Cytotechnology **10**: 75–89, 1992.

Multiple drug-resistance in variant of a human non-small cell lung carcinoma cell line, DLKP-A

Martin Clynes, Alice Redmond, Elizabeth Moran and Una Gilvarry
National Cell and Tissue Culture Centre/Bioresearch Ireland, Dublin City University, Glasnevin, Dublin 9, Ireland

Received 1 April 1992; accepted in revised form 4 September 1992

Key words: lung carcinoma cell line, multidrug resistance, MDR

Abstract

A 300-fold adriamycin resistant variant (DLKP-A) of the human lung squamous cell carcinoma line DLKP was established by stepwise selection in increasing concentrations of adriamycin. Different levels of cross-resistance were observed towards VP-16, VM-26, colchicine, vincristine and, somewhat unexpectedly, cis-platin. Resistance was stable for at least 3 months in culture in the absence of drug. P-glycoprotein overexpression was detected by immunofluorescence and Western Blotting, and a direct causal role for P-glycoprotein overexpression in the resistant phenotype was established by transfection with an *mdr1* specific antisense oligonucleotide. A modified cryopreservation procedure was necessary for the resistant variant line. The resistant population displays clonal heterogeneity with respect to resistance level. A higher frequency of double minute chromosomes was observed in DLKP-A when compared with the parental cell line.

Abbreviations: ADR: adriamycin; COLH: colchicine; C-PT: cis-platin; MDR: multidrug resistance; NSCLC: non-small cell lung carcinoma; VCR: vincristine; VP-16: etoposide; VM-26: tenoposide

Introduction

Chemotherapy has a significant role in control of metastatic cancers which cannot be cured by surgery because of their widespread dissemination within the body. The effective use of chemotherapy is limited, however, by both inherent and acquired resistance of many human tumours to chemotherapeutic agents. Combination chemotherapy was introduced in an attempt to overcome single agent resistance, but unfortunately many human tumours become multidrug resistant (MDR), that is they become resistant to a spectrum of drugs which are often chemically and mechanistically unrelated (Clynes *et al.*, 1990). An understanding of the mechanisms by which human cancer cells become multidrug resistant is important for design and selection of new drugs which either reverse the resistant phenotype or are directly toxic to MDR cells (Ford *et al.*, 1990; Citro *et al.*, 1991) and also for diagnostic purposes to assess in advance the likely response of a particular tumour to a specified chemotherapeutic regime (Dalton and Grogan, 1991).

The principal mechanisms of multidrug resistance reported to date have been:

1. Overexpression of a 170 kD membrane protein, P-glycoprotein (encoded by the *mdr1* gene) which acts as an ATP driven efflux pump for a variety of drugs including anthracyclines, vinca alkaloids and epipodophyllotoxins and thus reduces the effective cellular concentration of drug (Bradley *et al.*, 1988; Roninson, 1991). This glycoprotein is present also in many normal tissues (Gottesman *et al.*, 1991; Van der Valk *et al.*, 1990) and may function as an ATP-driven chloride selective channel (Valverde *et al.*, 1992). It has recently been suggested that P-170 may not act as a direct "channel" for drug efflux, but may instead enhance transit of substances already "dissolved" in the cell membrane (Higgins and Gottesman, 1992).
2. Reduced or aberrant expression of the enzyme topoisomerase II which is involved in resistance to adriamycin and epipodophylltoxins but not to vinca alkaloids (Beck, 1989).
3. Overexpression of glutathione-S-transferase (in particular the pi isoenzyme) and related enzymes (Moscow *et al.*, 1989).

Non-small cell lung cancer (NSCLC) is generally considered to be an unsuitable target for chemotherapy, even if surgery is ineffective. It has been observed clinically for many years, however, that chemotherapy causes tumour shrinkage in some patients with non-small-cell lung carcinoma. Recent work involving *in vitro* correlation with *in vivo* response has confirmed the existence of a range of different tumour sensitivities within the population of NSCLC patients (Volm *et al.*, 1991; Wilbur *et al.*, 1992). It seems therefore that further detailed study on mechanisms of inherent and acquired resistance in human NSCLC is warranted, since it could lead to improved therapy for this very serious type of cancer. Cell culture model systems are particularly important in the study of resistance mechanisms, since they allow detailed studies on cross-resistance patterns and direct investigations of resistance mechanisms (e.g., by antisense transfection and circumvention studies). The relevance of conclusions from *in vitro* studies must of course be validated by appropriate investigation (e.g., immunohistochemistry) in human tumour biopsies. This paper describes the selection and characterisation of a new multidrug resistant variant of a poorly differentiated squamous cell carcinoma line, DLKP, recently established in our laboratory (Law *et al.*, 1992) and compares its properties with those of some other published multidrug resistant human cell lines.

Materials and methods

Drugs. Doxorubicin hydrochloride (Adriamycin) was obtained from Farmitalia (Carlo Erba Ltd., (Barnet, Herts)) and cis-platin, VP-16, VM-26 and vincristine sulphate from Lederle (Laboratories Division, Fareham Road 1, Hampshire, PO13 0AS, UK).

Cells. DLKP is a cell line derived from a bronchoscopy of a 52-year-old male with a history of haemoptysis and dyspepsia. At bronchoscopy he had compression of the left upper lobe of the bronchus. Mediastinoscopy showed multiple enlarged nodes. Histology diagnosed poorly differentiated squamous cell carcinoma (Law *et al.*, 1992). CHrC5 was obtained from Dr Victor Ling, (Ontario Cancer Institute, University of Toronto, Ontario) and CHOK1 (CCL61) was obtained from the ECACC (European Collection of animal cell cultures, PHLS, Porton Down, Sailbury, UK). DLKP was cultured in DMEM/HAM's F12, CHOK1 was cultured in Ham's-F12 medium, and CHrC5 was maintained in MEM alpha. All cells were grown at 37°C in medium supplemented with 5% FCS and 20 mM Hepes (Sigma H-9139).

Adaptation of MDR variants

To generate the resistant variant, adriamycin was added (at a concentration permitting approximately 5% survival) until the cells appeared healthy and attaining high numbers; the drug concentration was then doubled. This process was continued, doubling drug concentration whenever the cells appeared to have adapted. The adapted cell lines are designated by an "A" after the name of the cell line, with the level of adriamycin resistance in

parenthesis. In comparison to other adapted cell lines established in our laboratory (Redmond *et al.*, 1990), DLKP-A selected very quickly. Subsequent experiments designed to investigate the clonal variation of parental cell lines showed that a resistant population was evident that accounted for approximately 4% of the total population. During the adaptation process it was observed that cells exposed to trypsin-EDTA (0.25% trypsin/0.01% EDTA) for greater than 10 min were very slow to recover in terms of both the growth of the cells and the resistance level. This phenomenon was previously observed with MDR variants of Hep-2 carcinoma cell line (Redmond *et al.*, 1990). Subculture in 0.05% EDTA or 3 min exposure in 0.25% Trypsin/0.01% EDTA were the methods ultimately found suitable for DLKP-A.

The time period for selection was as follows:

- 0.05 μg/ml – 0.8 μg/ml ADR November 1989 – September 1990;
- 0.8 μg/ml – 1.75 μg/ml ADR October 1990 – December 1990;
- 1.75 μg/ml – 2.1 μg/ml ADR January 1991 – March 1991.

Toxicity assays

A standard pretreatment procedure was adopted prior to the performance of any toxicity assay to ensure that the cells were in the exponential phase of growth, to improve the reproducibility of experiments. Pretreatment involved trypsinization and inoculation of 10^6 cells per 75 cm^2 flask 2 days prior to the performance of the assay. The cells were fed the following day and the cells plated and the assay started on day three. DLKP-A (2.1 μg/ml Adriamycin) was analyzed for cross-resistance profiles to a number of chemotherapeutic agents using the acid phosphatase procedure (Martin and Clynes, 1991). The drugs included in the study were adriamycin, VP-16, VM-26, vincristine, cis-platin and colchicine. Results are given for a typical experiment as mean ± SD (n = 4 – 8 wells). Each experiment was repeated at least three times and the IC50 varied by not more than ±20% for any results presented.

