defecnces and allow such tumors to grow unchecked. Clear evidence for the development of second cancers in man following cytotoxic therapy has now emerged from observations on the long term survivors of certain cancers (Hodgkins, some childhood maligancies and ovarian carcinoma) who received cytotoxics as part of their treatment. Cytotoxic agents may be grouped into three catergories; firstly those that have clearly been shown to be carcinogenic in clinical studies (these include nitrogen mustard, cyclophosphamide, chloroambucil, melphan and nitroureas, procarbazine and thiopea); secondly , agents which have demonstrated some carcinogenicity in laboratory tests but not as yet in clinical series (these include bleomycin, doxorubicin, actinomycin D, cis -platin and the vinca alkaoloids); finally, a number of drugs, which so far have shown no evidence of carcinogenicity in either laboratory or in patients (these include 5-Flurouracil, methotrexate and cytosine arabinoside). The model suggesting a series of mutation- affects by carcinogenic agents, offers some explanation for the development of multiple mechanisms of resistance. If this model is a true representation of events, it provides a further argument for adjuvant therapy, in that earlier cytotoxic treatment is given the less chance of resistant strains having emerged.

Mutagenic affects on a number of genes could be the causative reason for resistance to both 5-Fluorouracil and cis-platin. Resistance to 5-Flurouracil is an unusual finding in MDR cells, and

also in the clinical situation. Three fold resistance over the parental cell lines was evident in the case of DLKP-A, 1.86 fold resistance for SKMES1-A, 1.88 for DLRP-A and 1.54 for SKLU1-A. Increases in resistance to 5-FU is often associated with increases in the cellular levels of thymidylate synthetase, which converts deoxyuridylic acid to thymidilic acid this is an essential step in pyrimidine synthesis. Future studies estimating the thymidylate synthetase levels of DLKP-A would indicate if increased levels of this enzyme were the causative reason for 5-Fluoruracil resistance in DLKP-A. Resistance to Cis-platin is not always observed in the classical MDR phenotype. There are numerous reported mechanisms of resistance to platinum based drugs; Godwin et al.,(1991) implicated the alteration of DNA polymerase μ in 2780ADM ovarian carcinoma. Also increases in metallothionein levels have been reported in H69/C-PT (Kasahara et al.,1991) resistant cells. Finally increases in the GSTπ has been highlighted by Cho et al.(1991). To ascertain what mechanism(s) of C-PT resistance exists in DLKP-A, OAW42-A, SKMES1-A, HEP-2A and HEP-2B studies should include assays for the above stated enzymes.

Behavior which may be analogous to the phenomenon of tumor dormancy and senesence was noticed in a number of the MDR variants during the selection process DLRP-A, SLKU1-A, HEP-2A and HEP-2B. The concept of dormancy was invoked to account for the delayed local recurrences of tumors after surgical excisions and for the growth of metastatic tumors a long time after supposedly complete removal or destruction of primary growth. However this theory has never been discussed in terms of selecting of MDR variants in vitro. Several possible factors could be responsible for induction of the dormant state in vivo, such as mechanical, immunological or

nutritional restraints. Little is known about the dormant state or the progression of cell lines or tumor growth from it. Two reasonable possibilities are the emergence might be 'triggered' by some unidentified extrinsic stimulus or that it might be the consequence of the progression of the dormant tumor. However the relevance of the dormant period in the MDR variants is unknown. A related concept is synchronization of tumor cells as an ingenious method of increasing cell kill during cancer chemotherapy. Metabolic modulation alters the metabolism of the target tumor cell and renders it vunerable to the cytotoxic agent. An example is given by Vadlamudi et al. (1971) who treated mice carrying the L1210 leukemia with cytosine arabinoside (a specific S phase inhibitor) and vinblastine (an M phase inhibitor). When the two drugs were given simultaneously, a slight decrease in the therapeutic effect occurs in comparison to the effects obtained if each drug is used alone. A remarkable synergism is observed however if vinblastine is given first, followed by ara-C 16 hours later: increase in the median survival time exceeds 150%. What happens is, the vinblastine arrests the cells in M phase, when its effect fades, all blocked but viable cells are synchronized to enter the S phase at the same time. In the L1210 system maximum synchrony in S phase is achieved about 16 hours after vinblastine injection and ara-C has its greatest effect at that time. Synchronization has also been reported to be a useful method to overcome natural resistance. A good model is the modulation of 5-FU by high dose exogenous leukovorin. In the initial protocol, leukovorin was given by a two hour infusion at a dose of 500mg/m^2. Half way during the infusion, 5-FU was injected I.V. at the dose of 600mg/m^2 . This regime was repeated every week for 6 weeks, followed by a two week rest period before the six cycles were repeated. This regime

appeared to be extremely effective with a reported response rate above 40% in colon carcinoma (Madejewicz et al.,1984). Again, the modulation of 5-FU by previously administered methotrexate is also exploitable in chemotherapy ; also the timing of methotrexate administration in relation to cytarabine and L-asparaginase is important (Bertino et al.,1985). The regime of synchronised chemotherapy may be useful in the treatment of MDR tumors, particularly if the regime incorporates the use of a chemosensitizing agent. The synchronising of the tumor population should yield increased efficay of the treatment given and to some extent overcome the problems of dormancy.

4.5. Altered Biochemical and Biophysical Properties of MDR Cells.

The multidrug resistant variants displayed numerous altered biochemical and biophysical properties compared to their sensitive counterparts. The observation that MDR cells were sensitive to both standard freezing and standard subculture methods was an initial surprising finding, with neither of these previously reported in literature. It was noted that in all cases the best conditions for freezing of MDR variants was high cell number (10^7 cells) and high precentage FCS (50%) (See figures 3.3.1 to 3.3.6). Further increases in percentage FCS did not alter drug resistant profiles after freezing (data not shown). Hep-2A (figure 3.3.2) and Hep-2B (figure 3.3.3) proved to be the most sensitive to standard freezing procedures with a marked decrease in the IC50 from 2.5μg/ml to 1.3μg/ml of adriamycin. The assay procedure used was acid phosphatase activity. It was then decided to look also at clonal variation of HEP-2A (Figure 3.3.8) and HEP-2B (Figure 3.3.9) after freezing as an indication if one particular population of cells was most affected by the sub-optimal freezing conditions. It was suspected that the most resistant cells were not surviving the freezing process. The preferential sensitivity of one population of HEP-2A and HEP-2B was not however evident in the results. Suboptimal freezing conditions had an overall effect on the population ; with HEP-2B a decrease of 10% in the survival of the most resistant cells (at 3μg/ml Adriamycin) was evident under conditions of 10^6 cells/25% FCS . DLKP-A proved to be less sensitive to standard freezing procedures but an interesting observation is noted from the profile of the toxicity curve for DLKP-A , 10^6cells/25% FCS (Figure 3.3.1). At a concentration of 0.5μg/ml adriamycin cells frozen at 10^6 cells/25%FCS, a 10%

decrease in survival was noted compared to 10^7/50%FCS,additionally at a concentration of 1μg/ml for 10^6 cells/25%FCS a decrease of 93% to 63% was evident. Note, however that these are not clonal survival results but acid phosphatase which measures bulk cell survival. This provides suggestive evidence that a population of DLKP-A is susceptible to freezing, but under optimal conditions of increased cell number and percentage FCS it is possible to protect this population. Some decrease in IC50 was noted for SKMES1-A (1.65μg/ml to 0.95μg/ml ; figure 3.3.3), OAW42-A (1.3μg/ml to 1.075μg/ml ; figure 3.3.5.) and SKLU1-A (0.6μg/ml to 0.5μg/ml ; figure 3.3.6) under suboptimal freezing conditions, however as with DLKP-A an increase in cell number and percentage FCS present in the freezing medium ,ensured recovery of the MDR phenotype. It is noted that the cell lines expressing the highest levels of P-170 are most susceptable to standard freezing procedure, this would indicates the fragility the cell membrane containing high levels of of the P-170 glycoprotein. Increased serum protein concentration in the freezing medium was found to reduce the sensitivity of the cells, it is presumed that the protein and/or other substances present in the serum aid in stabilising the cell membrane.

Sensitivity to standard trypsin-EDTA (TV) treatment was a feature of a number of the cell lines. The reduction in IC50 of DLKP-A (figure 3.5.1) subcultured using scraping compared to T.V. is very marked, 1.81μg/ml to 0.72μg/ml adriamycin. Also, IC50 reduction by 2 fold is noted in HEP-2A (Figure 3.5.7) and HEP-2B (Figure 3.5.8). No adverse affect of T.V. was noted on the adriamycin toxicity profiles of SKMES1-A (Figure 3.5.5) and SKLU1-A (Figure 3.5.6) and only a slight affect was noted on the OAW42-A (Figure 3.5.3). In the cases of DLKP-A, HEP-2A and HEP-2B sensitivity to T.V.

correlates well with the level of P-170 expression. However, high levels of expression of P-170 were noted in SKMES1-A by western blotting, and immunofluoresence, but prolonged exposure to T.V. had no effect on toxicity profiles. This implies that the overexpression of P-170 must differ in some way in different cell lines ; for example the P-170 glycoprotein could be more protected in SKMES1-A, through extensive phosphorylation. Otherwise it could indicate that prolonged exposure to T.V. affects other cellular functions, that subsequently alter drug toxicity profiles. Barranco et al.(1980) highlighted time dependent changes in drug exposure expressed by mammalian cells after exposure to trypsin of CHO cells. It was observed that trypsin was shown to cause the release of glycopeptides, from the surface of plasma membranes. These glycopeptides, synthesized intracellularly and transported through the Golgi apparatus to the periphery may have a function in the control of cell proliferation. Enzymatic alteration of the cell surface can also initiate DNA synthesis in contact inhibited cells (Burger,1970 ; Sefton et al.,1970) and can cause changes in membrane components, changes that have been correlated with the expression of a transformed phenotype (Robbins et al.,1979). These findings suggest that exposure to T.V. can cause alterations in cell membranes and/or cell surface components, which normally act as physical diffusion barriers or receptors and thereby affect the transport of drugs into the cells.

