Abstract. Background: The H69-EPR, H69-CP, H69-VP and H69/R38 resistant sublines of the classic small cell lung cancer (SCLC) line have proven useful in studies of resistance and its circumvention with paclitaxel. Materials and Methods: The suppressor/oncogene profile of these sublines determined by Western and Northern blot was compared to the variant H82 SCLC cell profile. Two-dimensional electrophoresis/mass spectrometry was used to determine the effect of paclitaxel on protein expression. Results: The H69-EPR and H69-CP resistant sublines were similar to the variant H82 cells for bcl-2, p21waf1, p53, N-myc and c-myc expression while the H69-VP subline retained the classic H69 pattern. A 1-h treatment with 10ng/ml paclitaxel substantially reversed the resistance except for the H69/R38 subline and tended to reverse the resistance-associated changes in protein expression in the H69-EPR subline. Conclusion: Although some resistant sublines express a variant pattern of suppressor/oncogenes with low bcl-2, resistance is substantially reversed by paclitaxel treatment.

Small cell lung cancer (SCLC) accounts for 25% of lung tumours and is one of the most aggressive forms of the disease. The principal treatment for all stages of SCLC is combination chemotherapy involving cisplatin, etoposide and often an anthracycline and radiotherapy. This produces a good response in 90% of patients, however relapse and disease progression occur relatively quickly with a 5-year survival of only 5% (1, 2). The most significant reason for the failure of therapy is the development of resistance (3).

SCLC is divided into two major subtypes, the more differentiated and responsive classic subtype and the less differentiated, faster growing and less responsive variant subtype (4). Amplification and increased expression of the growth promoting oncogene c-myc is commonly found in metastatic lesions, is usually associated with the variant subtype and hence could contribute to increased growth rate. The classic subtype has low c-myc expression.

The loss of wild-type p53 activity occurs in over 80% of SCLC tumours and is associated with disease progression, poor prognosis and chemotherapy resistance (5, 6). This loss of p53 activity in turn prevents the induction of the cyclin-dependent kinase inhibitor p21waf1 which results in loss of cell cycle checkpoint control, increased growth rate and decreased access to the apoptotic pathway.

Bcl-2 expression also inhibits apoptosis and causes resistance to chemotherapy (7, 8) and radiation (5). Although increased bcl-2 expression is often reported in SCLC, paradoxically this correlates with enhanced survival (9), complicating the role of bcl-2 in SCLC progression (4, 9). In cellular models of drug resistance, bcl-2 expression is usually increased (8, 10); however, we recently reported that the drug- and radiation-resistant H69/R38 subline had decreased rather than increased bcl-2 (11). This resistant subline was generated by treatment with fractionated low dose X-rays and forms part of a panel of resistant cell lines, all generated from the classic H69 SCLC cell line with low but clinically relevant levels of X-rays and drugs. Repeated treatments of 40ng/ml epirubicin produced the H69-EPR subline (12), 100ng/ml cisplatin produced the H69-CP subline (13), 500ng/ml etoposide produced the H69-VP subline (13) and fractions of 0.75Gy X-rays to a cumulated dose of 37.5Gy produced the H69/R38 subline (11). One of our aims, therefore, was to examine the bcl-2 expression as well as myc and p53 expression in this series of stable resistant sublines in order to better understand their role in resistance.

We have shown that pre-treatment of resistant cells for 1h with a low dose (10ng/ml) of paclitaxel reverses the resistance of the epirubicin selected H82/E8 variant SCLC subline (14), the epirubicin selected H69-EPR subline (12) and the cisplatin selected H69-CP and etoposide selected...
H69-VP sublines (13). The second aim was to further characterise the reversal of resistance by paclitaxel and to determine the changes in gene expression caused by paclitaxel treatment.

**Materials and Methods**

**Cell cultures and sensitivity to drugs and radiation.** The H69 and H82 human SCLC cells were from ATCC (Rockville, MD, USA). The resistant sublines H69-EPR (12), H69-CP, H69-VP (13) and H69/R38 cells (11) and the H69 and H82 cells were grown in RPMI 1640 (Trace Biosciences, Sydney, Australia) containing 10% foetal bovine serum (Trace Biosciences), 20mM HEPES and 10mM NaHCO₃ in a 5% CO₂ atmosphere at 37°C. All cells and sublines were free of *Mycoplasma* and exponentially growing cells were used for all experiments. Sensitivity to drugs was determined in triplicate in 96-well plates using the MTT (3,-4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide; Sigma) cell viability assay as previously described (11, 13, 15). Radiation sensitivity was determined as previously described (11, 15) in 96-well plates exposed to a linear gradient of X-radiation using a 6MV X-ray linear accelerator treatment machine fitted with a linear field lead wedge. Control wells were not irradiated. Plates were incubated for 5 days and cell viability determined using MTT. The 50% inhibitory drug concentration or radiation dose (IC₅₀) was determined as that resulting in a 50% reduction in cell viability after 5 days. Relative resistance was calculated by dividing the IC₅₀ obtained for the resistant subline by the IC₅₀ obtained for the H69 parental cell line.