Stability of the adriamycin resistance. Stability was ascertained by the removal of drug-containing medium from the cells and subculture for up to 3 months, followed by the performance of an adriamycin toxicity assays to establish the stability of the MDR phenotype.

Sensitivity of the MDR variants to different cryopreservation procedures

An initial observation that the MDR cells were slow to recover from standard freezing procedures and that their drug resistance level appeared reduced, led to the study of variations in cryopreservation procedures. Cells were frozen at varying density (10^6, 2.5×10^6, 5×10^6, 1×10^7), varying serum (10%, 20%, 50% and 100%) concentration and varying concentration of cryoprotective agents DMSO (10%, 20%) (Sigma) or glycerol (10%, 20%). After freezing, the cells were stored in liquid nitrogen for 6 weeks. On thawing, a viability count was performed utilising trypan blue (0.4%; 1:5 dilution of dye in cell suspension). The cells were then grown in drug free medium for 7 days and a toxicity assay performed.

Cloning efficiency assay. Cells in exponential phase of growth were plated at 100 cells/500 μl per well in a 24 well plate (Costar 3524). Adriamycin at varying concentrations was added 24 h later. The plates were incubated for 10 days at 5% CO_2 at 37°C, then stained for exactly 10 min with 0.25% crystal violet and dried thoroughly. Colonies ≥25 μm in diameter were counted.

Immunofluorescence. A monoclonal antibody to an internal epitope of the C-terminus of the P-170 glycoprotein (C219) was used in this study (Georges *et al.*, 1990). Cells were trypsinized and diluted to 10^5 cells/ml. Fifty microlitres of this suspension was added to each well of a Dynatech multiwell slide and incubated at 37°C, 5% CO_2 for 24 h. Cells were washed three times in PBS, fixed and permeabilised in acetone (Pre-cooled at –20°C) for 20 min. Then 20 μl of primary antibody, (C219, 5 μg/ml) was added and the slides

incubated for 24 h at 4°C. Following three 30 ml PBS washes, 20 µl of FITC labelled second antibody (1:32 dilution) (Sigma F3008) was added to each well and incubated at 37°C, 5% CO_2 for 1 h. Slides were rinsed three times in PBSA, and then mounted in glycerol/DABCO (1 mg/ml) (Aldrich D2, 780, –2) (Johnson, 1982) sealed and viewed with a Nikon fluorescent microscope.

Sonication procedure for DLKP and DLKP-A

A Braun Labsonic 2000 sonicator was used for the preparation of the purified membrane fractions. The instrument was set on low power setting to administer a 70 watt power output at a repeating cycle of a pulse every 0.4 s. Lysis of the cellular membranes was assessed by microscopic examination.

Membrane protein purification and Western blotting.

Cell membranes were prepared by the procedure of Ronchi *et al.*, (1989). Resultant membrane-enriched fractions were freeze dried and stored at –20°C. Protein concentration was estimated by the Pierce Bincinchoninic assay, (Smith *et al.*, 1985). Using a discontinuous system, 0.6 µg/µl of membrane proteins was then separated on a 7.5% SDS polyacrylamide gels (Laemmli, 1970). Membrane proteins were transferred to Hybond-C (Amersham PPLC) using a Biorad Transblot apparatus. The Western blots were placed in blocking buffer (3% (w/v) BSA, 15 mM NaN_3 in PBSA) overnight at 4°C. C219 antibody was diluted with 2% (w/v) BSA and 1% (v/v) Tween in PBSA and incubated at 2 h at 37°C. After washing, the second antibody, (alkaline phosphatase conjugated anti-mouse IgG) was added. The blots were washed in PBS and colour was developed using 0.1% (v/v) Nitroblue tetrazolium (Sigma N-5514) in 0.5 M Tris-HCl, pH 8.9 (Soln. A), 10 mH 5-bromo-4-chloro-3-phosphate (BCIP) in N,N'-dimethylformamide (Sigma D-8654) (Soln. B), 1.0 M magnesium chloride in distilled H_2O (Soln. C) and 0.5 M Tris-HCl, pH 8.9 (Soln. D). The components for the substrate where mixed in the following proportions 88.6 ml Soln. D, 0.4 ml Soln. C, 4 ml Soln. B and 10 ml Soln. A. The addition of substrate gave rise to the development of blue/purple colour for positive antibody recognition.

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 of the human *mdr1* coding sequences. Cells were set up in 25 cm^2 flasks at a density of 4×10^6 cells per flask. All culture and assay medium was prepared with 10% heat inactivated FCS, (heat treated at 65°C for 15 min), to eliminate serum nucleases. Oligomers were added at a dose of 80 µg per flask on the first day and 40 µg per flask on the second and third days. Growth medium volume per flask was 3.0 ml. After pretreatment with oligonucleotide the cells were plated at a density of 2×10^3 cells/100 µl in each well of a 96 well plate. Adriamycin at varying concentrations was added 24 h after plating the cells. A 4 day incubation period was used to test for adriamycin sensitivity. Five 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 oligomer present in the toxicity assay. Oligomers were prepared at a concentration of 40 µg/3 ml and 2µl of this solution added per 100 µl on each of the 4 days of the toxicity assay.

Cytogenetic analysis. Cytogenetic analysis was carried out on DLKP parental cell line and the resistant variant DLKP-A (2.1 µg/ml ADR), (DLKP-A was analyzed after 2 days culture in the absence of adriamycin). Cells were inoculated at 6×10^6 per 75 cm^2 flask. The modal chromosome number was 56 for DLKP (Law *et al.*, 1992) and 59 for DLKP-A. For DLKP, colcemid at a final concentration of 0.02 µg/ml was added 2 h prior to harvesting and 18 h after seeding the cells,

while with DLKP-A colcemid was added 10 h before harvesting and 20 h after seeding. This difference in timing was due to the altered growth pattern of the drug resistant cells; in practise it was found to be much more difficult to obtain a good metaphase yield from the latter. Cultures were not trypsinized as a good mitotic yield was achieved by vigorous shaking of the flasks. The resultant cell-rich supernatant was decanted, centrifuged and resuspended in 0.075 M KCl, 37°C, DLKP for 25 min and DLKP-A for 45 min. The varying time in hypotonic solution was related to the ease with which the metaphases spread. The cell suspension was centrifuged at 3000 r.p.m. and the cell button gently resuspended in cold methanol-acetic acid fixative (3:1 (v/v)) for a minimum of 1 h. 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 suspension to a clean glass slide. Staining was with either Giemsa or Giemsa trypsin (Seabright, 1971). One hundred metaphases of both DLKP and DLKP-A were analysed for Double minute chromosomes. Additionally due to the large number of abnormal metaphase spreads encountered, 100 metaphases were reanalysed to quantify the presence of these abnormal metaphases. Abnormal metaphases were characterised by severe chromosome aberrations, fragmentation and chromosome condensation.

Circumvention assays

Pretreated cells were plated in 96 well plates at 2×10^3 cells/100 µl per well and then adriamycin and circumvention agents (C.A.) added 24 h later. Initial toxicity assays were performed with each of the test agents (Verapamil, quinidine, quinine and nifedipine) to ascertain nontoxic dose levels to use in the circumvention assay with adriamycin. 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 in conjunction with adriamycin. The assay was incubated for 6 days, cell growth was quantified by the acid phosphatase procedure (Martin and Clynes, 1991) and the resistance modulating ratio (RMR) of each test agent calculated.

$$\text{RMR} = \frac{\text{IC50 adriamycin alone}}{\text{IC50 adriamycin + C.A.}}$$

Results

Cross resistance patterns of DLKP-A at 2.1 µg/ml adriamycin resistance

DLKP-A was found to be cross resistant to all the agents tested, with highest cross resistance noted to the podophyllotoxins and vincristine (Tables 1 and 2). A surprising finding is the resistance to cis-platin, which is not normally associated with the classical MDR phenotype. DLKP-A was found to be stable with respect to adriamycin resistance after 3 months in the absence of drug, signifying a genetically stable population.

