Background: Recent studies have shown that paclitaxel (Taxol) is an active chemotherapeutic in the treatment of small cell lung cancer. Paclitaxel binds to tubulin and prevents depolymerization. This causes cells to arrest in the G2/M phase of the cell cycle, resulting in sensitization of cells to drug or radiation treatment.

Methods: A drug-resistant H69 small cell lung cancer subline was established. Cytotoxicity of cisplatin and chlorambucil was determined using the MTT cell viability assay and distribution of DNA in the cell cycle. DNA distribution was analyzed by flow cytometry after treatment with paclitaxel or the other tubulin-binding drugs, vinblastine and navelbine.

Results: The H69-EPR drug-resistant subline was resistant to epirubicin (sixfold) and was cross-resistant to cisplatin (7.5-fold) and chlorambucil (7.5-fold). Pretreatment with paclitaxel or vinblastine, but not navelbine, sensitized the subline to cisplatin and chlorambucil ($P < 0.05$), with no effect on parental H69 cells. Sensitization was dose dependent and occurred at doses below those that caused a G2/M block in the cell cycle.

Conclusion: Sensitization of drug-resistant cells by paclitaxel was not associated with its ability to cause a G2/M block in the cell cycle. Sensitization by paclitaxel and vinblastine, but not navelbine, which preferentially targets mitotic tubulin, suggests that sensitization may involve changes in the tubulin-dependent intracellular transport processes rather than changes in mitotic tubulin and the G2/M block. Cytometry 43:170–174, 2001.

Key terms: paclitaxel (Taxol); vinblastine; navelbine (vinorelbine tartrate); cell cycle; sensitization; H69 small cell lung cancer cells; multidrug resistance (MDR)
with the anthracycline epirubicin. The H82/E8 subline had increased MRP expression and was resistant to cisplatin, chlorambucil, and MDR drugs. The subline therefore expressed an extended-MDR phenotype, which reflects the broad type of resistance encountered in the treatment of resistant cancer (14). We have now extended these studies using H69 cells, representative of the classic form of SCLC, and have determined the ability of paclitaxel to sensitize drug-resistant H69 cells to cisplatin and chlorambucil. We determined the ability of other tubulin-binding drugs (e.g., vinblastine and navelbine [vinorelbine tartrate]) to sensitize the drug-resistant cells and examined the role of the G2/M block in the modulation of drug resistance by these tubulin inhibitors.

MATERIALS AND METHODS

Cell Lines

H69 SCLC cells (ATCC, Rockville, MD) were maintained in RPMI-1640 (Trace Biosciences, Sydney, Australia) medium supplemented with 10% fetal calf serum (FCS; Trace Biosciences), 20 mM HEPES, and 10 mM NaHCO3 at 37°C in a humidified atmosphere with 5% CO2. The MDR H69-EPR subline was developed by intermittent exposure of the H69 cells to 8 and then 40 ng/ml of epirubicin. Cells were maintained in the absence of drug, but were treated with epirubicin for 3 days every 6 weeks to maintain stability. Exponentially growing cells were used for all experiments. Cultures were free of Mycoplasma.

Cytotoxicity Assays

Etoposide, genistein, chlorambucil, and paclitaxel (Taxol) were purchased from Sigma (St Louis, MO); epirubicin and daunorubicin were purchased from Pharmacia (Sydney, Australia); vinblastine, navelbine, and cisplatin were purchased from David Bull Laboratories (Sydney, Australia); and amsacrine was kindly provided by Professor Bruce Baguley (University of Auckland Medical School, Auckland, New Zealand). Sensitivity to drugs was determined in triplicate using the MTT assay as previously described (15) with 6 × 104 cells per well. The 50% inhibitory concentration (IC50) was determined for the resistant subline by the IC50 obtained for the parental H69, (not shown). Consistent with MDR mediated by MRP, the accumulation of daunorubicin was significantly reduced (15%; P < 0.05; Fig. 1). P-glycoprotein was not detectable in either the H69 or H69-EPR cells (not shown) but MRP expression was approximately twofold increased in the H69-EPR subline (Fig. 1). The subline also remained sensitive to the antimetotic agents paclitaxel, vinblastine, and navelbine. However, the H69-EPR subline was resistant to the non-MDR agents, chlorambucil and cisplatin (approximately sevenfold; Fig. 1). P-glycoprotein was not detectable in either the H69 or H69-EPR cells (not shown) but MRP expression was approximately twofold increased in the H69-EPR cell line (not shown). Consistent with MDR mediated by MRP, the accumulation of daunorubicin was significantly reduced (15%, P < 0.05; Fig 2A) in the H69-EPR subline compared with the parental H69 cells. Drug accumulation was also increased in the presence of 10 μM verapamil (Fig. 2A). The accumulation of Rhodamine 123 (effluxed by P-glycoprotein more than MRP) was not reduced compared with the parental H69 cells.