The presence of a heterogeneous population as regards drug toxicity, is a very evident feature of the multiple drug resistant variants selected (Luo et al.,1991). The clonal variation of DLKP-A (Figure 3.4.1) is very broad with a large population of resistant

cells viable above the final selected concentration of adriamycin. This accounts for 72% in the case of DLKP-A (1.5) and 81% for DLKP-A (2.1). A drug resistant population in DLKP-A is very evident with extended growth in the presence of drug up to 3.5μg/ml of adriamycin, with 30% of the population surviving at this concentration. Above that concentration the morphology of the cells was altered, they appeared large, dark and granular with a large number of cellular projections evident. It is doubtful if these cells were viable. The presence of a resistant population explains why the DLKP-A cell adapted to adriamycin toxicity so quickly. Very similar observations were noted with SKMES1-A (3.4.4.) , whereby a large population of resistant cells are present above the final selective adriamycin concentration,this accounts for an 80% survival at 1μg/ml adriamycin. Cells grew to 2.5μg/ml adriamycin without displaying any altered morphological alterations. On analysis of the toxicity profile of the parental cell lines DLKP and SKMES-1 a resistant population was evident in both DLKP (4%) and SKMES-1 (1-2%), the increased drug resistance above the final adriamycin selected concentration in DLKP-A and SKMES1-A could be attributed to the cloning out of the resistant sub-population. In OAW42-A (Figure 3.4.2) such a range of clonal variation was not noted with a decline in resistance levels very evident from 90% to 50% at 0.75-1.0μg/ml of adriamycin. There is an indication of two predominant drug resistant populations one which is present from 0-0.75μg/ml of resistance with 90% survival and the second from 1μg/ml Adriamycin (with 50% survival) which gradually reduces to 22% at 2.5μg/ml adriamycin. The same is evident in HEP-2A, HEP-2B (Figure 3.4.3) and SKLU1-A (Figure 3.4.5) where a sharp decrease in the level of resistance occurs. This decline in

the level of drug resistance is very evident in HEP-2B where a decrease in resistance from 88% to 24% occurs between 1.5μg/ml and 2μg/ml of adriamycin.

During the preparation of cell membranes from the sensitive cell lines for western blotting procedures, it was consistently found that the resistant variants were slower to lyse on sonication. In view of our earlier results on sensitivity to cryopreservation and to TV, this finding was surprising. This could indicate altered lipid composition of the plasma membrane increasing the fluidity of the membrane, making it more resistant to mechanical force. Little is known about differences in the lipid composition of cells sensitive and resistant to anticancer drugs. In mouse leukemia P388 cells Ramu et al.,(1983) have found a greater triglyceride and sphingomyelin content along with a lower phophatidylcholine (PC) content than in the sensitive parental cell line. In contrast no major differences in the content of neutral lipid, phospholipid or ganglioside family was found by Holleran et al.(1986) while Tapiero et al.,(1986) have found a greater PC to sphingomyelin and PC to phosphatidylethanolamine ratios in resistant than in sensitive cells. Finally Wright et al, (1985) have found a greater sphingomyelin, cardiolipin and lower phosphatidylserine in resistant acute lymphoblastic T cells than in their sensitive counterparts. An indication was noted in this report that these cells were generally more mechanically sturdy, this could indicate that an increase in the levels of sphingomyelin and cardiolipin in our resistant variants could be the causative reason for the increased sonication time required for cell lysis. Also alterations in the fatty acid content of the membrane has been reported. Adriamycin resistant rat glioblastoma cells are reported (Vrignaud

et al.,1986) to incorporate fatty acids from the media more rapidly than in sensitive counterparts resulting in greater relative amounts of oleic acid than in the sensitive line. In P388 cells no difference between adriamycin resistance and sensitive cells in the fatty acid distribution was detected (Holleran et al,1986). It was observed that the fatty acid composition of adriamycin resistant Friend leukemia cells is more saturated, has a greater 18:0 content and has a larger neutral lipid component than the adraimycin sensitive cells. Additionally low density lipoprotein transport has been found to be altered in ovarian resistant A2780-DX3 cells indicating altered membrane lipid concentration (Mazzoni et al.,1991). To confirm this hypothesis for a mechanism of resistance to sonication, future work involving analysis of lipid composition of the MDR variants is necessary.

4.6 Analysis of P-170 in Sensitive and Resistant Cell lines by Western Blotting and Immunofluorescence.

Multidrug resistant cells are reported to show characteristic changes in surface glycoproteins. The most prominent of these is the enhanced expression of an M.W. 170,000 to 180,0000 glycoprotein, the amount of which has been shown to be related to the degree of resistance (Riordan et al.,1985 ; Debenham et al., 1982; Hamada et al.,1986, Bradley et al.,1989; Chen et al.,1987; Redmond et al.,1991; Croop et al.,1988; Endicott and Ling 1989). A new membrane antigen associated with drug resistance in lung cancer of size 46kD, (SQMI) was reported by Bernal et al.(1990). The cDNA and predicted amino acid sequence showed no significant similarities with P-170. SQM1 has been identified as a differentiation-related membrane protein that appears to be associated with a new mdr phenotype particularly noted with resistance to MTX and C-PT. Several other membrane proteins unique to MDR cells has been observed in addition to P-170, such as an 85kD glycoprotein in K562/ADM cells (Hamada et al.,1988) and a 150KDa protein immunologically distinct from P-170 (Mc Grath et al.,1988). The C219 monoclonal antibody was used for the analysis of the resistant variants by western blotting and immunofluoresence. Western blots results illustrated the highest levels of P-170 were present in the prototype resistant cell line CHrC5, a second band was evident at 70 kD. This is possibly another member of the P-glycoprotein gene family. It could represent a truncated form of the P-170 protein, P-170 being known to consist of two homologous halves this second band could represent an unphosphorylated form of one half of P-170. Immunological reactivity was very evident for both DLKP-A and

SKMES1-A with one very discrete band evident for both of these variants. With HEP-2A, HEP-2B and OAW42-A very lighty stained bands were evident that were not as discrete as in SKMES1-A. This could indicate heterogeneity in the rate of phosphorylation of P-170 in these cell lines giving a broader M.W. range of the P-170 hence the larger band, or partial degradation of the membrane glycoprotein during the preparation procedure. The immunological reactivity of HEP-2B membrane glycoproteins were lower compared to HEP-2A, these two cell lines have been found to react very similarly in antisense oligomer transfection and immunofluorescence. The significance of the lesser reactivity of HEP-2B P-170 in the western blotting procedure is unknown. Consistent with the remainder of the results SKLU1-A showed no immunological reactivity to P-170.

Immunofluorescence staining using C219 as the primary antibody and antimouse FITC as the secondary antibody yielded a number of different patterns of staining. CHrC5 staining was very evenly distributed across the population, indicating the presence of a very homogeneous population in their expression of P-170 glycoprotein. The pattern of staining in DLKP-A was relatively homogenous, signifying the dominance of a resistant population, there was less intense staining which accounted for less than 10% of the population and were only evident in some fields. The staining of SKMES1-A, OAW42-A, HEP-2A and HEP-2B were very heterogenous with a number of populations evident. Metabolic cooperation, which is a common feature of epithelial cells, is a possible mechanism which could allow a less resistant population to survive at higher drug concentrations. The technique of

immunohistochemistry has been used in the clinical situation for the analysis of a large number of malignancies, particularly haematological disease (Carulli et al.,1990), however there have been a number of short comings in the basic methodology and the manner with which positivity is scored. In earlier studies the role of P-170 utilised bulk tissue, no tissue sections were taken and analysed to confirm the presence and what percentage of neoplastic tissue was present. Another factor in which inconsistancies are definitly present is the manner in which positivity is scored as a means of determining P-170 expression (Kumazuru et al.,1990). In the study of AML, differences in scoring positivity have been very evident. Mattern et al.,(1989) looked at nine cases using 5-50% positive cell on staining as the criteron for P-170 expression. However, Ito et al.,(1989) described 14 patient with AML. Using flow cytometric analysis with FITC fluorescence, more than 20% of fluoresence positive cells were defined as positive, from 10-20% as intermediate, and less than 10% as negative. Needless to say it is very difficult to compare the results of these two experiments. It is necessary to set a standard level of positivity for the study of P-170 in the clinical situation, until this is done it will be impossible to compare different investigators results. The primary advantages of using immunohistochemistry in the clinical situation include the ability to detect P-170 in a single cell, to determine subcellular distribution of P-170 and to discriminate between P-170 expression in normal and neoplastic tissue (Dalton et al.,1991). The inconsistencies noted in the immunohistochemical procedures have been the high levels of false posistives and negatives noted from different investigators for the same tumor type. The level of false positives tends to be quite high given the nature of the protein under investigation and its distribution in normal tissue.

Introduction of routine H+E staining in conjunction with staining for the presence of P-170 is a definite prerequisite necessary for routine examination of clinical samples. This could possibly cut down on the number of false positives and negatives received. Another possible way in which this could be overcome is to use a panel of antibodies which may prove to be more efficient than the use of a single one. Alternatively, competitive immunohistochemical staining may be preformed with epitope specific peptides to ensure specificity of detection (Marquardt et al,1990). The use of bulk tissue for DNA, RNA and protein analysis should be firstly examined to ascertain the proportion of neoplastic cells present. The introduction of the above stated factors into routine clinical practice for examination of neoplastic tissue should improve the consistancy of results and should overall paint a clearer picture as to the clinical role of P-170. (See section 4.10.3. for details of possible use of anti MDR antibodies for therapeutic purposes and section 4.10.1. for details of RT-PCR).

4.7. Analysis of the Role of mdr1 Over Expression in MDR using Antisense Technology.

Currently there is much excitment about antisense oligonucleotides, both as drugs of the future for the treatment of cancer and viral infections (Tidd et al.,1991). The present state of the art is that antisense oligodeoxynucloetides or oligodeoxy nucleotide analogues can apparently inhibit gene expression when presented exogenously to cell lines, but its efficacy as a therapeutic tool has not as yet been ascertained. Two studies have been presented relevant to the MDR area. It was reported by Vsanthakumar et al.,(1989) that the K562/111 cell line positive for MDR1, on exposure to non-ionic oligonuceoside methylphosphonates, complementary to the initation codon and 15 bases upstream of the mdr1 gene, can completly inhibit the synthesis of P-170 and partially decrease the toxicity of daunorubicin. Additionally, Rivoltini et al.,(1990) used antisense oligonucleotides in the modulation of MDR. The aim of their study was to test the susceptability of Lovo/Dox cells to lysis by different types of immune effectors, modulation of toxicity with the use of antisense oligonucleotides, and the reversal of resistance by verapamil. The effect of verapamil and the modulation by antisense oligomers were found to be very similar. Our experiments involved the transfection of an 18mer downstream of the mdr1 promoter. Both sense and antisense oligomers were transfected. No alteration in adriamycin toxicity profiles was noted after culture in the presence of sense oligomers for any of the cell lines analysed. Only one of the parental cell lines (SKMES-1) showed any affect in the presence of antisense oligomers with a slight decrease in IC50