Table 1. Cross resistance patterns of DLKP-A

IC50 nM[a]	DLKP	DLKP-A
Drug		2.1 µg/ml
ADR	16.6 ± 3.7	5349 ± 72.7
VP16	42.5 ± 6.8	1539 ± 134
VM26	84.8 ± 7.2	1128 ± 139
VCR	0.83 ± 0.01	66 ± 8.6
C-PT	1433 ± 200	4783 ± 216
COLH	17 ± 0.3	69 ± 7.8

[a]Toxicity is expressed as the 50% inhibitory concentration (nanomolar).

Table 2. Fold resistances[a]

Drug	DLKP-A (2.1 μg/ml)
ADR	322.2
VP16	36.2
VM26	13.3
VCR	79.5
C-PT	3.37
COLH	4.05

[a]IC50 Variant/IC50 DLKP for each drug.

Sensitivity of DLKP-A to cryopreservation procedures

DLKP-A was found to be sensitive to standard freezing conditions of 10^6 cells per ml/10% FCS. This observation was also noted with other MDR variants (Redmond *et al.*, 1990), with low viability noted in addition to some loss of drug resistance. The optimum set of conditions proved to be 10^7 cells per ml /50% FCS/10% DMSO proved to be the optimum set of conditions which favoured high cell viability after revival in addition to maintenance of the drug-resistant phenotype. Alteration of cryoprotectant concentration (DMSO or glycerol) did not alter viability and resistance profile on thawing.

Clonal variation. Cloning assays indicated marked heterogeneity in terms of drug resistance in DLKP-A. Figure 1 indicates the presence of several populations with different adriamycin sensitivity, and also marked survival above the final selective concentration of adriamycin, indicating the presence of a very resistant subpopulation.

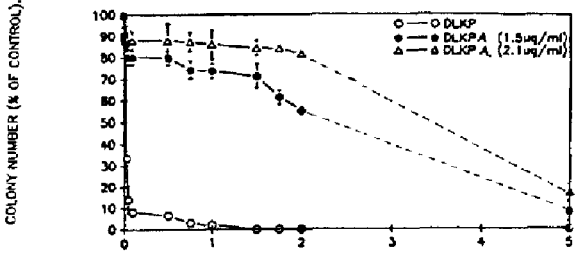

Fig. 1. Clonal variation of DLKP and DLKP-A.

Immunohistochemical detection of P-170. C219 monoclonal antibody recognizes an internal epitope of the C-terminal region of the P-glycoprotein polypeptide. The positive control cell line used was CHrC5 and the negative control cell line CHOK1. A pattern with numerous positively stained cells with fluorescence that appeared to be very evenly distributed, was seen in CHrC5 (Fig. 2) and DLKP-A (Fig. 3). This indicated the presence of a very homogenous population in terms of P-glycoprotein expression in these cell lines. In addition, a basal level of fluorescence was noted on the parental cell line DLKP (Fig. 4).

Analysis for P-170 glycoprotein by Western blotting. The cellular membrane preparations were made by sonication followed by differential centrifugation. The MDR variants required more sonication before cell lysis was obtained (as judged by microscopic examination). DLKP required 10 min sonication to obtain cell lysis while DLKP-A required 27 min. The increase in sonication time required for lysis of the resistant cells could be indicative of altered lipid composition of the resistant cells rather than a P-170 related property. Immunoreactive P-170 was evident in CHrC5 and DLKP-A (Fig. 5) but not in the sensitive cell lines. The presence of a second band was evident at 70 KD M.W. in CHrC5. The significance of this band is unknown.

Transfection of antisense and sense oligonucleotides. The direct role of P-170 overexpression in adriamycin resistance was studied by the transfection of antisense and sense oligonucleotides, as described in materials and methods. No change in adriamycin resistance was seen after culture with sense oligomers for DLKP-A or the control resistant cell line CHrC5 (Fig. 6a and 9a respectively). In the two resistant cell lines tested, CHrC5 and DLKP-A. Increased adriamycin sensitivity with antisense oligomers was noted, but to markedly varying degrees. The greatest altered resistance was noted for CHrC5 (the resistant control cell line) with a 12 fold reduction in IC50 (Fig. 7b); the reduction in IC50 noted for DLKP-A was smaller (Fig. 9b). No alteration in the adriamycin

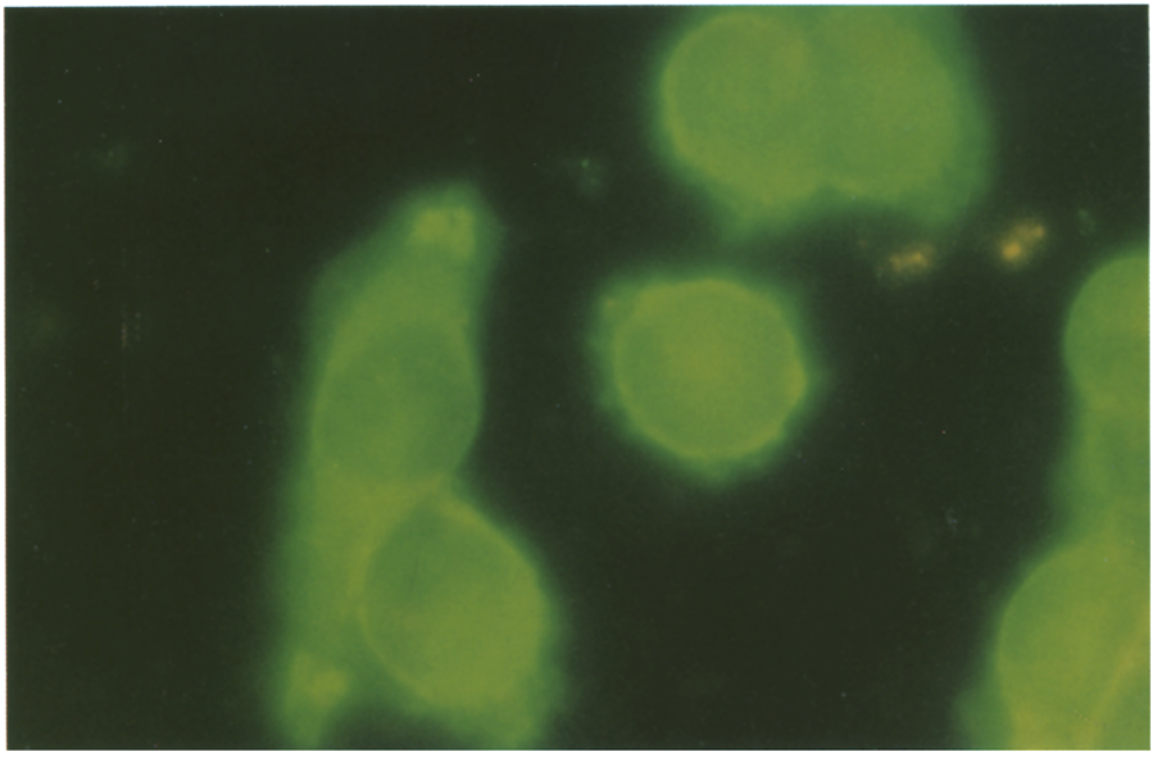

Fig. 2. Immunofluorescence of CHrC5 cell line (magnification ×250).