Daunorubicin and Rhodamine 123 Accumulation

Cell-associated daunorubicin and rhodamine 123 were determined as previously described (16). Cells (5 × 105/ml in RPMI-1640) were incubated in duplicate with 1 μM drug for 1 h in the absence or presence of 10 μM verapamil. Cell-associated drug was determined by flow cytometry (FACScan, Becton Dickinson, Sydney, Australia) and the mean fluorescence index (MFI) calculated using CellQuest software (Becton Dickinson). Results are presented as percent drug accumulation of H69 cells. All determinations were performed in duplicate and each experiment was repeated at least twice.

Western Blot Analysis

Plasma membrane preparations were electrophoresed and transferred to a nitrocellulose membrane (BioRad Labs, Sydney, Australia) as previously described (17). Blots were developed for P-glycoprotein, using C219 antibody (Centacore, Malvern, PA) at a dilution of 1:500 and MRP, using B5 antibody (kindly donated by Dr. D. Keppler, Heidelberg, Germany) at a dilution of 1:1,000.

Cell Cycle Analysis

All experiments were repeated at least twice and statistical analysis performed using Student’s t-test. Statistical significance of P < 0.05 is indicated by an asterisk.

RESULTS

Characterization of the Drug-Resistant Cell Line H69-EPR

Intermittent treatment with low doses of epirubicin produced a stable subline, H69-EPR. It was sixfold resistant to the selecting drug and cross-resistant to daunorubicin (fourfold) and etoposide (10-fold), but not to the other topoisomerase II inhibitors, amsacrine and genistein (Fig. 1). The subline also remained sensitive to the antimetotic agents paclitaxel, vinblastine, and navelbine. However, the H69-EPR subline was resistant to the non-MDR agents, chlorambucil and cisplatin (approximately sevenfold; Fig. 1). P-glycoprotein was not detectable in either the H69 or H69-EPR cells (not shown) but MRP expression was approximately twofold increased in the H69-EPR cell line (not shown). Consistent with MDR mediated by MRP, the accumulation of daunorubicin was significantly reduced (15%, P < 0.05; Fig 2A) in the H69-EPR subline compared with the parental H69 cells. Drug accumulation was also increased in the presence of 10 μM verapamil (Fig. 2A). The accumulation of Rhodamine 123 (effluxed by P-glycoprotein more than MRP) was not reduced compared with the parental H69 cells (Fig. 2B).

Paclitaxel Sensitizes Drug-Resistant H69 Cells

The ability of paclitaxel to modulate the drug resistance of the H69-EPR cell line to cisplatin and chlorambucil was determined after exposure of the cells to various doses of paclitaxel for 1 h, followed by a 24-h incubation in drug-free culture medium. Figure 3A shows that after treatment with a
noncytotoxic dose of paclitaxel (10 ng/ml), the H69-EPR cells were more sensitive to treatment with cisplatin. The IC\textsubscript{50} of cisplatin was reduced from 1,000 ± 105 ng/ml to 280 ± 24 ng/ml (P < 0.01), similar to the IC\textsubscript{50} of cisplatin in the H69 cells. Treatment of the H69 cells with 10 ng/ml paclitaxel had no effect, suggesting that the effect of paclitaxel pretreatment was related to the mechanism of resistance in the H69-EPR cells. Similar results were obtained for modulation of H69-EPR resistance to chlorambucil (data not shown). The modulation of cisplatin resistance by paclitaxel was dose dependent. Although paclitaxel treatment had no effect on the H69 cells, doses between 10 and 15 ng/ml significantly reversed the resistance of the H69-EPR cells to cisplatin (Fig. 3B). Higher and lower doses of paclitaxel failed to cause any significant change in the resistance of the H69-EPR cells.

To determine if the modulation of cisplatin resistance was associated with a G\textsubscript{2}/M block, DNA distribution in the cell cycle was analyzed after paclitaxel treatment. The response of both the parental H69 and resistant H69-EPR cells was similar, with a G\textsubscript{2}/M block detectable at paclitaxel doses of 12.5 ng/ml and higher. No cell cycle aberrations were found after exposure to 10 ng/ml paclitaxel, the dose that caused maximum sensitization (Fig. 4).

**Sensitization of Drug-Resistant H69 Cells by Vinblastine and Navelbine**

Although both vinblastine and navelbine belong to the *Vinca* family of natural product drugs, vinblastine, but not navelbine, was able to sensitize the H69-EPR cells to cisplatin treatment. Figure 5A shows that the IC\textsubscript{50} of cisplatin was reduced from 1,000 ± 105 ng/ml to 540 ± 20 ng/ml (P < 0.05) by pretreatment with vinblastine. In contrast, pretreatment with navelbine failed to cause any significant change in cisplatin resistance in the H69-EPR cells (Fig. 5A). These doses of vinblastine and navelbine had no effect on the growth of the H69-EPR cells or any effect on cisplatin cytotoxicity in the H69 cells (Figs. 5A,B). The reversal by vinblastine occurred at doses between 2.5 and 10 ng/ml (not shown). However, vinblastine caused a G\textsubscript{2}/M block in the cell cycle at doses of 15 ng/ml or greater in both the H69 cells and the drug-resistant subline (Fig. 5B). Navelbine required higher doses than vinblastine.
tine (30 ng/ml) to cause a G2/M block in both H69 and H69-EPR cell lines (Fig. 5B).