noted from 0.03μg/ml to 0.02μg/ml of adriamycin (figure 3.10.9); this could indicate the presence of a subpopulation of P-170 expressing cells in the SKMES-1 population. The greatest reversal of resistance was noted in the resistant control cell line CHrC5 (figure 3.10.11) with a reduction in IC50 form 4.1μg/ml to 0.02 μg/ml of adriamycin, this highly sensitizing affect noted in the presence of antisense oligomers indicates that P-170 expression is a definite and major mechanism of MDR in CHrC5, as indicated in literature. An important observation noted in all the antisense assays is the requirement to include oligonucleotide into the assay medium during the course of the adriamycin toxicity assay. When the cells are only pretreated with oligomer a blocking affect of P-170 expression is noted, but not to such an extent ; this indicates the instability of the oligomers and the necessity to incorporate them into the toxicity assay to obtain a definite effect. A reduction in IC50 of 1.4μg/ml to 0.4μg/ml was seen for SKMES1-A (Figure 3.10.11), with both HEP-2A (figure 3.10.17) and HEP-2B (3.10.19) a four fold decrease in IC50 was noted on addition of antisense oligonucleotides. In the case of OAW42-A (Figure 3.10.13) only a 1.6 fold reversal of resistance was noted. A reduction in IC50 of 3.8μg/ml to 1.6μg/ml was noted with DLKP-A (Figure 3.10.7). SKLU1-A did not show positivity for P-170 in either western blotting or immunofluorescence, no affect was noted with the addition of antisense oligomers. Results from western blotting and immunofluoresence indicated high levels of expression of P-170 in SKMES1-A and DLKP-A, with lower levels present in HEP-2A, HEP-2B and OAW42-A. A greater blocking affect with antisense oligomers was expected particularly for DLKP-A and SKMES1-A. This would suggest that P-170 expression might not be the only mechanism of resistance in DLKP-A and SKMES1-A ;

additionally the lack of complete block of P-170 expression in cells exposed to antisense oligmers could be due to the short time of treatment, or a too low concentration of antisense oligonucleotide used. The half life of the P-170 has been found to be variable depending on the cell line analysed, 24 hours for SW620 (Richert et al.,1988) and 72 hours for KB-V1 (Rivolitini et al.,1990). Further work to estimate the half life of the P-170 in the MDR variants developed, is necessary to use antisense technology technology to its full potential in the analysis of P-170 as a mechanism of resistance. However, P-170 expression, through subsequent decreased adriamycin toxicity, is a definite mechanism of resistance in CHrC5, DLKP-A, HEP-2A, HEP-2B and OAW42-A. The data presented gives hope for the use of antisense technology as a diagnostic tool and future valuable applications in therapeutic control of drug resistance.

4.8. Cytogenetic Analysis of DLKP-A, HEP-2A and HEP-2B.

Numerical heterogeneity and varying degrees of staining of Double minute chromosomes (DMs) were cytogenetic hallmarks of the cell lines characterised. DMs were very faintly stained in HEP-2B (figure 3.8.7). HEP-2A (figure 3.8.6) shows a large number of DMs varying in size and intensity of staining and possibly the presence of a large HSR. The percentage of metaphases analysed with DMs present was 47% for HEP-2A and 39% for HEP-2B. HEP-2A and HEP-2B were both found to be markedly similar in their spectrum of the number of DMs present in the metaphases analysed. In DLKP-A (Figure 3.8.2.) four pairs of DMs are present, varying in uptake of stain and size, and the presence of a ring chromosome. It has been suggested by Sandberg et al.,(1983) that DMs are derived from HSRs with the location and the size of the HSR differing from one cell to the next. Ring chromosomes have been reported to be an intermediate stage in the progression of DMs to HSRs (Barker et al.,1982) this could indicate the presence of HSRs in DLKP-A (although we did not attempt to analyse for the presence of HSRs), this could also explain the fact that DLKP-A has less DMs than HEP-2A and HEP-2B despite the fact that DLKP-A is the more resistant of the two cells line. Generally either double minute chromosomes or homogenously staining regions are evident on analysis of a MDR population of cells (See table 1.3 for details). Only three cell lines have been reported were the presence of DMs and HSRs are found concomitantally (Quinn et al.,1979, Meyers et al.,1989 and Slovak et al.,1987). Double minute chromosomes are generally found at lower levels of resistance and at this stage the number of double minute chromosomes correlates well with the degree of resistance. HSRs number tends to increase with increased levels

of resistance and as can be seem from table 1.3 the location of HSRs is not limited to chromosome 7 (the site of the P-170 gene), indicating that amplification of other genes are also implicated in the MDR phenotype. The reason why numerical heterogengenity of DMs exists and the unusual coexistance of DMs and HSR are unknown. The numerical heterogenity of DMs in a population implies that DMs segregate differently from chromosomes. Underlying mechanisms of heterogeneity might include disproportionate replication of DMs, unequal distribution to daughter cells at anaphase of mitosis, or intra cellular transfer of DMs by processes other than mitosis,numerical heterogenity of DMs is due to unequal distribution of DMs at mitosis resulting from the lack of centromers. Replication studies indicated that DMs replicate early in S phase in certain systems (Levin et al.,1976). In other DMs replicated proportionately with chromosomal DNA during S phase of the cell cycle (Barker et al.,1980). Schimke et al.,(1984, 1985, 1986) has proposed a different theory that HSRs and DMs arise from the same events. First overreplication of DNA occurs by multiple initations of DNA replication. The initial reduplication of DNA is apparently followed by a step involving DNA recombination. When the recombination event occurs chromosomally, an HSR may develop at the site of the integrated gene. When this event occurs extrachromsomally, a DM is formed. It has been suggested that homologous recombination of the amplified DNA sequences that are transiently formed may result in a number of other cytogenetic aberrations in addition to HSRs and DMs, including sister chromatid exchanges, discentric chromosomes, and chromosomal inversions. This theory adds some explanation for the large number of chromosomal abnormalities encountered in the three MDR variants

analysed (Table 3.8.1). Alternatively the abnormal metaphase spreads in HEP-2A, HEP-2B and DLKP-A could be the result of high level drug exposure.

On reviewing the human gene map of chromosome 7, several observations are noteworthy. First, no currently recognised cellular oncogene sequence is localised to the distal arm of chromosome 7 where the P-glycoprotein is localised. However, the ß-subunit of the T-cell receptor has been localised to this approximate chromosome region (Isobe et al,1985). Secondly it has been shown that the distal arm of chromosome 7 is a hot spot for rearrangement in normal lymphocytes (Scheres et al.,1986), although in general this region is infrequently altered in neoplastic tissue (Mitelman et al.,1984). The third alteration of chromosome 7q14-36 is in fact a very common karyotypic alteration found in two forms of cancer that display profound resistance to chemotherapy 1. Secondary myelodysplastic syndromes and 2. Acute non-lymphocytic leukemia occuring in patients with documented exposure to various leukemogenic agents (eg chemicals, solvents, insecticides) (Rowley et al.,1983). It is suggested that chromosomal alteration involving 7q may alter expression of P-glycoprotein in some cases and this may lead to development of resistance to chemotherapy.

Future work in this area should include a study of the HSRs present in HEP-2A, HEP-2B and DLKP-A, it is impossible to make any conclusions from the above data regarding the degree of resistance proportionate to either the number of DMs or the length of HSR present. However, the definite presence of DMs indicate cytogenetic manifestations of gene amplification in HEP-2A, HEP-2B and DLKP-A.

4.9. Pharmacological Reversal of MDR.

Circumvention of resistance at clinically relevant levels was achieved with all the agents tested, verapamil, nifedipine, quinine, quinidine, chloroquinine, caffeine and aspirin. However some were found to be much more efficient; relative efficiency was however quite dependent on the cell line in question. Analysis of circumvention agents and their effects will be discussed under their group classification and compared to observations from the literature. All of the compounds used in this study were used at clinically relevant concentrations at which toxicity to normal tissue would be minimal.

4.9.1. Calcium Antagonists.

These are categorised into three subclasses,

1.Dihydropyridines, eg nifedipine

2.Verapamil, diltiazem

3.Diphenylalkylamines eg Flunarizine.

Verapamil is the most widely tested calcium channel blocker in the area of circumvention. The listing of cell lines on which it has been tested and proven to reverse resistance either partially or completly, is extensive (see table 4.2 and 4.3 for further details). The two compounds tested from this classification were verapamil and nifedipine.

The calcium channel blocker verapamil is traditonally used in the treatment of coranory heart disease, arterial hypertension and supraventricular arrhthmias, has vasodilatory effects on vascular smooth muscle, has negative ionotropic effects on myocardial cells and lengthens atrioventricular conduction time (Fleckenstein et al.,1977). Verapamil is almost completly absorbed after oral administration and is affected by extensive first pass hepatic metabolism, with P-450-mediated N-dealkylation and O-demethylation accounting for the majority of identified metabolites (Eichelbaum et al.,1979). The bioavailability of verapamil is low (10-35%), it has a high systemic clearance and its half life of 3-7 hours (Echizen et al.,1986). The majority of metabolites lack cardiovascular activity. Whether they posses chemosensitizing activity is unknown . The effect of verapamil on cross resistance to chemotherapeutic drugs other than those used for selection has yielded some very interesting results, with respect to both the chemosensitizing effect and in defining the mechanisms by which P-170 transport structurally unrelated compounds.

In our studies verapamil proved to be most effective agent in the reversal of resistance to the control cell line CHrC5; with a RMR of 14.9, this was the largest RMR noted for any of the circumvention agents. Verapamil has been consistantly noted to be a very effective circumvention agent for the reversal of the P-170 mechanism of resistance (Ford et al.,1990). There is a correlation between the level of P-170 expression and extent of effect by verapamil, with the most marked effect noted for cell lines expressing highest levels of P-170, CHrC5 in the cases of our studies. Additionally verapamil proved to be the most effective agent for the reversal of resistance in SKMES1-A with a RMR of 3.9.

This is consistant with the high level of expression of P-170 in this cell line. Verapamil was also found to be an effective agent in the reversal of resistance to DLKP-A with an RMR of 3.5. In the case of HEP-2A and HEP-2B RMRs of 1.5 and 2.4 were found respectively with no circumvention effect noted on SKLU1-A.