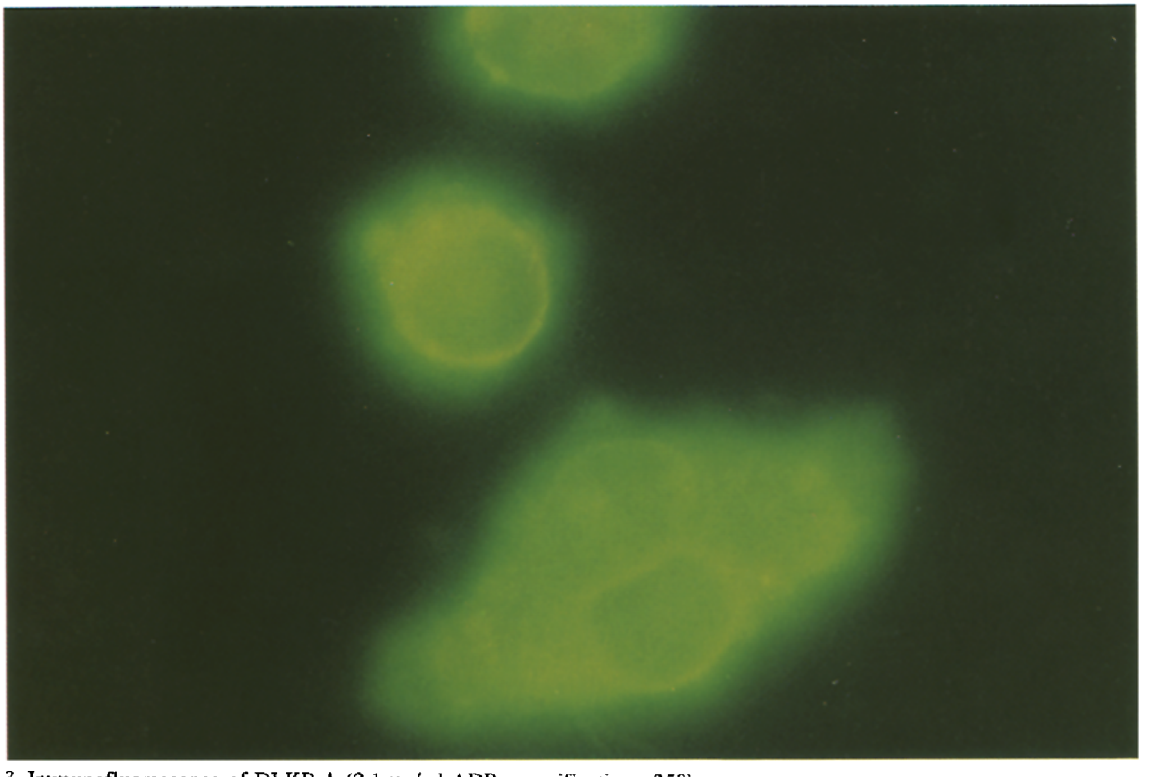

Fig. 3. Immunofluorescence of DLKP-A (2.1 μg/ml ADR magnification ×250).

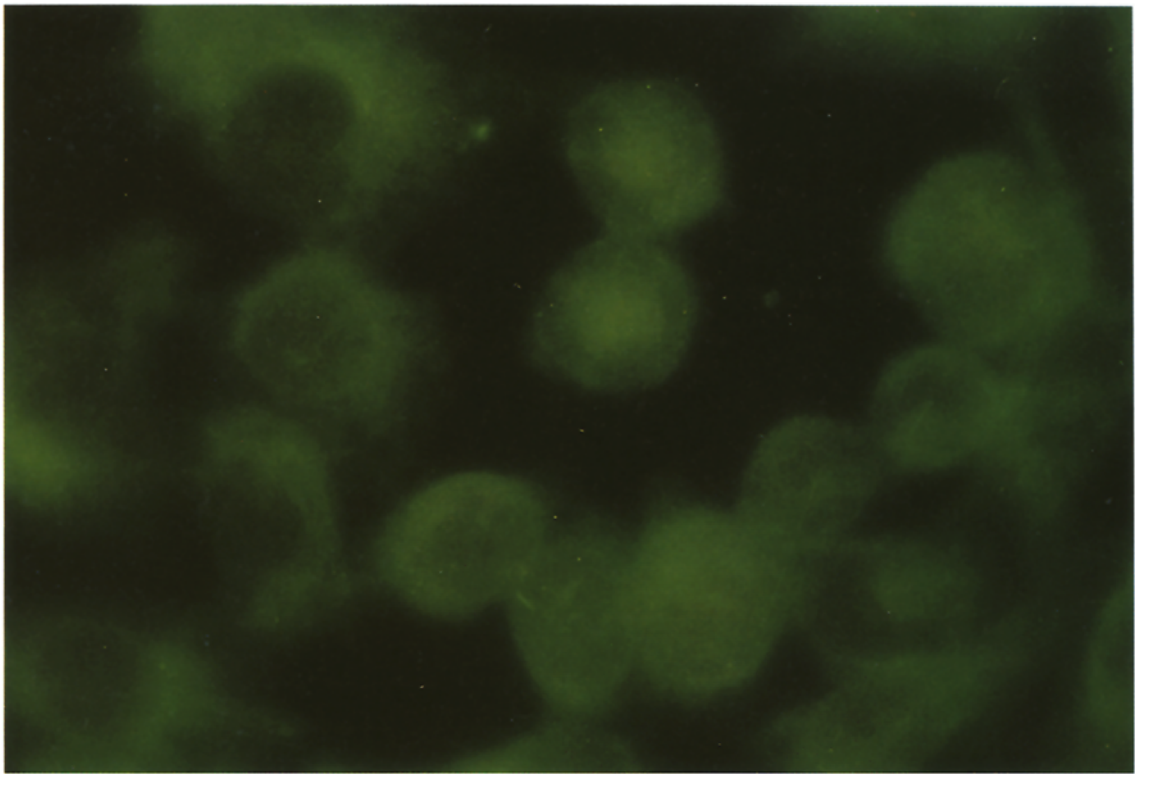

Fig. 4. Immunofluorescence of DLKP parental cell line (magnification ×250).

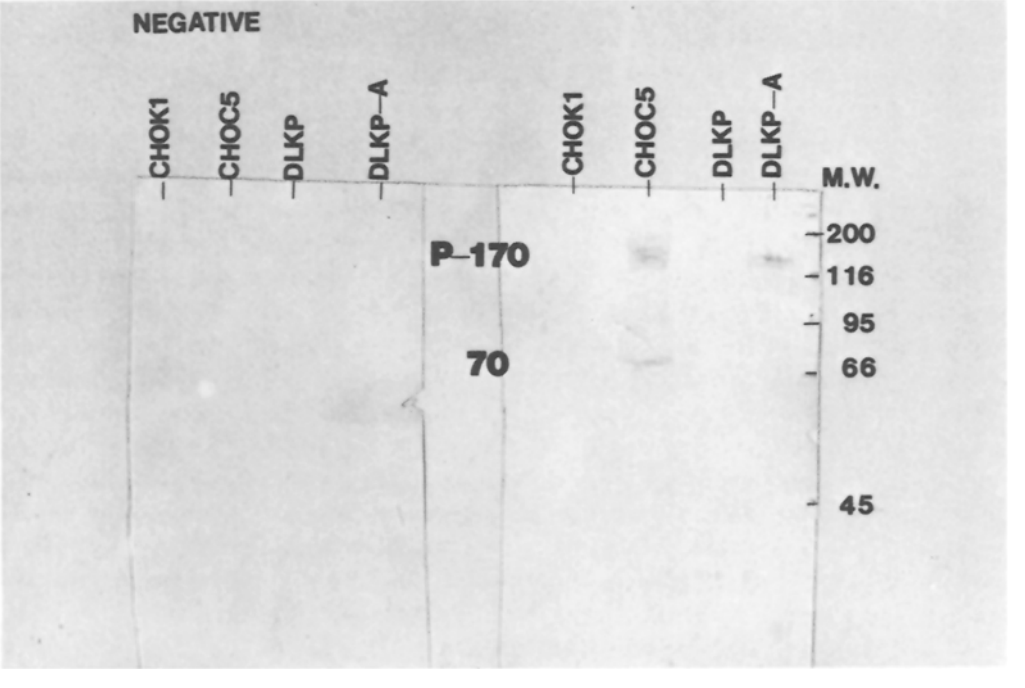

Fig. 5. Western blot of CHrC5, CHOK1, DLKP and DLKP-A (2.1 μg/ml).