**Western blot analysis.** Cells (5 x 10⁶) were washed in phosphate-buffered saline, pH 7.2 and resuspended in 100 μl lysis buffer (50mM Tris-HCl, 1mM PMSF), sonicated and 20 μg total protein was subjected to electrophoresis on Tris-glycine 4-20% gradient gel (Gradipore, Sydney, Australia) or 10% polyacrylamide/SDS Laemmlı gels (16). The blots were developed using the following antibodies: bcl-2 (clone 124, Dako, Sydney, Australia); p53 (DO-7 Dako); p21<sup>wt</sup> (Santa Cruz Biotech, Santa Cruz, CA, USA), topoisomerase I (2011-1 Topogen, Columbus, OH, USA), c-myc (sc40 Santa Cruz) and N-myc (sc-142 Santa Cruz). To allow for protein loading, membranes were either stained before blocking with Ponceau-S (Sigma, P7170) or were re-developed using anti-actin antibody (Sigma). The blots and protein were quantitated using the Microtek ScanMaker III and the Molecular Analyst program (BioRad, Sydney, Australia).

**Northern blot.** RNA was extracted from 10⁷ cells using the GTC-phenol method (17) and 20 μg RNA was electrophoresed, transferred and hybridised with a 400 bp Pst1 fragment of c-myc (Oncogene Science, Cambridge, MA, USA; Cat #: HP111). Membranes were exposed on a BioRad imaging screen cassette (BI-β imaging) for 4 to 24h and scanned by the GS-250 Molecular Imager (BioRad) and quantitated. Sample loading was assessed using 28S RNA.

**2-Dimensional protein electrophoresis/mass spectrometry.** Proteins were extracted from washed cells, separated by 2-dimensional electrophoresis and the silver-stained spots of interest were digested with trypsin and the peptides isolated as previously described (18). The amino acid sequence was determined using a C18 microcapillary column (Michrom BioResources, CA, USA) coupled to a TSQ7000 Tandem mass spectrometer (Thermofinnigan, CA, USA) as previously described (19).
Results

Drug and radiation sensitivity. Figure 1 shows the fold resistance of the H69 sublines to a variety of drugs and radiation relative to the H69 cells. The H69-EPR cells showed the greatest resistance to etoposide (11-fold) and were significantly resistant to all drugs tested except paclitaxel and vinorelbine. They were also 1.5-fold resistant to X-rays. The H69-CP cells were significantly resistant to cisplatin (3-fold), gemcitabine (4-fold), vinorelbine (1.5-fold), etoposide (1.5-fold) and X-rays (1.8-fold), while the H69-VP only showed resistance to the selecting drug, etoposide (2-fold). The H69/R38 cells were resistant to X-rays (2-fold) and to all drugs except paclitaxel, gemcitabine and etoposide.

Expression of tumour suppressor and oncogenes. The expression of bcl-2, p53, p21wafl and myc was determined by Western and Northern blot assays for the resistant sublines and compared to the H69 cells representative of the classic subtype of SCLC and the H82 cells representative of the variant subtype of SCLC. Figure 2 shows that relative to the H69 cells, both the H69-EPR and H69-CP sublines had decreased bcl-2, p21wafl and N-myc expression and increased p53 and c-myc expression, giving these sublines an expression profile similar to the variant H82 cells. The pattern of expression of the H69-VP cells was similar to the classic H69 cells. The H69/R38 cells had decreased bcl-2 expression and increased N-myc expression making them similar to the variant H82 cells, but they had low level expression of c-myc similar to the classic H69 cells (Figure 3).

Reversal of resistance and gene expression by paclitaxel treatment. The resistant sublines and the sensitive H69 cells were treated with 10ng/ml paclitaxel for 1h, after which they were washed and incubated in fresh culture media for 24h before their sensitivity to a variety of drugs and X-rays was determined as described in Materials and Methods. Figure 4 shows that pre-treatment with paclitaxel reduced the resistance of the H69-EPR cells to X-rays and to all drugs except gemcitabine. The H69-CP cells also showed this reversal with the paclitaxel pre-treatment reducing the resistance to cisplatin, chlorambucil, etoposide and X-rays, while for the H69-VP cells it caused reduced resistance to etoposide, the only drug to which these cells were resistant. Paclitaxel treatment had no effect on cisplatin or radiation resistance of the H69/R38 cells and it had little effect on the sensitivity of the H69 cells, except for vinblastine where there was a slight but significant increase in sensitivity.