In some cases, verapamil produced as great effect on cross resistance to drugs as on primary resistance to the selecting agent. Sikic et al.(1989) using the MES-SA Uterine carcinoma cell line (100 fold resistance to adriamycin), which has been shown to have elevated levels of mdr1 gene expression, achieved seven fold reversal of MDR with 6μM verapamil. It was also noted that adriamycin uptake was increased. Similar reversal of resistance with different drugs (Daunomycin, Actinomycin D and Mitroxantrone, Harker et al.,1986) plus 6μM verapamil, was achieved at the same magnitude. An interesting factor noted with vinblastine was that verapamil increased accumulation of the drug without altering MES-SA/Dx5 resistance to vinblastine. Similarly using an NIH3T3 cell line transfected with the mdr1 gene 12μM verapamil completely reversed resistance to colchicine (40 fold) and cross resistance to adriamycin (20 fold). In certain studies, verapamil was more effective in reversing resistance to the drug used in selection rather than cross resistant drugs. For example Becks et al.(1986) reported that 10μM verapamil produced a 75 and 87 fold decrease in the 244 and 1163 fold resistance to vinblastine and vincristine in the vinblastine selected CEM/VLB100 cell line but only two to five fold reversal to daunorubicin and doxorubicin resistance, to which the cells were 100 fold resistant. Similary although 5μM verapamil caused a 13 fold reversal of 200 fold doxorubicin resistance and 33 fold reversal of 100 fold vinblastine resistant MCF-7 ADRr cells,

it resulted in only a four fold change in the 400 fold cross resistance to colchicine (Ford et al.,1990). Why MDR cell lines differ in the effect in which verapamil has on cirumventing resistance to various drugs is unclear. An interesting possibility is that alterations in the MDR at the genomic level (Choi et al.,1988) or posttranslation modification in P-170 may cause changes in the affinity of cytotoxic drugs for the putative binding site(s). Alternatively, multiple mechanisms of resistance to various chemotherapeutic agent operating within a single cell line, could explain this phenomenon. Although verapamil has been widely studied and is a potent and effective circumvention agent, it possesses potentially life threatening cardiovasicular effects in humans at plasma concentrations in the 2 to 6 μM range required for circumvention of MDR (Candall et al.,1979). It is cytotoxic at higher doses to normal tissue (Lampidis et al.,1986). Therefore more potent and less toxic chemosensitizing agents need to be studied. Alteration in calcium transport or distribution may not be involved in the circumvention process (Naito et al.,1989) and many compounds with calcium blocking activity are inactive in reversing MDR. Verapamil is bound to α- acid glycoprotein (AAG) in human serum but this problem is absent from culture media (FCS). Chatterjee et al.(1990) studied the presence of absence of AAG on the circumventing ability of verapamil. The presence of AAG was found to increase the circumventing affect. These results suggest that in addition to a plasma memebrane site, there may be a major endosomal site of action of verapamil in MDR cells. Kessel et al.(1985b) examined 14 analogues of verapamil and found that tiapamil caused a partial reversal (15 fold), in 100-fold doxorubicin resistant P388/ADR cell line, whereas the analogue DMDP was only seven fold less potent and was 10 fold more effective

in reversing resistance (Radel et al.,1988). Also, nor verapamil has been effectively used as a circumvention agent (Merry et al.,1989) Yamaguchi et al.(1986) reported that SDB - ethylenediamine, a synthetic isoprenoid with a structurally similar backbone to verapamil containing a 9 carbon isoprene side group produced a two to five fold increase in toxicity to adriamycin in addition to partial reversal of resistance to vincristine, vinblastine and daunomycin. In the papers cited, the effect of verapamil on MDR has used racemic mixtures. Because the S-enantiomer of verapamil selectively binds to calcium channels recent studies have compared the effects of S- and R-enantiomers of verapamil and its analogues desmethyoxyverapamil and emopamil on MDR (Keilhaurer et al.(1990); Qian et al.(1990); Pirker et al.(1990) and found them to be equally active chemosensitisers in MDR CEM/VLB and KB-C1 cells. The closely related analogues gallopamil and devapamil were more potent calcium channel blockers but less potent chemosensitizers in MDR cells (Pirker et al.,1990). Therefore the use of less cardiotoxic enantiomers of verapamil and its analogues may provided clinically useful compounds. A number of other calcium channel blockers structurally dissimilar to verapamil, have been found to be effective circumvention agents. Nifedipine proved to be an effective agent in the reversal of resistance to a number of cell lines in vitro (Tsuruo et al.,1986; Onada et al.,1989).This compound proved effective for the reversal of resistance to DLKP-A (RMR of 2.988), OAW42-A (RMR of 2.85) and to a lesser extent for HEP-2A, HEP-2B and SKLU1-A. It is noticable that nifedipine did not affect the reversal of resistance in CHrC5, this indicats that the mode of action of nifedipine is markedly different to that of verapamil. However the noticable advantage of nifedipine over verapmil is the marked reduction in cardiac

toxicity. Other dihydropyridines that have been proven to be particularly effective are Diltazem (Kolhs et al.,1986), Niludapine (Yoshinari et al.,1989), Nicardipine (Tsuruo et al.,1986). Also a number of dihydropyridine analogs have been found to be particularly potent circumvention agents. NK-138 and NK-194 (Nogia et al.,1989), BS300 and BS304 (Yoshinari et al.,1989), Carovine (Tsuruo et al.,1983), PAK-104P (Shudo et al.,1990), N-Solanesyl-N, N'bis(3,4 -dimethyoxybenzyl) Ethylenediamine (Kamiwatari et al.,1989) Prenylamine (Tsuruo et al 1983a), Bepridil (Schuurhuis et al.,1987), (Shinoda et al.,1990), NK-242, (Kiue et al.,1990) and AHC-52 (Shinoda et al.,1989). As can be noted a number of calcium channel blockers from different classes have been identified as agents capable of circumventing MDR. Unfortunately, because of the cardiovascular effects of these agents their clinical use is limited. However several of the dihydropyridine chemosensitizers that lack calcium channel blocking activity hold promise as relatively specific agents. Of those calcium channel blockers in clinical use, bepridil and nifedipine appear to be the most promising for continued pre clinical developmental research on for the in vivo reversal of drug resistance.

Table 4.2. Summary of Studies Using Verapamil to Reverse MDR.

Verapamil (μM)	Cell Line	Cytotoxic -Fold Resistance	-Fold Resistance	Reference
6.6	P388/VCR	VCR(31)	122	Tsuruo et al.,
		VLB(7)	7	1981.
2.2		VCR(31)	34	
		VLB(7)	7	
3.3	P388/VCR	VCR(20)	26	Tsuruo et al., 1982.
10	P388/VCR	VCR(20)	40	Tsuruo et al., 1985.
3.3	P388/ADM	DOX(43)	12	Tsuruo et al.,
6.6			13	1982.
10	P388/ADR	DOX(27)	9	Tsuruo et al.,
		DAU(19)	9	1985.
30	P388/ADR	DOX(40)	28	Ramu et al., 1984.
10	P388/ADR	DOX(51)	11	Klohs et al.,
		DAU(38)	9	1986.
5	P388/DOX	DOX(100)	6	Ford et al., 1990.
2	P388/ADR	DOX(69)	9	Yoshinari et al.,1989.
40	EA/DAU	DAU(5)	4	Slater et al., 1982.
5	EA/DAU	DAU(5)	5.6	Schested et al.,1990.
6.6	K562/VCR	VCR(17)	61	Tsuruo et al.,
		DOX(3)	4	1982.
6	C6/DOX	DOX(20)	10	Huet et al., 1988.
1	CHO/B30	DAU(100)	8	Cano-Gauci et al.,1987.
10	8226/DOX40	DOX(50)	40	Bellamy et al.,1988.
1	8226/DOX6	DOX(9)	4.5	Lehert et al.,
0.5	8226/DOX6	DOX(9)	3.8	1991.
0.1	8226/DOX6	DOX(9)	2.4	
16	MGH-U1R	DOX(20)	2.2	Long et al., 1990.
10	MCF-7ADRr	DOX(100)	1	Fine et al.,
		VCR(100)	10	1988.
20	MCF7-ADRr	DOX(100)	7	Kramer et al., 1988.
5	MCF-7ADRr	DOX(200)	13	Ford et al.,
		VLB(100)	33	1990.
		VCR(74)	15	
		COLH(40)	4	
2	2780-ADM	DOX(100)	4	Schuurhuis et al.,1987
1	2780-ADM	DOX(170)	6	Rogan et al., 1984.
1	1847-ADM	DOX(5)	6	
10	CEM/VLB100	VLB(420)	22	Zamora et al., 1988.

Table 4.2. Summary of Studies Using Verapamil to Reverse MDR. (Cont'D)

Verapamil (μM)	Cell Line	Cytotoxic -Fold Resistance	-Fold Resistance	Reference
10	CEM/VLB100	VLB(930)	21	Beck et al., 1988.
10	CEM/VLB.10	VLB(420)	17	Cass et al.,
5	HL60/ADR	DOX(NR)	1.2	1989.
10	HCT-8	DOX(NR)	1.2	
	Colon 26	DOX(NR)	4	
	LOVO	DOX(NR)	3	
10	HT29	I.R.	1	Spoelstra et
	Colon 26		7.6	al.,1991.
	COLO 320		4.6	
	SW1116		2.1	
0.7	Lewis Lung	I.R.	1.6	Tsuruo et al.,
6.6	Lewis Lung	I.R.	2.6	1983.
6.6	B16	I.R.	2.5	
6.6	C-26 Colon	I.R.	12	
6.6	C-38 Colon	I.R.	6.4	
10	HT1080/DR4	ADM(55)	7.4	
10	HT69AR	ADM(NR)	4.9	
6.6	NCI-H69/Cx	DOX(85)	19	Twentyman et
		VCR(750)	72	al.,1986.
	MOR/DOX	DOX(12)	5	
	COR-L23/DO	DOX(12)	4	
20	KB-CHr			Fojo et al.,
	(8-5-11-24)	COLH(22)	63	1990
		DOX(21)	23	
		VLB(21)	38	
		VCR(73)	183	
5	KB-VI	VLB(500)	4	
		VLB(500)	15	Ford et al.,
		VCR 2000	8	1990.
		DOX(200)	30	
		COLH 166	20	
6	MES-SA/Dx5	DOX(100)	7	Harker et al., 1986.

NR - Not Reported I.R.-Intrinsically Resistant

Table 4.3. Summary of In Vivo Studies with Verapamil.

Concentration Verapamil : Drug ug/ml		Tumor	Schedule	ILS %	Reference
0	VCR 0.03	P388/VCR	QD X 10	0	Tsuruo et
0	0.2			0	al.,1981
50	0.1			34	
50	0.2			29	
100	0.03			29	
100	0.1			45	
0	DAU 0.4	Ehrlich	QD X 5	8	Slater et
25	0.4	Ascites		120	al.,1984
50	0.4	EA/DR		106	
0	DOX 0	P388/DOX	QD X 8	2	Randal et
0	1			7	al.,1988.
75	1			25	
0	BOU 0.6	Sarcoma	QD X 4	0	Chitnis et
10	0	180		5	al.,1985.
10	0.6			45	
100	VCR 0.1	P388/VCR	QD X 10	45	Tsuruo et
125	ADM 1.0	P388/ADM		37	al.,1983.

Abbrevations : BOU-Bouavidin,EA/DR-Ehrlich ascite /daunomycin resistant,QD -1 time/day,ILS - Increased Life Span.