toxicity profiles was noted for the two parental sensitive cell lines on the addition of either sense or antisense oligonucleotides (Fig. 6a, 6b, 8a and 8b). The highly sensitizing effect noted with CHrC5 in the presence of antisense oligomers indicates that P-170 is a major mechanism of MDR in these cells, as indicated in the literature. The lower magnitude of resistance reversal in DLKP-A suggests that P-170 expression may not be the only mechanism of resistance in DLKP-A; alternatively it could be due to lack of optimisation of time of treatment, or concentration of antisense oligonucleotide used. The half life of the P-170 has been found to be variable depending on the cell lines analysed, e.g. 24 h (Richert *et al.*, 1988) and 72 h (Rivoltini *et al.*, 1990). Further investigations to estimate the half life of P-170 in DLKP-A would be required to permit use of antisense technology to its full potential. An important observation noted in the antisense transfections of CHrC5 and DLKP-A is the enhanced reversal of resistance observed, when in

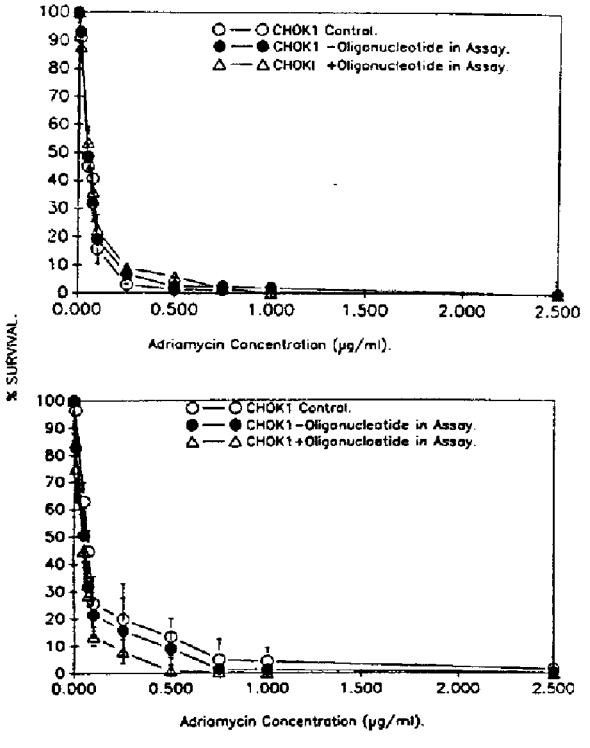

Fig. 6. Effect of: a) sense oligomers on CHOK1; and b) antisense oligomers on CHOK1.

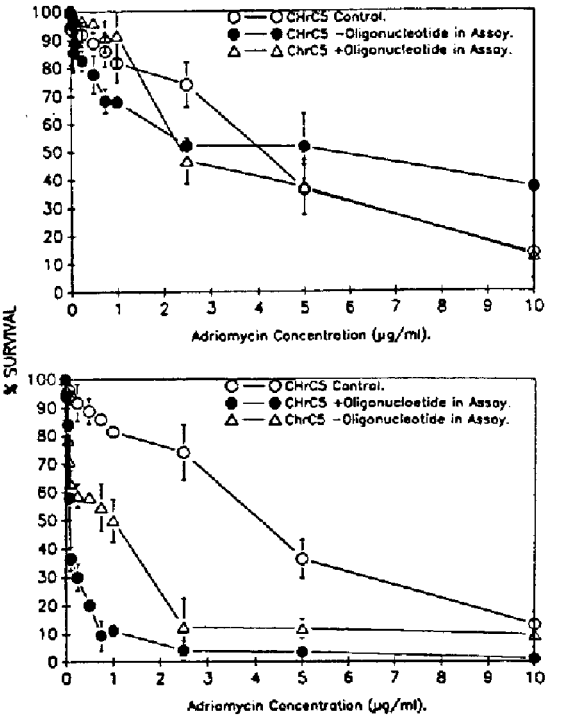

Fig. 7. Effect of: a) sense oligomers on CHrC5; and b) antisense oligomers on CHrC5.

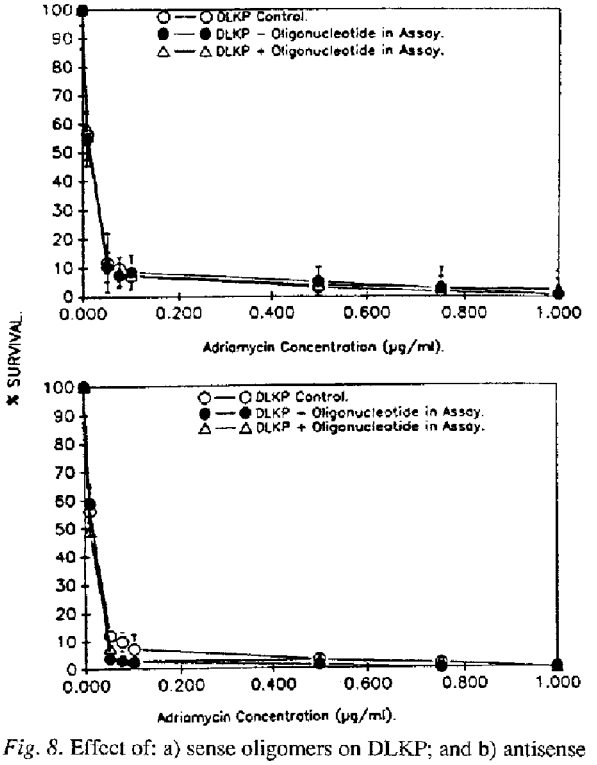

Fig. 8. Effect of: a) sense oligomers on DLKP; and b) antisense oligomers on DLKP.

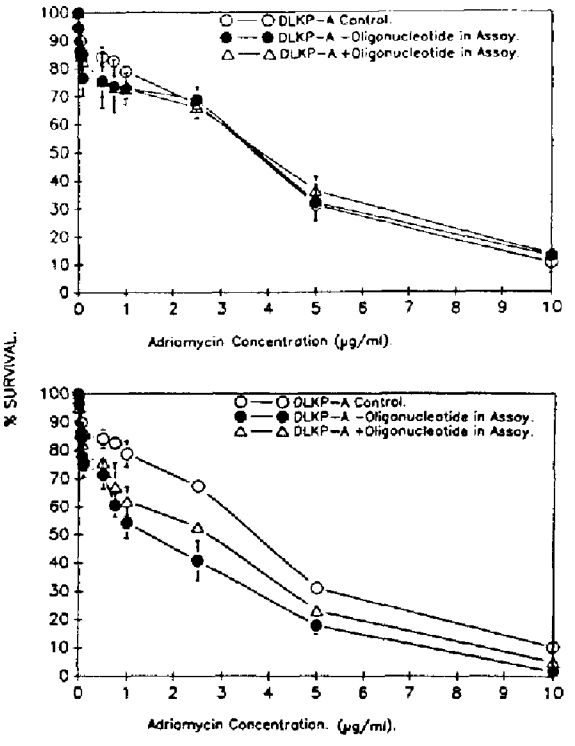

Fig. 9. Effect of: a) sense oligomers on DLKP-A; and b) antisense oligomers on DLKP-A.

addition to pretreatment, the oligonucleotide is included in the assay medium during the adriamycin toxicity assay.

