Non-P-glycoprotein-mediated Multidrug Resistance in a Small Cell Lung Cancer Cell Line: Evidence for Decreased Susceptibility to Drug-induced DNA Damage and Reduced Levels of Topoisomerase II


ABSTRACT

Data obtained from clinical samples suggest that non-P-glycoprotein mechanisms of multidrug resistance are likely to be important in small cell lung cancer. The H69AR cell line was derived from the H69 small cell lung cancer cell line by selection in doxorubicin (adriamycin) and does not overexpress P-glycoprotein as detected by monoclonal antibody cell lung cancer. The I169AR cell line was derived from the H69 small cell lung cancer cell line by selection in doxorubicin (adriamycin) and does not overexpress P-glycoprotein. Further, transport studies with radiolabeled daunomycin, VP-16, and vinblastine demonstrate that differences in net drug accumulation or efflux are not part of the resistance phenotype of H69AR cells. To determine if H69 and H69AR cells differ in their susceptibility to drug-induced DNA damage, DNA single-strand breaks (SSB) generated by VP-16 and Adriamycin were measured using the alkaline filter elution assay. Readily detectable SSB were produced in intact H69 cells by 5 μM VP-16, but 100 μM drug was required to cause similar damage in H69AR cells. H69AR cells were also resistant to SSB induction by Adriamycin. The formation of SSB by VP-16 was similarly reduced in isolated H69AR nuclei, indicating that resistance to this drug resides, at least in part, in the nucleus. No significant differences were observed in the rate or extent of repair of VP-16-induced DNA SSB in H69 and H69AR cells. The reduced susceptibility to drug-induced SSB may result from alterations in topoisomerase II, since less immunoreactive topoisomerase II was found in H69AR cells compared to H69 cells. However, changes in topoisomerase II cannot explain the resistance of H69AR cells to such drugs as the Vinca alkaloids and gramicidin D, indicating that multiple mechanisms contribute to drug resistance in this small cell lung cancer cell line.

INTRODUCTION

Lung cancer continues to be a significant health problem throughout the world (1). From a therapeutic viewpoint, this disease may be classified as either SCLC or NSCLC, the major distinction being the sensitivity of SCLC to chemotherapy and radiotherapy. Chemotherapy is not generally useful in NSCLC because of the intrinsic drug resistance observed in these types of tumors (2). On the other hand, SCLC often responds well to chemotherapy, but almost inevitably resistance develops (3). The impact of acquired drug resistance on the clinical usefulness of doxorubicin (ADM), VP-16, and cisplatin is particularly striking in SCLC, where these drugs (usually in combination with others) elicit a very high response rate in previously untreated patients but are much less effective in relapsed patients. The P-glycoprotein drug efflux pump is a product of the MDR1 gene (4) and appears to play a role in multidrug resistance in a number of human malignancies (5-7); however, there is no compelling evidence that P-glycoprotein is widely involved in either the intrinsic resistance of NSCLC tumors or the acquired resistance of SCLC tumors (8).

To study the problem of acquired resistance in SCLC, we (9) and others (10-13) have developed drug-resistant SCLC cell lines in vitro by selection in a number of chemotherapeutic agents. A variety of biochemical mechanisms may contribute to resistance in these SCLC cell lines, including the overexpression of P-glycoprotein (10, 11, 13), enhanced DNA repair (12), elevated levels of the detoxification enzyme GSH-S-transferase (14), and reduced activity of the DNA-unwinding enzyme topoisomerase II (15).

The H69AR cell line derived in this laboratory (9) was selected in ADM and is 40-fold resistant to this drug. It is cross-resistant to a wide variety of compounds (9, 16) including the Vinca alkaloids and topoisomerase II-targeting drugs such as VP-16 and mitoxantrone. This pattern of resistance associated with overexpression of P-glycoprotein, yet chemosensitizers such as verapamil and other calcium-modulating agents have little or no effect on ADM cytotoxicity in H69AR cells (17). The mechanism(s) underlying resistance in this cell line are unknown, but it does not overexpress P-glycoprotein as detected by the monoclonal antibody C219 (9) or by Northern blotting with the MDR1 cDNA probe, pCHP1 (18). In a previous study, we reported that GSH-S-transferase activity was elevated 10-fold in H69AR cells (14). However, although the resistance of the H69AR cells compared to H69 cells is very stable, the relative difference in GSH-S-transferase activities is not, indicating that other biochemical alterations must be responsible for drug resistance. In the present study we have used a sensitive PCR technique to verify that P-glycoprotein is not overexpressed in H69AR cells. Consistent with this finding, we show that these resistant SCLC cells do not display decreased drug accumulation. Lastly we have found that H69AR cells are less susceptible to drug-induced DNA damage than the parental H69 cells, an observation that may be explained by diminished levels of topoisomerase II.