4.9.2. Calmodulin Antagonists.

These agents possess the ability to inhibit calmodulin (CaM) mediated processes such as activity of the Ca^{++}/CaM dependent form of cyclic nucleotide phosphodiesterase (Levin et al.,1976). Nontoxic concentrations of the phenothiazine antipsychotic, trifluroperazine, caused a 5 to 10 fold increase in VCR and DOX toxicity 20-fold and 40-fold resistant P388/VCR and P388 /ADM cells respectively (Tsuruo et al.,1982) and fully reversed 17 fold K562/VCR resistance to vincristine. A large range of other CaM antagonists has been proven to be effective circumvention agents: Thioridazine (Akiyama et al.,1986), Chlorpromazine (Ford et al.,1989), Prochloroperazine (Ford et al.,1989), Fluphenazine (Ford et al.,1990), cis-Flupentixol (Ford et al.,1989), trans-Flupenthixol (Ford et al.,1990), cis- and trans-Chlorprothixene (Ford et al.,1990) and cis- and trans-Clopenthixol (Ford et al.,1990). By the examination of the structure function relationship of a series of 22 phenothiazines for circumvention of adriamycin resistance (Ford et al .,1989), hydrophobicity of the tricyclic ring and the structural features of the amino side chain were found to be independently important for circumventing ability. The compound that appears to be the most suitable for <u>in vivo</u> use is trans-fluxpenthixol. Clinical trials have shown that cis-flupenthixol was far more effective than trans-flupenthixol but that the latter drug was far less toxic (Johnson et al.,1978). It has been found that cis-flupenthixol is a potent antagonist of dopamine receptors (Huff et al.,1984). In mouse trials trans-flupenthixol has been found to have 100-1000 fold less potency than the active cis-isomer. In addition, <u>in vitro</u> studies demonstrate that [3H]trans-flupenthixol completly lacks the

specific dopamine receptor of [3H] cis-flupentixol (Hyttell et al.,1984), hence resulting in lack of extrapyramidal side effects. Because extrapyramidal side effects have proven to be a limiting factor in phase 1 trials that combined trifluroperazine with bleomycin (Hait et al.,1989a) or adriamycin (Miller et al.,1988). Further preclinical trials are necessary to establish the efficacy of this compound in the clinic.

4.9.3. Non Toxic Antracycline and Vinca Alkaloid Analogues.

Soon after the discovery that MDR cells accumulate less drug because of an active transport (Skovsgaard et al.,1978), Skovsgaard et al.(1980) tested the hypothesis that a specific drug transport pump would be competitively inhibited by an excess of a nontoxic substrate. A suitable anthracyline structural analog for this purpose was N-acetyl-daunorubicin, which lacks the free amino group of daunomycin essential for DNA intercalation and thus has a lower affinity for DNA resulting in less nuclear accumulation, less cytotoxicty and higher cytoplasmic concentrations. This compound has only been tested in Ehrich ascites cells (Skovsgaard et al.,1980) and it was found that a 1:20 combination of n-acetyl-daunorubicin with daunomycin, increased the life span in mice with MDR Ehrlich ascites innoculated intraperitoneally compared to no effect with either drug alone. Inaba et al.(1984) analysed the chemosensitizing effect of three additional anthracycline analogs on MDR cells and found enhancement of vincristine toxicity in P388 cells in vitro when used in 100 fold excess. One of these analogs also partially reversed the cross resistance to daunomycin, indicating that either that a similar drug transport mechanism effects both the anthracyclines and vinca

alkaloids or else that dauomycin analogs compete with both anthracycline and vinca alkaloids for seperate binding sites on the P-170. Similary a number of nontoxic vinca alkaloid analogs have been studied, such as vindoline. This was found to effectively reverse the primary resistance and the cross resistance of MDR P388/ADM and P388/VCR (Inaba et al.,1986). In order to understand the mechanism of these effects,it would be desirable to establish whether these non toxic analogs competitively inhibit the photoaffinity labelling of the photoactive vinblastine analog 125 [I]-NASV (Akiyama et al.,1988) or the photoactive verapamil analog 125 [I]NASAV (Safa et al.,1986). This class of circumvention agent is of particular interest because the modifiers themselves possess cytotoxic activity. The combination with other chemotherapeutics, both at subtoxic doses may produce a combination with increased therapeutic index of more relevance clinically.

4.9.4. Steroid and Hormonal Compounds.

Prompted by the discovery of high levels of MDR1 mRNA in the pregnant murine uterine uterus (Arceci et al.,1988), Yang et al.(1989) recently studied the effect of various steroids on MDR cells. They found that progesterone and deoxycorticosterone, but not estradiol, caused an increase in labelled vinblastine accumulation in MDR J7.V1-1 murine macrophages, similar in degree to equivalent doses of verapamil. Furthermore progesterone was the most potent in a series of steroids tested for inhibition of azidopine labelling of endometrial P-170 as well as inhibition of labelled vinblastine or vincristine binding to MDR cell membranes (Naito et al.,1989). This suggests the possibility that certain hormones may be the natural substrates for P-170 and that the less

physiologically active steroid hormones may be capable of circumventing drug resistance. It has been that found the antiestrogens tamoxifen, toremifene and other structurally related triparol analogs can partially overcome resistance in P388/DOX cells and MCF-7 ADRr cells independently of their effects on estrogen receptor (Foster et al.,1988; DeGregorio et al.,1989; Berman et al.,1991).

4.9.5. Miscellaneous Hydrophobic Cationic Compounds.

A large number of compounds found to be effective for circumvention of MDR cannot be grouped in the classification of calcium channel blockers or CaM antagonists and are not otherwise related pharmacologically. Most of these compounds are amphipathic and lipophilic in nature and share a broad structural similarity that includes a heterocyclic ring nucleus separated at a distance from a cationic,amino group (Hofsli et al.,1990). Chemosensitising agents as varied as the antiarrhythmics amidorone (Chaffert et al.,1986) , an inhibitor of cyclic AMP dependent protein kinase C, H-87 (Miyamot et al.,1990), the lysomotropic agents atropine,propranol and amantadine (Shiraishi et al.,1986; Zamora et al.,1988),the alkaloid derivative cepharanthine (Shiraishi et al.,1987), Cremphor EL (Woodcock et al.,1990) a common vehicle for water insoluble vitamins and drugs, the antimalarial agent quinacrine (Inaba et al.,1988 ; Zamora et al.,1988), the indole alkaloids reserpine and yohimbine (Beck et al.,1988; Pearce et al.,1989), NC-190 , A benzophenazine derivative (Tsuruo et al.,1990) the platlet anticoagulant dipyridomole (Howell et al.,1989; Ramu et al.,1989), Solutol H515 (Coon et al.,1991), Erthroid differentiation factor (Activin A) the efficacy of this compound suggests that

differentiation inducers may be useful in chemotherapy of Leukemic cell lines (Okabe-Kado et al.,1991) and the antibiotics erthromycin (Hofsli and Nissen-Meyer ,1989a,b), cefoperaone and ceftriaxone (Gosland et al.,1989) have been reported to partially circumvent drug resistance and to increase intracellular accumulation of drug. Whether many of these compounds are acting in a similar pharmacological manner to circumvent MDR or whether numerous drug target interaction are capable of producing this effect, (or indeed depending on the degree of the hydrophobicicty of the agent and its ability to cause membrane pertubations leads to decreases drug accumulation) is as yet unclear. Four agents from this classification were analysed for their effectivness in the reversal of resistance, quinine, quinidine (Dextroisomer of quinidine) and the lysosomothropic agents, chloroquinine and caffeine. Quinidine and quinine have previously reported to be effective circumvention agents both in vivo and in in vitro (Tsuruo et al.,1984; Pommeremke et al.,1990; Lehert et al.,1991). In our studies quinine was found to be an effective agent for the reversal of resistance to DLKP-A (RMR 3.25), SKMES1-A (RMR 3.03) and SKLU1-A (RMR 2.27). Quinidine was found to be effective in the reversal of resistance to CHrC5 (RMR of 4.39), HEP-2A (RMR of 3.3), HEP-2B (RMR of 3.5) and SKLU1-A (2.69). As highlighted by Pommerenke et al.(1990) a higher concentration of quinidine is required to elicit a circumvention effect (25 fold in the cases of our studies and 33 fold in the cases of Pommerenke et al.(1990). It is noticable that quinidine and quinine display different circumventing affects, both compounds are effective on different cells lines. This could be due to different mechanism of action because of their differing steroisomeric characteristics. Both quinine and quinidine have been found to affect PKC activity, this could also give an indication to

their mode of action as circumvention agents. A very interesting procedure was highlighted by Mickisch et al.,(1991) that transgenic mice that express the mdr1 gene in bone marrow represent a rapid system of identification of agents that are able to reverse resistance. The drugs that were tested in this model were verapamil, quinidine and quinine. Verapamil and quinine, both at levels suitable for human trials produced only partial sensitization but when these compounds were used in combination complete reversal of resistance was noted. This observation could imply high clinical efficacy for the use of a combination of these agents at clinical non toxic levels. These observations indicate the need for clinical trials using both these compounds,at concentrations that are not toxic in vivo. Another system (Horio et al.,1991) indicates the efficacy of quinidine, using an in vitro ATP dependent multidrug transport system using vesicles isolated from resistant cells. The further efficacy of quinidine and quinine need to be ascertained in the clinical situation.

The lysosomotropic agent chloroquinine was found not to be a very effective circumvention agent for any of the cell line in our study, with highest efficay noted for HEP-2A and HEP-2B with RMRs of 2.043 and 1.7 respectively. This agent was effectively used for the circumvention of resistance to KB-CHr-24 cells with a RMR noted of 5.6, this level of circumventing ability was noted with any of the cell lines in our study. The affect of caffeine was only ascertained on one cell line DLKP-A with an RMR of 2.17. Further work with this compound is necessary on a number of cell lines, exhibiting different mechanisms of resistance.

4.9.6. Cyclosporins.

Structurally and pharmacologically cyclosporins are very different to other classes of circumvention agents. CsA consists of a hydrophobic cyclic peptide ring of 11 amino acids. Although there were several reports that CsA could potentiate various cytotoxic drugs in non resistant cells (Kloke et al.,1985). It was found that CsA caused a three to four fold circumvention of MDR in Ehrilch ascites (Slater et al.,1986). However, there are some conflicting reports, whereby CsA was found to circumvent MDR in resistant cells only by Twentyman et al.(1987), Twentyman et al.(1988b), Hait et al.,(1989b), but was reported to also produce a drug potentiation to certain sensitive as well as resistant cells (Chambers et al.,1989; Gaveriaux et al.,1989). The mechanism of action of CsA appears to be more complex than the chemosensitizers described to date. Other cyclosporins have been investigated for their ability to circumvent MDR. Cyclosporin C, cyclosporin G and cyclosporin H have been found to be very effective (Twentyman et al.,1987, Gaveriaux et al.,1989) in the circumvention of Small Cell Lung Carcinoma (SCLC). In summary, although the cyclosporins are clearly agents that can circumvent drug resistance, it is uncertain whether the mechanism is through a direct effect on the P-170, through an indirect effect on the cellular metabolism, through direct potentiation of chemotherapeutic drug toxicity, or a combination of all these.