Cytogenetic analysis

The presence of Double minute chromosomes (DMs) and homogeneously staining regions are both indicative of gene amplification and are a common cytogenetic manifestation of MDR. DLKP and DLKP-A were both analysed for the presence of DMs (Table 3; Figs. 10, 11, 12). Additionally, on analysis of the slides a number of very abnormal spreads were encountered with vast cytogenetic damage evident. The number of abnormal spreads noted per 100 metaphases is reported. DLKP-A had a much higher proportion of cells with DMs and with a high degree of chromosome breakage (Table 3). *(Note: No abnormal spreads were counted in the DM analysis).*

Circumvention of multidrug resistance. The emphasis of this study was on compounds and concentrations that could be used *in vivo*. Initially toxicity assays were performed with each of the agents to ascertain nontoxic dose levels to use in circumvention assays. In each case, a dose was chosen atwhich no toxicity or growth inhibition was observed. This concentration of agent was then incorporated into an adriamycin toxicity assay to assess possible reversal in IC50 of adriamycin due to the presence of circumvention agent. The details of the concentration of the circumvention agents used and the results of the circumvention experiments are shown in Tables 4, 5 and 6. None of the chemicals tested had any significant modulating affect on resistance in the parental cell line. Verapamil increased adriamycin sensitivity significantly in DLKP-A but not in the parental cell line. The reversal effect noted in DLKP-A with verapamil supports a contributory role for P-170 in the mechanism of resistance in DLKP-A. Quinine, quinidine (and to a lesser extent nifedipine) also increased sensitivity in both resistant cell lines.

Discussion

The results presented here show that the DLKP-A squamous cell carcinoma line can be adapted to

Table 3. Cytogenetic analysis of MDR

Cell line	% of cells with DMs					% of cells with high degree of chromosome damage
	0	1	2	3	>4	
DLKP	97	3	0	0	0	2
DLKP-A	66	8	6	7	3	18

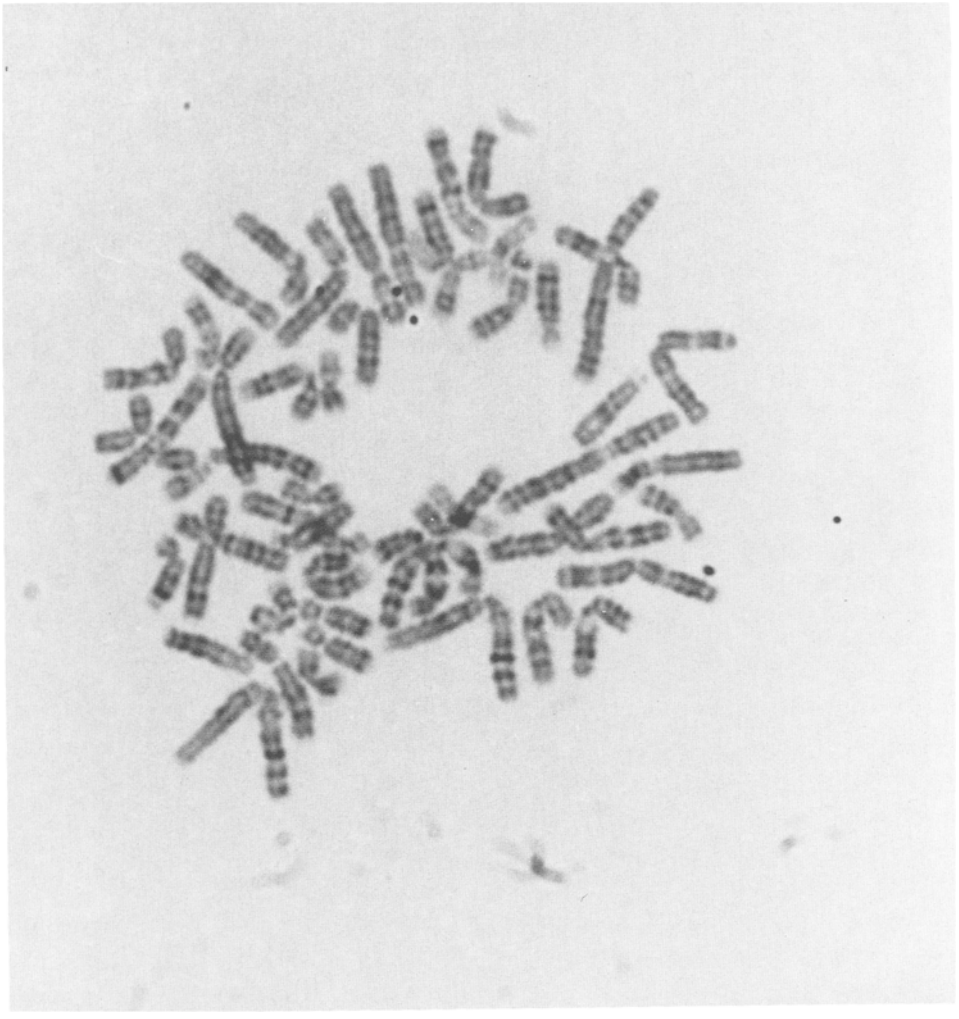

Fig. 10. G-banded DLKP metaphase spread.

Table 4. Concentration of agents used in circumvention assays

Circumvention agent	Concentration
Verapamil	1 μg/ml
Quinidine	2.0 μg/ml
Quinine	50 μg/ml
Nifedipine	4.5 μg/ml

grow in high levels of adriamycin, demonstrating approximately 300 fold resistance to the selective agent (adriamycin). Significant levels of cross resistance to VP-16 (36-fold), VM-26 (13-fold), vincristine (80-fold) and colchicine (fourfold) could be consistent with a P-glycoprotein mechanism. The very high relative level of resistance to adriamycin, however, and the unexpected cross resistance to cis-platin (threefold) indicate that one or more other mechanisms may be involved, or alternatively that a mutant form of P-glycoprotein is being expressed. Immunofluorescence and Western blotting clearly indicate overexpression of P-glycoprotein in the resistant variant, and reversal of adriamycin toxicity by transfection with an antisense oligonucleotide specific for the *mdr1* gene establishes that P-glycoprotein has a direct causal role in resistance. The fact that

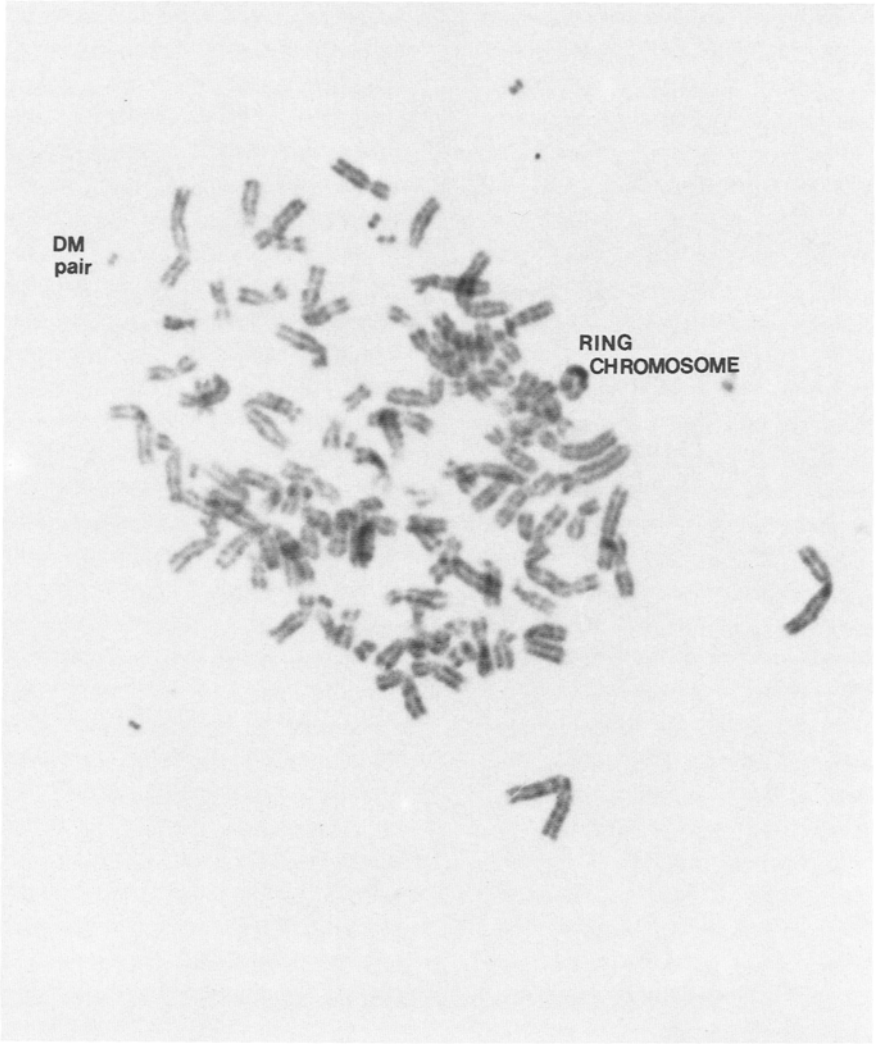

Fig. 11. DLKP-A metaphase showing the presence of DMs and a ring chromosome.