MATERIALS AND METHODS

Drugs and Chemicals. [3H]VP-16 (specific activity, 900 mCi/mmol) was obtained from Moravek Biochemicals, Brea, CA. [3H]Daunomycin (specific activity, 3.1 Ci/mmol), [14C]thymidine (specific activity, 58.3 Ci/mmol).
mCi/mmol), [3H]ATP (specific activity, 3000 Ci/mmol) and [methyl-3H]thymidine (specific activity, 6.7 Ci/mmol) were obtained from NEN-Dupont, Markham, Ontario, Canada. [3H]Vinblastine sulfate (specific activity, 10.1 Ci/mmol) was obtained from Amersham Corp., Oakville, Ontario, Canada. VP-16, ADM, and DNR were obtained from the Kingston Regional Cancer Center pharmacy. VBL, BSA, and ATP were obtained from Sigma Chemical Co. (St. Louis, MO) as was alkaline phosphatase-conjugated goat anti-rabbit IgG. Random hexanucleotide primers, T4 poly nucleotide kinase, and RNAguard ribonuclease inhibitor were obtained from Pharmacia (Baie d’Urfé, Quebec, Canada. AMV reverse transcriptase was obtained from Life Sciences (St. Petersburg, FL), and Taq DNA polymerase was from Perkin-Elmer Cetus (Madison, WI). Proteinase K was from BDH Chemicals (Toronto, Ontario, Canada), and tetrapropylammonium hydroxide was from Aldrich Chemical Co. (Milwaukee, WI).

Cell Lines. The SCLC NCI-H69 (H69) cell line was obtained from Dr. A. Gazdar and Dr. J. Minna (Naval Medical National Cancer Institute, Bethesda, MD), and its ADM-selected multidrug-resistant variant, H69AR, was derived in this laboratory and has been described previously (9, 14, 16). These cell lines were maintained in RPMI 1640 medium (GIBCO, Burlington, Ontario, Canada) supplemented with 4 mM l-glutamine and 5% heat-inactivated defined-supplemented bovine calf serum (Hyclone Laboratories, Logan, UT) (RPMI 1640/5% HC medium). The H69AR cell line was challenged once a month with 0.8 μM ADM, and it was rearg to be at least 48 h before each experiment. The K562 human chronic myelogenous leukemia cell line was obtained from the American Type Culture Collection (Rockville, MD). The P-glycoprotein-overexpressing multidrug-resistant human myeloma cell line 8226/R40 was kindly provided by Dr. W. Dalton (Arizona Cancer Center, Tucson, AZ) (19). These cell lines were cultured in Opti-MEM (GIBCO) supplemented with 2% heat-inactivated FBS. The murine leukemia L1210 cell line was obtained from Dr. L. Erickson ( Loyola University, Maywood, IL) and was cultured in RPMI 1640/5% HC medium. All cell lines were cultured at 37°C in a 5% air/5% CO2 atmosphere without antibiotics and were negative for Mycoplasma contamination.

Oligonucleotides. The oligonucleotides (Table 1) used as amplifiers and probes in this study were synthesized on a Biosearch 8750 DNA synthesizer and purified as described (20). To avoid the possibility of amplifying genomic DNA, amplifier pairs for MDR1 were chosen that span several introns. Potential primer sequences were analyzed for the presence of complementary multidrug-resistant human myeloma cell line 8226/R40 was kindly provided by Dr. W. Dalton (Arizona Cancer Center, Tucson, AZ) (19). These cell lines were cultured in Opti-MEM (GIBCO) supplemented with 2% heat-inactivated FBS. The murine leukemia L1210 cell line was obtained from Dr. L. Erickson (Loyola University, Maywood, IL) and was cultured in RPMI 1640/5% HC medium. All cell lines were cultured at 37°C in a 5% air/5% CO2 atmosphere without antibiotics and were negative for Mycoplasma contamination.

Table 1 Oligonucleotides used in amplification and detection of cDNA

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplifiers</td>
<td></td>
</tr>
<tr>
<td>MDR1-U (upstream)</td>
<td>5' ACACCGGACCTACAGATGAGTGTGCT3'</td>
</tr>
<tr>
<td>MDR1-D (downstream)</td>
<td>5' GGAGCACTGAAAATGTGAGAACAGACT3'</td>
</tr>
<tr>
<td>ETRD-U (upstream)</td>
<td>5' GGACGTCTCCCCCAATCATAAGTGGCC3'</td>
</tr>
<tr>
<td>ETRD-D (downstream)</td>
<td>5' GCATGATGTGTTGTTGTCGTA3'</td>
</tr>
<tr>
<td>Probes</td>
<td></td>
</tr>
<tr>
<td>MDR1-1</td>
<td>5' CCCAGTGAAAATGTGAGAACAGACT3'</td>
</tr>
<tr>
<td>ETRD-1</td>
<td>5' GCATGATGTGTTGTTGTCGTA3'</td>
</tr>
</tbody>
</table>