4.9.7. Agents That reverse At-MDR.

Much of the emphasis to date has been reversal of P-170 mediated resistance. New approaches to circumvention of At-MDR will come from a better understanding of the mechanisms of action of these classes of Topo 11 acting drugs and the biochemistry of this form of resistance (Kaufmann et al.,1990). Preliminary results indicate (Wolverton et al.,1990) that At-MDR CEM/VM1 cells are collaterally sensitive to the cytotoxic effects of ultraviolet radiation , nitrogen mustard and 3-aminobenamide indicating that abberant mechanisms DNA repair may determine resistance in some At-MDR cells. The present strategies for circumvention of Topo 11 mediated MDR is the use of agents that are not substrates for DNA topoisomerase 11, one such class of compounds is aminoacridine analogs (Baguley et al.,1990). Similarly five anthracyline analogues are as cytotoxic in the At-MDR cells as they are in drug sensitive parents: N-benzyladriamycin-14 valterate (Sweatman et al.,1990), morpholino- and methomorpholinodoxorubicin (Grandi et al.,1990), 4'-ido-doxrubicin and 4-demethoxydaunorubicin (Robert et al .,1990). In contrast to these results , Pearce et al.(1990) have found that an N for C substitution in the 2' moiety of VP-16, creating "aza-VP" completly reduced the cytotoxic and topo 11 inhibitory activity of VP-16. This reveals the significance of the electronic and stereochemical aspects of epipdophyllotoxin-topo 11 mediated DNA interactions. These results demonstrate the importance of rational drug design, molecular modeling and structural activity studies in At-MDR research. Reversal of Topo 11 At-MDR has also been reported with 3'DEamoino-3'-Morpholino-3-deox-10 Hydroycorinmycin, (Horichi et al.,1990). BSO has been described in

a large number of systems which have GSTπ mechanisms of MDR as an effective agent in vivo (Ford et al.,1990). However, because of endogenous activity of BSO in destruction of GSTs, its use in the clinical situation could be limited, due to the large number of essential GST dependent processes.

4.9.8. Non Steroidal Anti-Inflammatory Agents (NSAIAs)-Aspirin.

Both standard aspirin and generic aspirin were found to be effective in the reversal of resistance for all the cell lines tested. Both these agents were used at clinically relevant concentrations; a maximum tolerated dose of aspirin of 4g/day (for most adults) has been reported. The effect of standard aspirin on CHrC5 was substantial; with an increase in the concentration in aspirin the RMR also increases (2.4 to 3.44). The same affect was not noted with generic aspirin; with an increase in concentration of aspirin from 0.1 to 0.25mg/ml, an increase in the RMR is noted but a subsequent decrease in RMR is noted at concentration of 0.5mg/ml; the reason for these observations is unknown. The initial studies with aspirin were preformed with generic aspirin. To ensure that the effect being noted was due to aspirin and not to one of the agents used in the formulation of generic aspirin, standard aspirin was incorporated into the study. It was observed for a number of the parental cell lines, that the adriamycin toxicity profile is also affected. This indicates the broad range of action of aspirin in that it can circumvent resistance both in sensitive and resistant cells,which have been shown to have altered biochemical and pharmacological features. A similar affect was noted with DLKP-A, with a increase in concentration of both

standard and generic aspirin. An increase in the RMRs of both DLKP and DLKP-A is noted in the presence of aspirin (Table 3.11.3). The greatest circumvention affect is noted with CHrC5 and DLKP-A with a lesser decrease in IC50 noted for SKMES1-A,SKLU1-A,HEP-2A and HEP-2B and OAW42-A.

The mechanisms of action of aspirin are very varied. It has been shown that the effectiveness of the compound is largely due to its capacity to inhibit prostaglandin synthesis (Abramson et al.,1989); however this hypothesis does not account for all the effects of aspirin at all dosages. Higher doses of aspirin are required to suppress inflammation than are required to inhibit prostaglandin synthesis; moreover sodium salicyclate is an effective inhibitor of cycloxygenase at all concentrations used clinically (Morris et al.,1985). Alternative hypotheses have therefore been proposed to explain the antiflammatory affects of aspirin. Additionally a number of mechanisms of action of aspirin could contribute to its chemosensitizing affect. When exposed to ligands such as the chemoattractants C5a of FMLP (Abramson et al.,1990) , neutrophils are activated as part of a general stimulus-response coupling to phospholipid remodelling (Weismann et al.,1988). The twin signals (See figure 4.1) are provided by mobilization of intracellular calcium and the activation of protein kinase C. Aspirin inhibits the aggregation of neutrophils *in vitro* and *in vivo* , and shows inhibitory effects additive to those of stable PGs on the generation of superoxide anion, a product of inflammatory cells. The activity of aspirin in terms of PKC may be important as regards its circumventing affect. PKC is known to affect the phosphorylation of P-170, this may in turn alter the function of P-170 producing a circumventing affect (Aquino et al.,1988) Aspirin

has also been found to disrupt a variety of processes at the plasmalemma that regulate the generation of twin signals. These effects are most likely due to the capacity of aspirin,which is a planar lipophilic molecule, to insert into the lipid bilayer, this process results in the disruption of signal transduction through the membrane. Aspirin was also noted to affect the viscosity of neutrophil plasma membranes (Abramson et al.,1990). This could be a possible mechanism of circumvention of resistance, by the altering of the fluidity of the membrane, this could possibly lead to increases intracellular concentration of drug. Aspirin also interferes with the chemical mediators of the kallikrein system, inhibits granulocyte adherence to damaged vasculature, stabilises lysosomes, and inhibits the migration of polymorphonuclear leukocytes and macrophages to the site of infection. Finally, aspirin was shown to be effective in the increase in intracellular accumulation of Ca^{2++} (Flescher et al.,1991). However, increases in intracellular Ca^{2++} is not a specific requirement of a circumvention agent (Nokia et al.,1990) calcium antagonising ability is a feature of a number of useful circumvention agents. This feature of the mechanism of action of aspirin could also contribute to its circumvention affect.

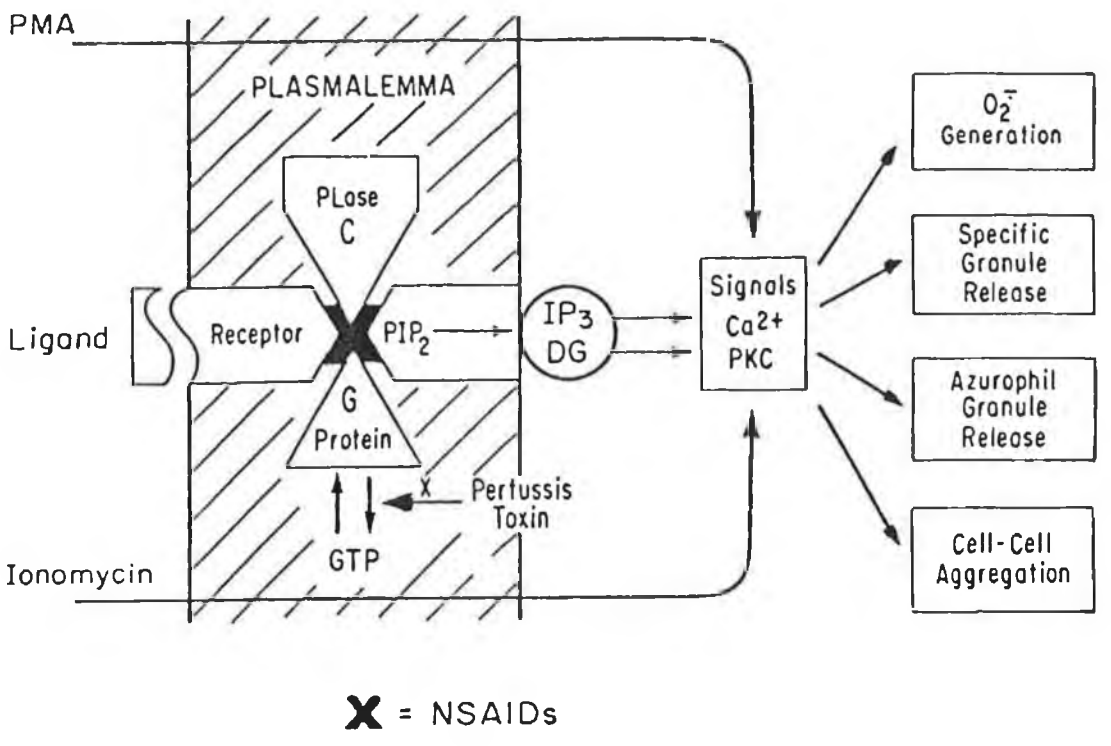


Figure 4.1 :Stimulus -Response Coulping 'Twin Signals' in the neutrophil. X represents the insertion into the lipid bilayer and the potential uncoulping of protein-protein interactions by non-steroidal antiinflammatory drugs (NSAIDs)-Aspirin.

A tremendous amount has been learned about MDR at the molecular, biochemical, and cellular levels, but little as yet as been translated into clinical therapeutic gains. However much of the data arising from literature relevant to circumvention of MDR suggests that chemosensitizers will play an important role in future chemotherapy regimes. Most of the *in vivo* studies have used verapamil, which has been proven to be very cardiotoxic, thus it is not a suitable chemosensitizing agent for routine use in the clinic. Clearly, the indentification of more potent and less toxic chemosensitizers remains a critical parameter to the routine use of chemosensitizers. Our studies indicate that a number compounds including aspirin (both standard and generic), nifedipine, quinine and quinidine are be suitable candidates for futher *in vivo* study. The *in vivo* use of quinine, quinidine and nifedipine have all been proven in animal systems ; there is a need for future studies to evaluate the use of these compounds in human clinical trials. Aspirin was never reported previously as possessing any circumventing ability, however it proved to have an affect on all the cell lines tested. This indicates the need for future *in vivo* studies with animal model systems to see if a similar effect is noted *in vivo*. If these results prove positive, further studies in the clinical situation would be necessary to ascertain the true value of aspirin as a circumvention agent. So much information is available on the *in vivo* uses of aspirin, if this compound was proven effective in clinical trials it could be introduced into standard chemotherapeutic regimes very quickly and make a major impact on standard chemotherapeutic regimes.

4.10. Future Developments.

4.10.1. Development of Quantitative PCR.

To improve the ability to detect low levels of P-170 expression in clinical samples, a number of investigators (Noonan et al.,1990; Murphy et al.,1990; Horikoshi et al.,1991; Sugawara et al.,1991) are starting to use PCR. For application to the MDR area, PCR has been found to be a highly sensitive, specific and quantitative procedure for measuring the levels of mdr1 mRNA in clinical samples. Noonan et al.,(1990) used PCR to analyse mdr1 gene expression in normal tissue, MDR cell lines and more than 300 clinical samples. The most critical parameter in the PCR procedure relevant to MDR, is that accurate quantification requires the demonstration of an exponential range that varies among samples, hence it is of the utmost importance to determine the exponential range for each sample. Normalisation of results can be determined by the independent amplification of a control gene such as ß2-microglobulin, where by the latter is also evaluated in exponential range. The results have indicated to date that low level mdr1 expression undetectable by conventional assays, frequently occurs in tumors before chemotherapy. As in the case of immunofluorescence procedures, adequate histology must be preformed with each sample to ascertain the quantity of neoplastic tissue present, additionally no vascular tissue could be present in the sample to be analysed, as it highly positive for P-170. Provided adequate controls are a routine part of the PCR procedure and the tissue under analysis is scrutinised thoroughly before RNA extraction PCR technology could serve as a very important diagnostic tool for the detection of mdr gene products in human tumor samples.