To determine the effect of the 1-h paclitaxel treatment on protein expression in the H69, H69-EPR, H69-CP and H69-VP cells, protein extracts were prepared 24h after treatment and expression was determined by Western blot. Paclitaxel treatment had no effect on topoisomerase IIα in the H69-EPR and H69-CP cells, but for the H69-VP cells which had decreased topoisomerase IIα it increased the expression back to the H69 level (not shown). There was no change in p53 or bcl-2 expression except for a similar slight decrease in bcl-2 in the H69 and H69-VP cells (not shown).
The effect of the 1-h paclitaxel treatment on protein expression was characterised further in the H69-EPR cells using 2-dimensional electrophoresis of protein extracts from paclitaxel-treated and control cells 24h after treatment and this was compared to the protein pattern in the H69 cells. A set of silver-stained protein spots, that were either differentially expressed between the H69 and H69-EPR cells or were altered by paclitaxel treatment, were analysed and identified by mass spectrometry. Figure 5 shows there was a trend for paclitaxel treatment to reverse the protein change associated with the development of resistance. Examples of this were malate dehydrogenase, thioredoxin peroxidase 2, triosephosphate isomerase, Hsp27, glutathione-S-transferase and Hsp70. Other proteins, such as the two isoforms of HSP60, did not show this trend.

**Discussion**

We have previously shown that drug and radiation resistance develops readily when the H69 classic SCLC cell line is treated with low but clinically relevant levels of epirubicin, cisplatin, etoposide and X-rays (11-13). We show here that, while the H69-VP subline had increased bcl-2 levels consistent with resistance (5, 7, 8), the other resistant sublines all expressed decreased bcl-2 relative to the sensitive H69 cells (Figures 2, 3). Although decreased bcl-2 is not normally associated with resistance, there are reports that increased bcl-2 is associated with a good response to treatment (4, 9, 20, 21). This suggests that decreased bcl-2 expression may be associated with a more aggressive and resistant phenotype and this would be supported by our findings for three of our four resistant sublines with decreased bcl-2.

The pattern of bcl-2, p53, p21^{waf1}, N-myc and c-myc expression in the H69-EPR and H69-CP sublines was similar to the pattern in the H82 variant SCLC cell line, suggesting that the development of resistance was accompanied by a change to a variant pattern of expression which probably reflects a more aggressive phenotype. The ease with which resistance developed in these sublines and the transition from a classic to a variant expression pattern parallels the clinical scenario and suggests that treatment failure may involve progression from a classic to a variant subtype. It also suggests that this progression can be easily induced by epirubicin and cisplatin. It is interesting to note that etoposide treatment did not cause a change from the classic to variant suppressor/oncogene expression pattern consistent with the high efficacy of etoposide in the treatment of SCLC.

Despite the development of a variant and more aggressive phenotype associated with the resistance, a 1-h paclitaxel treatment was able to effect a substantial reversal of resistance in the H69-EPR and H69-CP sublines (Figure 4), suggesting that paclitaxel may have a role as a second-line drug in the treatment of refractory SCLC (22). None of the resistant sublines were resistant to paclitaxel (Figure 1), consistent with recent clinical findings of good responses to paclitaxel-containing regimens (23). We demonstrated that the 1-h paclitaxel treatment also substantially reversed the changes in protein expression associated with the resistance of the H69-EPR subline (Figure 5). Further investigation of the molecular changes associated with the reversal of resistance by paclitaxel using these clinically relevant cellular models may reveal new treatment strategies for SCLC.
Figure 4. Effect of paclitaxel on drug and radiation sensitivity. The H69 cells and resistant sublines were incubated in 10ng/ml paclitaxel for 1h after which the media was removed and replaced with fresh media and the cells were incubated for 24h. Drug and radiation sensitivity was then determined as described in Materials and Methods. All determinations were in triplicate and all experiments were repeated at least twice. Error bars are the standard deviation and * indicates significantly decreased (p<0.05).
Acknowledgements

We thank Valerie Wasinger and Garry Corthals for their mass spectrometry analyses, Regina Bromley and Philip Vial for irradiating cell cultures and the Dust Diseases Board, NSW, Australia, for financial support.

References


Figure 5. Effect of paclitaxel on gene expression in the H69-EPR cells. Total cell extracts were prepared from H69 cells, H69-EPR cells and H69-EPR 24h after a 1-h treatment with 10ng/ml paclitaxel, separated by 2-dimensional electrophoresis and the silver-stained pattern was analysed by eye and quantitated by PDQuest (BioRad) to detect differences. Spots were cut out of the gel, digested with trypsin and the amino acid sequence of the peptides was determined by mass spectrometry as described in Materials and Methods. Expression levels and the mean of at least two experiments is presented. The error bars are standard deviations and * indicates a significant change with paclitaxel treatment (p<0.05).


Received September 17, 2003
Accepted January 5, 2004