reversal by the antisense oligonucleotide (and also by verapamil) was only partial may indicate that P-glycoprotein is only one of a number of mechanisms involved in resistance or alternatively it may relate to technical limitations of the experimental procedure and short half life of the free

Table 5. Effect of circumvention agents on IC50 (nM) of adriamycin for DLKP and DLKP-A

Cell line ± C.A.	DLKP		DLKP-A	
	–	+	–	+
Verapamil	5.33 ± 0.2	6.43 ± 0.8	5749 ± 657	1648 ± 145
Quinidine	6.9 ± 0.78	4.59 ± 0.6	5748 ± 762	1548 ± 189
Quinine	4.04 ± 0.05	4.49 ± 0.3	5546 ± 435	1703 ± 187
Nifedipine	5.7 ± 0.76	7.78 ± 0.7	5751 ± 557	1924 ± 236

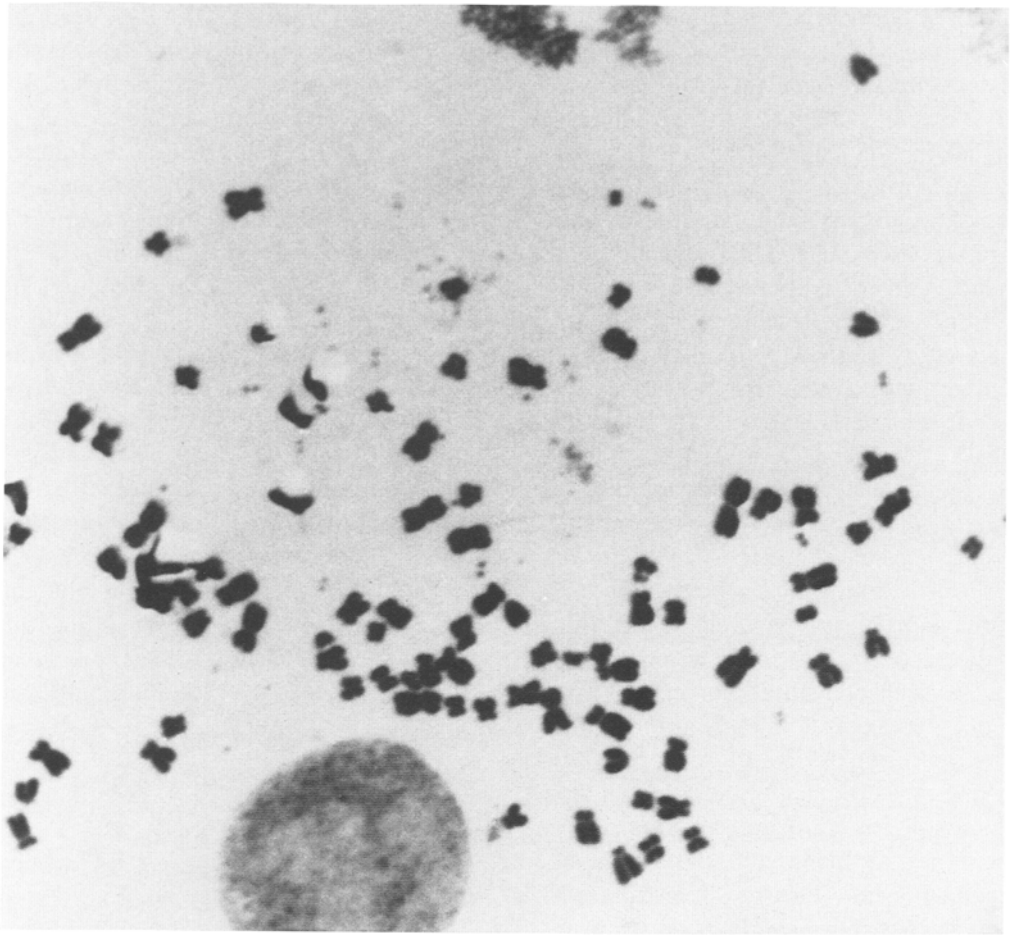

Fig. 12. DLKP-A abnormal metaphase, showing numerous DMs, abnormal breakages and chromosome condensation.

oligonucleotide within the cell; the fact that extended exposure to the oligonucleotide increased the magnitude of resistance reversal lends some support to the latter explanation. It is interesting to note that the cross-resistance pattern varies at different stages of the selection (A. Redmond and M. Clynes, unpublished), indicating involvement of different genes and/or different cellular subpopulations.

The molecular mechanisms of multidrug resistance in human lung carcinomas are not fully understood. Low levels of *mdr1* mRNA are found

Table 6. Resistance modulating ratios for DLKP and DLKP-A

Cell line ± C.A.	DLKP RMR[a]	DLKP-A RMR[a]
Verapamil	0.828	3.48
Quinidine	1.5	3.71
Quinine	0.899	3.25
Nifedipine	0.73	2.98

[a]RMR = (IC50 adriamycin alone)/(IC50 adriamycin + C.A.).

in a proportion of both normal and malignant lung biopsies (Lai *et al.*, 1989) but recent work using immunohistochemistry (and therefore allowing evaluation of expression in a sub-population of cells) established a correlation between expression of P-glycoprotein and glutathione-S-transferase π, smoking history, and adriamycin sensitivity in a rapid *in vitro* test. The correlation with smoking history did not apply to adenocarcinoma. No relationship between sensitivity and topoisomerase II activity was found (Volm *et al.*, 1991). There is, nevertheless, interest in a possible role for reduced or altered Topoisomerase II expression in NSCLC; reduced expression appears to correlate with increased resistance in a number of small cell lung carcinoma lines *in vitro* (de Jong *et al.*, 1990; Long *et al.*, 1991; Kasahara *et al.*, 1992). It is worth noting that topoisomerase II-related mechanisms do not generally affect vinca alkaloid resistance, and in some cases reduced activity causes hypersensitivity to cis-platinum (Long *et al.*, 1991). Cole *et al.* (1990) have described a multidrug resistant small cell lung carcinoma line cross resistant to adriamycin, epirubicin, daunomycin, mitoxantrone and vinca-alkaloids but not to bleomycin, cis-platin and 5-fluorouracil; this line does not overexpress P-glycoprotein, but has significantly increased levels of glutathione-S-transferase (probably class π) and related enzymes. Coley *et al.* (1991) have reported on a variant of an adriamycin resistant human large cell lung carcinoma variant which is cross-resistant to daunorubicin, vincristine, colchicine and etoposide, but which does not express P-glycoprotein and in which resistance is not affected by verapamil; a non-P-glycoprotein ATP-driven drug efflux pump may be involved in this cell line. Versantvoort *et al.* (1992) also report analogous mechanisms of drug resistance in human non-small cell lung carcinoma cell lines. In the cell line DLKP-A described in this present paper, adriamycin resistance is partially reversed by verapamil treatment. Multidrug resistant variants of human lung carcinomas overexpressing P-glycoprotein have also been reported (Baas *et al.*, 1990).

The MDR variant DLKP-A described in this paper was significantly more resistant to sonication than was the parental cell line, DLKP; the cells were also more sensitive to cryopreservation, and high serum levels and densities were needed to ensure recovery of the variants at a high level of resistance. Cloning experiments in different concentrations of adriamycin indicate the existence, in the DLKP-A population, of different cellular population heterogenous with respect to adriamycin resistance. A possible explanation is that the less resistant cells survive by metabolic co-operation with those cells which are actively extruding drug. This process would also reduce the effective resistance level of the more highly resistant population. This phenomenon warrants further investigation.