4.10.2. Human Gene Therapy as a future Goal in Clinical Studies.

Human gene therapy involves the introduction of genes which are frequently unselectable into a stable expressing cell type or tissue to correct an inborn error of metabolism or provide a therapeutic gene product. This technology could have a direct effect on the routine treatment of patients with tumors that fall into the MDR classification of drugs. Because bone marrow supression is a common consequence of chemotherapy, it might be possible, using gene therapy, to make the bone marrow of patients who will be subjected to cancer treatment resistant to chemotherapeutic agents. This could be achieved by removing the bone marrow, introducing the MDR gene into the marrow, and returning the bone marrow to the patients. Retroviral vectors carrying the mdr1 gene could be used for this purpose;in fact, the mdr1 cDNA has already been introduced into the mouse bone marrow cells and shown to protect them from vinblastine and colchicine (Mc Lachlin et al.,1990). To develop such therapies, transgenic mice were created in which the human mdr1 gene is expressed in bone marrow cells (Mickisch et al.,1990). These mice are resistant to the bone marrow suppressive effects of adriamycin and other drugs in the MDR group,this study suggests that bone marrow replacement therapy may be successful in humans. In addition these mice provide a very useful model for the study of efficacy of certain compounds to reverse MDR (See section 4.9 for details). It is relatively simple to show that a compound has the ability to reverse resistance in in vitro cell culture systems, but it is essential to know if these agents will work in animals before human trials can begin. The method in which these experiments would work would involve the monitoring of the white blood cell count of a

transgenic mouse that is drug resistant because of expression of the mdr1 gene in its marrow. The addition of reversing agents causes the white cell count of such mice to fall in the presence of cytotoxic agents and provides proof that reversing agents work in animals (Mickisch et al.,1990).

4.10.3. Anti-MDR antibodies.

Another strategy for circumvention of MDR is the possibility of inhibiting protein function by the use of immunotoxins, this could be the basis of new therapeutic protocols. The in vivo use of immunotoxin conjugated anti P-170 antibody would seem to be impossible due to its wide expression in normal tissue, but this approach could be used for purging autologous bone marrow until subsequent transplatation. Some experimental procedures carried out with the MRK16 antibody (Willigham et al.,1987) suggest that P-170 is not efficiently internalised or rapidly recylced. However since only a small amount of immunotoxin needs to enter cells to be toxic, a MRK16 toxin conjugated antibody could be effective in killing cells expressing P-170. Dinota et al (1989) used incubation with MRK16 followed saporine-6 conjugated antimouse serum in order to kill the LOVO/DOX MDR cell line. The inhibition of the clonogenic growth of this cell line was 96% and 91% with saporine concentrations of 10^{-7} and 10^{-8} M respectively. Using a cellular suspension made up of normal bone marrow cells (90%)and LOVO/DOX mdr cells (10%) saporine conjugated antibodies killed 99% of the tumor cells; myeloid precursor cells were not affected. Also Aihara et al.,(1991) used a combined approach for purging MDR leukemic cell line bone marrow using a monoclonal antibody and chemotherapy. MAB Moab17F9 was effectively in the selective

purging of MDR cells from bone marrow. Using two different cell lines, K562/DOX and CEM/VLB, it was found that three rounds of 17F9 and complement (C') treatment results in the 96.4% kill of K562/DOX and 1005 kill of CEM/VLB. Mixtures of malignant and normal bone marrow results in 99.85% removal of K562/DOX and 99.91% removal of CEM/VLB.

4.11. Conclusions.

Seven multidrug resistance variants were established ; HEP-2A, HEP-2B, DLKP-A, OAW42-A, SKMES1-A, SKLU1-A and DLRP-A. All the variants were found to be multiply drug resistant but to markedly varying degrees. Generally the most resistant cell lines exhibited the greatest diversity and highest range of cross resistance. The MDR variants displayed significantly altered biochemical , biophysical and genetic characteristics from their parental counterparts. The MDR variants were found to be heterogenous in the level of drug resistance with more than one drug resistant population present. A resistant population in the parental population of DLKP possibly accounted for the ease of adaptation of the DLKP-A variant. MDR cells were found to be more sensitive to standard freezing procedures and standard subculture procedures. HEP2-A and HEP-2B the most sensitive to standard freezing procedures. From the cloning experiments before and after freezing it was evident that suboptimal freezing conditions had an overall affect on the whole population rather than on just the most resistant population. It was evident that the cell lines expressing the high levels of P-170 were most susceptible to standard freezing, this indicates the fragility of the cell membrane containing high levels of P-170. Sensitivity to trypsin was a feature of a number of the cell lines. In the cases of DLKP-A, HEP-2A and HEP-2B sensitivity to T.V. correlated well with the degree of resistance. However high levels of P-170 were evident in SKMES1-A but prolonged exposure of this cell line to T.V. had no adverse effect on the adriamycin toxicity profiles. This could indicate that over expression of P-170 must differ in some way in

SKMES1-A to DLKP-A, HEP-2A and HEP-2B; P-170 in SKMES1-A could possibly have a different effect on membrane structure , maybe through extensive phosphorylation. However, somewhat contradicatory to the above findings, the MDR variants were found to more mechanically sturdy to standard sonication. Alterations in the lipid composition of the plasma membrane of the MDR variants could possibly contribute to the increased mechanically strength of the MDR variants. A P-170 related mechanism of resistance was suggested in CHrC5, DLKP-A, SKMES1-A , OAW42-A, HEP-2A and HEP-2B from Western blotting, immunofluorescence and antisense techniques. However, one conflicting result was evident; HEP2-A and HEP2-B displayed very similar properties pharmacologically and by antisense techniques and immunofluorescence, but on Western Blotting HEP-2B displayed less immunologically reactive P-170, although this could be a technical artifact. There are indications of additional mechanisms of resistance to P-170 in a number of the cell lines. This was suggested by cross resistance to 5-flurouracil and cis-platin and also incomplete reversal of resistance of adriamycin by antisense oligomers. Further work on alternative mechanisms of MDR to P-170 is required in order to explain fully all the findings of this thesis. Cytogenetic manifestations of MDR (DMs) were evident in DLKP-A, HEP-2A and HEP-2B. Finally, drug resistance cell lines were generated by transfection with a complete murine cDNA for mdr1, pHAMDR1A. The drug resistance patterns observed were significantly different from those of the variants obtained by adaptation from the corresponding parental lines. It was found possible to circumvent MDR with a number of compounds at clinically relevant doses , verapamil, nifedipine, quinine, quinidine, caffeine, chloroquinine, standard aspirin and generic aspirin. The effect of each of the

compounds varied depending on the cell line in question, the most consistantly efficient compounds were found to be verapamil, standard aspirin, generic aspirin and quinidine. In the case of DLKP-A every circumvention agent under study was found to have an affect on reduction of RMR; this is a somewhat surprising finding. This could possibly indicate the presence of multiple mechanisms of resistance in DLKP-A. This was concluded from the diversity of the mechanisms of action of the circumvention agents under study.

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CHAPTER 5

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CHAPTER 6

APPENDICES

6.1. APPENDIX OF BUFFERS.

6.1.1. Carnoys Fixative

Acetic acid : Methanol
1 : 3

6.1.2. P-NP Substrate

0.1M Sodium Acetate, pH5.5
0.1% Triton X-100
10mM P-Nitrophenyl Phosphate.

6.1.3. DNA Gel Loading Buffer

12.5% Ficoll, by weight
0.2% Bromophenol Blue,by weight
6.7% 10 X Electrophoresis buffer,by volume.

6.1.4. 2 X Hepes -Buffered Saline (2X HBS)

1.63g NaCl
1.19g Hepes
0.023g $Na_2HPO_4.2H_2O$
Ultrapure $H_2O$to 100ml.

Adjust the pH to exactly 7.1 with 0.5M NAOH. Filter sterilise and store at 4°C

6.1.5. Lysis Buffer

10 mM KCL
1.5mM MgCl2
10 mM Tris/HCL ,pH 7.4
2 mM PMSF (added fresh just before use)

6.1.6. Sucrose Cushion

38% (w/v) Sucrose
10 mM KCL
1.5mM MgCl2
10mM Tris/HCL,pH 7.4
2mM PMSF

6.1.7. Washing Buffer

5 mM Tris/HCl ,pH 7.5
2mM PMSF

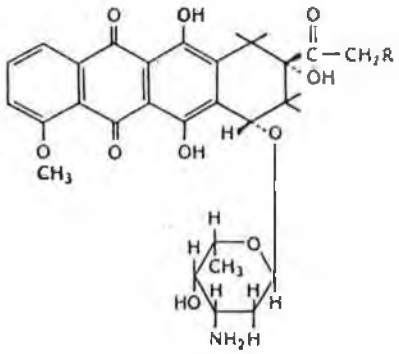
6.1.8. Storage Buffer

5 mM Tris/HCL ,pH7.5
0.25 M Sucrose
2mM PMSF

6.1.9. Acrylamide Stock Solution

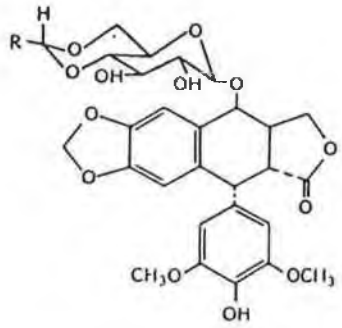
29.1 % (W/V) acrylamide
0.9 % (W/V) NN'-methylenebis -acrylamide

6.2.1. Adriamycin (Doxorubicin).



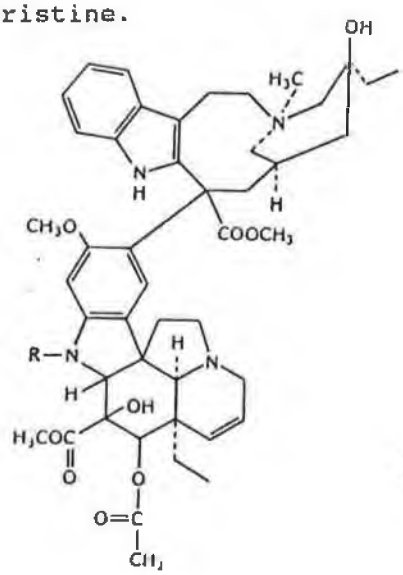
	R
Adriamycin	OH
Daunorubicin	H

6.2.2. VP16 and VM26.



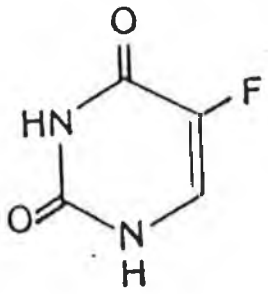
Epipodophyllotoxin analogs	R
VM 26 (NSC-122819)	
VP 16-213 (NSC-141540)	CH_3