**DISCUSSION**

The intermittent treatment of H69 SCLC cells with low, clinically relevant doses of epirubicin produced the H69-EPR subline. The subline expressed an extended MDR phenotype, with resistance to some MDR and non-MDR drugs (14). Resistance to the anthracyclines and etoposide is most likely due to the increased expression of MRP in the H69-EPR cells compared with the parental H69 cells. This is because these cells contain functional MRP as demonstrated by the decreased daunorubicin accumulation (Fig. 2A). However, it is unlikely that MRP confers resistance to the non-MDR drugs, cisplatin and chlorambucil, although increased expression of the closely related MRP2/cMOAT has been associated with resistance to cisplatin (18). Therefore, other resistance mechanisms must also be induced to cause the low-level resistance. The sensitization of the H69-EPR cells by paclitaxel may provide an understanding of the mechanism of this low-level drug resistance.

Paclitaxel binds to tubulin and stabilizes microtubules. Under normal cellular conditions, tubulin is organized in the form of microtubules and a dynamic equilibrium exists between soluble tubulin dimers and microtubules. These microtubules play a critical role in mitosis. This is reflected in the G2/M block caused by all the tubulin inhibitors, paclitaxel (Fig. 4), vinblastine, and navelbine (Fig. 5B). However, the sensitization of the resistant subline was independent of the G2/M block. Whereas the parental cells and the drug-resistant subline blocked at similar drug concentrations, only the resistant subline was sensitized. Further, navelbine did not sensitize the resistant subline, but caused a G2/M block. The lack of dependence of resistance reversal by paclitaxel on the G2/M block has also been reported in a drug-resistant HL60 subline where paclitaxel caused sensitization to radiation in the absence of a G2/M block (10).

Microtubules play a critical role, not only in mitosis, but also in intracellular transport and in the maintenance of cell structure. Paclitaxel has been shown to have different cellular effects at low doses that do not cause the G2/M block (19). It is possible that the resistance of the H69-EPR cells involves the tubulin-dependent intracellular transport of drugs and that the sensitization caused by paclitaxel is due to the inhibition of intracellular transport. This is consistent with maximal sensitization at 10 ng/ml (Figs. 3A,B), whereas the G2/M block is first evident at paclitaxel doses greater than 12.5 ng/ml (Fig. 4). This hypothesis is also supported by the fact that the drug-resistant cells may also be sensitized by treatment with vinblastine, but not with navelbine. Although the binding of vinblastine to

**Fig. 3.** Reversal of cisplatin resistance by pretreatment with paclitaxel. A: H69 (open and closed circles) and H69-EPR (open and closed triangles) cells were exposed to paclitaxel for 1 h and incubated overnight in drug-free media before the cytotoxicity of cisplatin was determined. The graph is a representative cytotoxicity assay without (solid lines) or with (broken lines) exposure to 10 ng/ml of paclitaxel. Error bars indicate the SD of triplicate wells. B: H69 cells and H69-EPR cells were exposed to 7.5, 10, 12.5, 15, or 20 ng/ml paclitaxel and the cytotoxicity of cisplatin determined after 24 h incubation in drug-free culture media. Bars represent the mean of the IC50 of at least two experiments and the error bars show SD (*P < 0.05).

**Fig. 4.** The effect of paclitaxel dose on cell cycle. Cells were exposed to paclitaxel for 1 h at the indicated doses (10–20 ng/ml) and the cell cycle profile (DNA content-FL2-height) was determined by flow cytometry after 24 h in drug-free culture media. A representative experiment is shown.
tubulin causes depolymerization at high drug concentrations, at low doses, vinblastine also causes tubulin stabilization (20). Navelbine, in contrast, appears to bind more specifically to tubulin in the mitotic spindle than to cellular tubulin (21), which could account for the inability of navelbine to sensitize the H69-EPR drug-resistant cells.

The mechanism by which tubulin inhibitors affect drug resistance is unknown. However, tubulin inhibitors have been reported to alter the accumulation of many drugs including anthracyclines, etoposide, and cisplatin (22). Navelbine to sensitize the H69-EPR drug-resistant cells. This modulation of cisplatin resistance is dose dependent, but does not correlate with the required concentration to cause a mitotic block.

Understanding the development of low-level drug resistance and methods of preventing its development offers a potential means for treating refractory disease. A combination of paclitaxel and cisplatin has been found to have an increased efficacy in the treatment for SCLC (1). However, the results here suggest that greater success may be possible with different doses of paclitaxel or by altering the treatment schedule.

LITERATURE CITED