The DLKP-A line is being characterised in more detail, and should be a useful addition to the panel of cell lines available as *in vitro* models for multidrug resistance in human lung cancer.

Acknowledgements

We would like to thank Dr E. Law for helpful discussions on the cytogenetic work; Dr Becky Shadduck, Department of Cell Biology, Vanderbilt University, Nashville, Tennessee for assistance in checking the unique nature of the antisense oligonucleotides and Mr Mike Powell, Delta Biotechnology, Nottingham, England for synthesis of the antisense oligonucleotides.

References

Baas F, Jongsma A, Broxterman H, Arceci R, Housman D, Scheffer G, Riethorst A, Van Groenigen M, Nieuwint A and Joense H (1990) Non P-glycoprotein mediated mechanism for MDR precedes P-glycoprotein expression during *in vitro* selection for doxorubicin resistance in human cancer cell line. Cancer Res 50: 5392–5398.

Beck WT (1989) Unknotting the complexities of MDR; the involvement of DNA topoisomerases in drug action and resistance. J Natl Cancer Inst 81: 1683–1686.

Bradley G, Juranka P and Ling V (1988) Mechanisms of multidrug resistance. Biochim Biophys Acta 948: 87–128.

Citro G, Cucco C, Verdina A and Zupi G (1991) Reversal of adriamycin resistance by lonidamine in a human breast cancer cell line. Br J Cancer 64: 534–536.

Clynes M, Redmond A and Heenan M (1990) Review: Recent developments in research on Multidrug Resistance in cancer cells. Cancer J 3: 34–39.

Cole SP, Downes HP, Mirski S and Clements J (1990) Alterations in glutathione and glutathione related enzymes in a multidrug-resistant small cell lung cancer cell line. Mol Pharmacol 37: 192–197.

Coley HM, Workman P and Twentyman P (1991) Retention of activity by selected anthracyclines in a multidrug resistant human large cell lung carcinoma line without P-glycoprotein hyperexpression. Br J Cancer 63: 351–357.

Dalton WS and Grogan TM (1991) Does P-glycoprotein predict response to chemotherapy, and if so, is there a reliable way to detect it? J Natl Cancer Inst 83: 80–81.

de Jong C, Zijlstra J, de Vries E and Mulder N (1990) Reduced DNA Topoisomerase 11 activity and drug-mediated DNA cleavage activity in an adriamycin-resistant human small cell lung carcinoma cell line. Cancer Res 50: 304–309.

Ford JM and Hait WN (1990) Pharmacology of drugs that alter Multidrug Resistance in cancer. Pharmacol Rev 42: 155–199.

Georges E, Bradley G, Gariepy J and Ling V (1990) Detection of P-glycoprotein by gene-specific monoclonal antibodies. Proc Natl Acad Sci USA 87: 152–156.

Gottesman M, Willingham MC, Thiebaut T and Pastan I (1991) Expression of the *mdr1* gene in normal human tissues. In: Ronninson I (ed.) "Molecular and Cellular Biology of Multidrug Resistance in tumor cells" Plenum Press, New York, pp. 279–290.

Higgins CF and Gottesman MM (1992) Is the multidrug transporter a flippase? Trends Biochem Sci 17: 18–21.

Johnson GD, Davidson RS, McNamee KC, Goodwin RD and Holborow EJ (1982) Fading of immunofluorescence during microscopy: A study of the phenomen and its remedy. J Immunolog Meth 55: 231–242.

Kasahara K, Fujiwara Y, Sugimoto Y, Nishio K, Tamura T, Matsuda T and Saijo N (1992) Determinants of response to the DNA Topoisomerases 11 inhibitors Doxorubicin and etoposide in human lung cancer cell lines. J Natl Cancer Inst 84: 113–118.

Laemmli EK (1970) Clevage of structural proteins during the assembly of bacteriophage T4. Nature 227: 680–695.

Lai SL, Goldstein LH, Gottesman M, Pastan I, Tsai C, Johnson B, Mulshine J, Ihde C, Kavser K and Gazdar A (1989) MDR1 expression in Lung Cancer. J Natl Cancer Inst 81: 1144–1150.

Law E, Gilvarry V, Grant G, Gregory B and Clynes M (1992) Cytogenetic comparison of two poorly differentiated human lung squamous cell carcinoma cell lines. Cancer Genet Cytogenet 59: 111–118.

Long B, Wang L, Lorico A, Wang R, Brattain M and Casazza A (1991) Mechanisms of resistance to etoposide and tenoposide in acquired resistant human colon and lung carcinoma cell lines. Cancer Res 51: 5275–5284.

Martin A and Clynes M (1991) Acid-phosphatase endpoint for *in vitro* toxicity tests. In Vitro Dev Biol 27A: 183–184.

Moscow JA, Fairchild CR, Madden MJ, Ransom DT, Wieand HS, O'Brien EE, Poplack DG, Cossman J, Myers CE and Cowan KH (1989) Expression of anionic Glutathione-S-Transferase and P-glycoprotein genes in human tissues and tumors. Cancer Res 49: 1422–1428.

Redmond A, Law E, Gilvarry U and Clynes M (1990) Establishment of two multidrug resistant variants of the human tumor cell line Hep-2. Cell Biol Toxicol 6: 293–302.

Richert ND, Aldwin L, Nitecki D, Gottesman MM and Pastan I (1988) Stability and covalent modification of P-glycoprotein in multidrug resistant KB cells. Biochemistry 27: 7607–7613.

Rivoltini L, Colombo MP, Supino R, Ballainari D, Tsuruo T and Parianti G (1990) Modulation of multidrug resistance by verapamil or MDR1 antisense oligonucleotides does not change the high susceptibility to lymphokine-activated killers in MDR activiated carcinomas (Lovo) line. Int J Cancer 46: 727–732.

Ronchi E, Sanfilippo O, di Fronzo G, Bani MR, Torre P, Catania S and Silvestrini R (1989) Detection of P-170 glycoprotein in neoplastic and normal tissue. Tumori 75: 542–546.

Roninson IB (1991) Molecular and Cellular biology of Multidrug resistance in tumor cells. Plenum Press, New York.

Seabright M (1971) A rapid banding technique for banding human chromosomes. Lancet 2: 971–972.

Smith PK, Krohn RI, Hermanson AK, Mallia FH, Gartner MD, Provenzano EK, Fujimoto NM, Goeke NM, Olson BJ and Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76–85.

Van der Valk P, Van Kalken C, Ketelaars H, Broxterman HJ, Scheffer G, Kuiper C, Tsuruo T, Lankelma J, Meijer C, Pinedo H and Scheper R (1990) Distribution of multidrug associated P-glycoprotein in normal and neoplastic human tissue. Ann Oncol 1: 56–64.

Valverde MA, Diaz M, Sepulveda FV, Gill DR, Hyde SC and Higgins CF (1992) Volume regulated chloride channels associated with the human multidrug resistant P-glycoprotein. Nature 355: 830–833.

Versantvoort C, Broxterman H, Pinedo H, de Vries E, Fellor N, Kuiper C and Lankelma J (1992) Energy dependent processes involved in reduced drug accumulation in multidrug resistant human lung cancer cell lines without P-glycoprotein expression. Cancer Res 52: 17–23.

Volm M, Mattern J and Samsel B (1991) Overexpression of P-glycoprotein Glutathione-S-Transferase pi in resistant non-small cell lung carcinomas of smokers. Br J Cancer 64: 700–704.

Wilbur DW, Camacho ES, Hillard DA, Dill PL and Weisenthal LM (1992) Chemotherapy of non-small cell lung carcinoma guided by an *in vitro* drug resistance assay measures total tumor cell kill. Br J Cancer 65: 27–32.

Address for correspondence: Martin Clynes, National Cell and Tissue Culture Centre/Bioresearch Ireland, Dublin City University, Glasnevin, Dublin 9, Ireland