6.2.3. Vincristine.

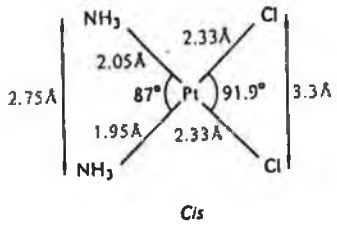


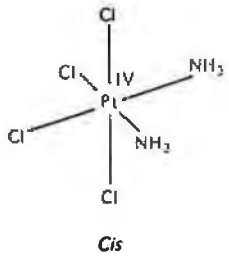
	R
Vincristine	CHO
Vinblastine	CH_3

6.2.4. **5-Fluoruracil.**

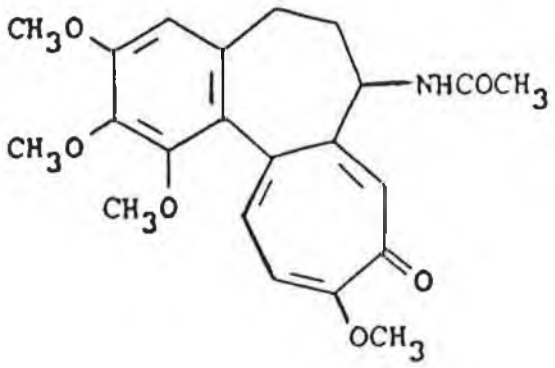


6.2.5. **Cis-Platium.**

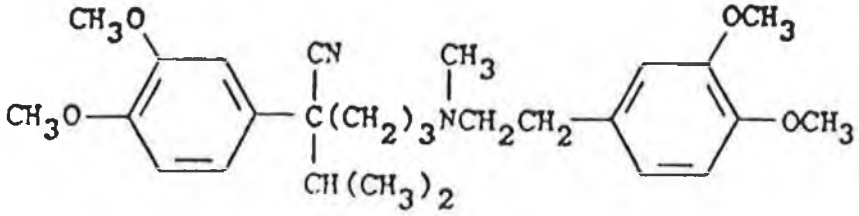




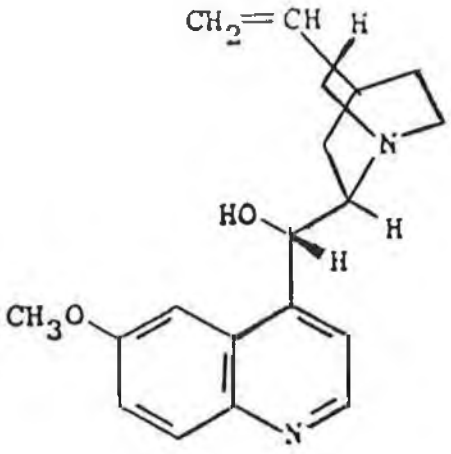
6.2.6. Colchicine.



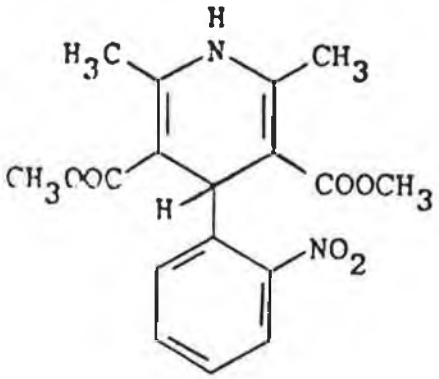
6.2.7. Verapamil.



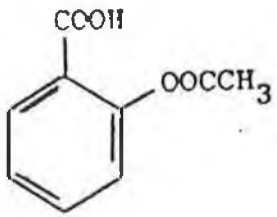
6.2.8. **Quinine/Quinidine (Steroisomers).**



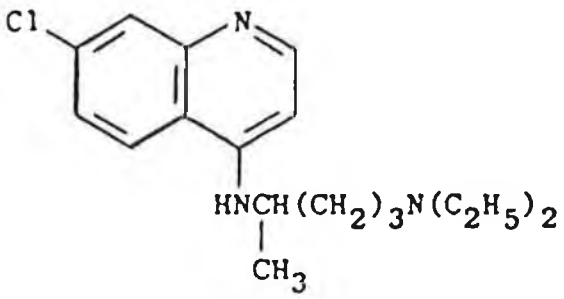
6.2.9. Nifedipine.



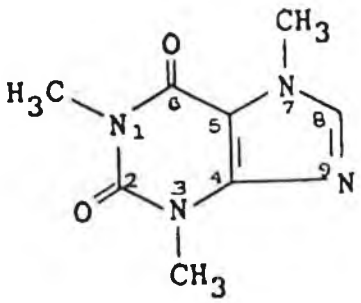
6.2.10. Aspirin.



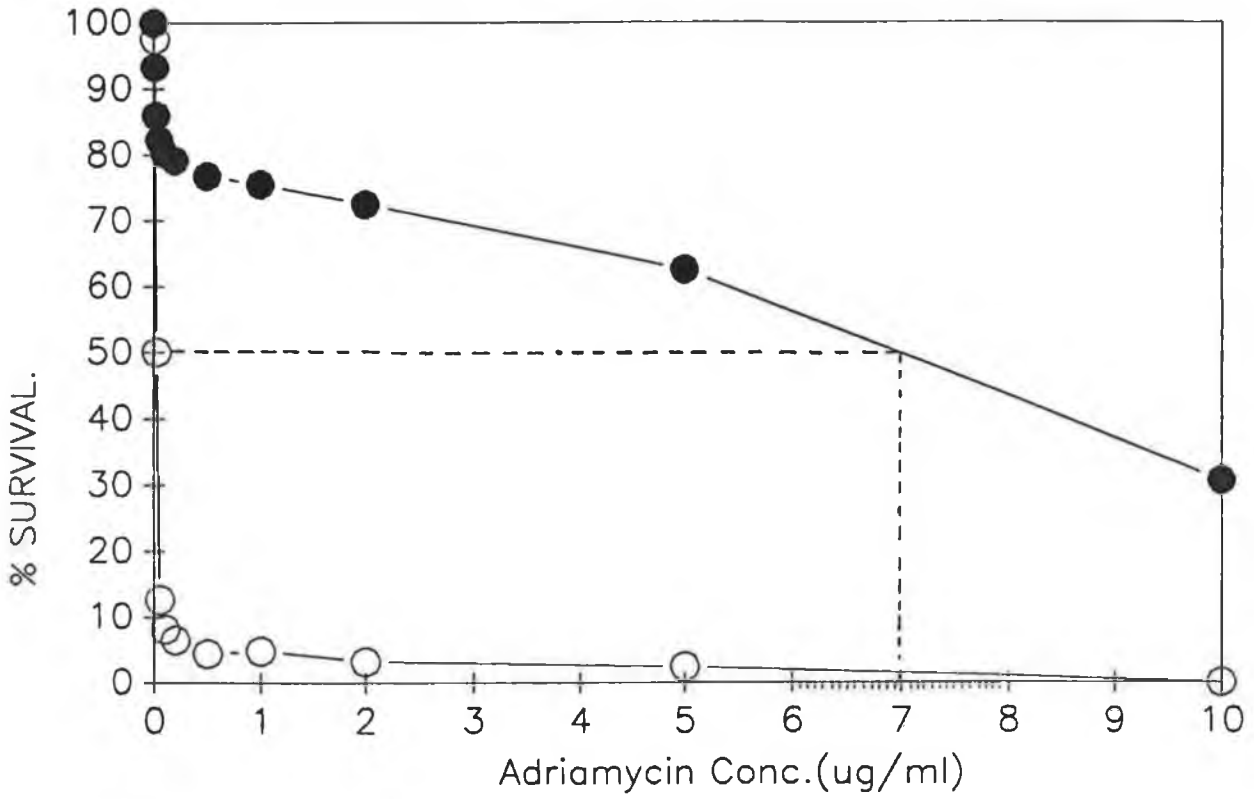
6.2.11. Chloroquinine.



6.2.12. Caffeine.



APPENDIX 6.3. SAMPLE CALCULATION OF IC50nM



Molcular Weight of Adriamycin = 580

IC50 μg/ml from above graph = 0.0025μg/ml

$$\text{Calculation} = \frac{7 \times 10^6}{580}$$

$$= 12068 \text{ nM}$$

APPENDIX 6.4. ABBREVIATIONS

ADR	Adriamycin
A.T.	After transfection
A.T.C.C.	Amercian Type Culture Collection
BCA	Bincinchonic acid
BOU	Bouavidin
bp	Base pair
B.S.A.	Bovine Serum Albumin
B.T.	Before Transfection
BSO	Buthionine Sulfoximide
C.A.	Circumvention Agent
COLH	Colchicine
C-PT	Cis-Platin
Conc.	Concentration
DEPC	Diethylpyrocarbonate
DME	Dulbeccos Modified Eagles Medium
DM	Double minute chromosome
DMSO	Dimetyl Sulfoximide
EA/DR	Ehrlich ascites/dauxorubicin resistant
EDTA	Ethlene diamino tetra-acetic acid
ECACC	European Collection of Animal Cell Cultures
FCS	Foetal Calf Serum
5-FU	5-Fluorouracil
GST	Gluathione-S Transferase
HBS	Hepes Buffered Saline
HCL	Hydrochloric Acid
HEPES	4-(2-hydroxethyl)-piperazineeethansulphonic acid

HSR	Homogenously Staining regions
IC50	Inhibitory concentration 50 percent
ILS	Increased life span
kb	Kilobase
MAB	Monoclonal Antibody
MDR	Multidrug Resistance
MEM	Minumum Essential Medium
M.W.	Molecular weight
n	Number of replicates
NBCS	New Born Calf Serum
NAOH	Sodium hydroxide
NSCLC	Non Small Cell lung Cancer
O.D.	Optical Density
PBSA	Phosphate Buffered Saline A
P-170	Glycoprotein M.W. 170,000
p.s.i.	Pounds per square inch
RMR	Resistance Modfying Region
r.p.m.	Revolution per minute
S.D.	Standard deviation
S.D.S.	Sodium Doceyl Sulfate
TE	Tris-EDTA
T.V.	Trypsin Versene
Topo 11	Topoisomerase 11
VCR	Vincristine
VM26	Teniposide
VP16	Vepesid (Etoposide)