PCR procedures were set up in a laminar flow hood, using positive-displacement pipettors. Reagents, tubes, and tips were UV irradiated for 10 min in a Stratalinker (Stratagene, La Jolla, CA). To synthesize cDNA, 1 μg of total RNA or 20 ng of mRNA were heated at 65°C for 5 min and subsequently chilled on ice. The RNA was incubated at 37°C for 30 min in a 20-μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 8 mM MgCl2, 50 mM KCl, 2 μM of each dNTP, 20 units of RNAGuard, 20 units of AMV reverse transcriptase, and 100 ng of random hexanucleotide primers, followed by the addition of 9 volumes of distilled water. To amplify the cDNA, the diluted cDNA mixture (1/10, 20 μl) was placed in a 500-μl microcentrifuge tube containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 3.5 mM MgCl2, 0.1% gelatin, 0.1% Triton X-100, 0.2 μM of each dNTP, 20 pmol of each primer (U primer and amplicons of both MDR1 and ETRD) (Table 1), and 2.5 units of Taq DNA polymerase (final volume, 50 μl). The reaction mixture was overlaid with light mineral oil, and 35 cycles of amplification were performed on a PHC-2 Dri-Bloc thermocycler (Technne, Cambridge, United Kingdom) as follows: one cycle of denaturation at 94°C for 1.5 min, annealing at 58°C for 1.0 min, and extension at 72°C for 3.0 min; 33 cycles of denaturation at 94°C for 0.75 min, annealing at 58°C for 1.0 min, and extension at 72°C for 1.0 min; and a final cycle of denaturation at 94°C for 0.75 min, annealing at 58°C for 1.0 min, and extension at 72°C for 5 min.

Blotting and Probing of PCR-amplified Samples. After PCR, the mineral oil was removed by two extractions with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0)-saturated chloroform. Ten μl of each sample were separated in a 3% Wide-Range agarose (Sigma), 0.75% agarose gel, 1% agarose gel in 89 mM Tris base, 89 mM boric acid, 2 mM EDTA or 1% agarose, 0.5% Tris-HCl, 2% Tris base, 89 mM boric acid, 2 mM EDTA gel. The gel was UV nicked for 120 s on a transilluminator, and the gel was equilibrated for 20 min in alkaline transfer buffer (0.4 N NaOH, 0.6 M NaCl) and blotted onto Zeta-Probe membrane (BioRad, Mississauga, Ontario, Canada) by an 8-h capillary transfer. The membrane was neutralized by immersion in 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) for 5 min and baked in a vacuum oven for 2 h at 80°C. PCR-amplified DNA was probed with MDR1-1 and ETRD-1, internal oligonucleotides specific for sequences lying between their respective PCR amplimers (Table 1). These oligonucleotides were end labeled with [γ-32P]ATP for 30 min at 37°C (20) and passed over a NAP-5 column (Pharmacia). Specific activities in excess of 7×106 cpm/μg of DNA were obtained. The membrane was washed at 1 h for 42°C in 0.1× SSPE and prehybridized at 55°C for 3 h in hybridization buffer consisting of 5× SSPE, 0.5% SDS, 5× Denhardt’s solution (1× Denhardt’s solution = 0.02% polyvinylpyrrolidone, 0.02% BSA, 0.02% Ficoll 400, and 100 μg/ml of sheared salmon testis DNA. Hybridization was carried out in hybridization buffer containing 1×106 cpm/ml of the appropriate oligonucleotide probe for 4 h at 55°C without agitation. The blot was washed twice for 5 min at room temperature in 20 μl of 1× SSPE, 0.1% SDS, once at 60°C in 5× SSPE, 0.1% SDS, and twice for 5 min at room temperature in 1× SSPE, 0.1% SDS. Autoradiography was carried out using Kodak X-Omat AR film. The blot was stripped by four 20-min washes at 37°C in 0.1× SSPE, 0.1% SDS and then reprobed with the other internal oligonucleotide under the same conditions.

Drug Accumulation and Efflux. To measure drug accumulation, cells (5×106/ml) were incubated with 9 μM [3H]VP-16 (1 μCi/ml) or 5 μM [3H]DNR (0.25 μCi/ml) or 2 μM [3H]VBL (0.25 μCi/ml) at 37°C in RPMI 1640/5% HC supplemented with 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. At selected times, aliquots of cell suspension were removed and added to ice-cold 1% BSA in phosphate-buffered saline (1% BSA/PBS) or ice-cold PBS to stop accumulation. After centrifugation and two washes at 4°C, the cell pellet was solubilized with 1% SDS. Cell-associated radioactivity was determined using liquid scintillation cocktail (Ready Gel; Beckman, Mississauga, Ontario, Canada) and a Beckman LS1701 liquid scintillation counter equipped with a disintegrations per minute calculation program.

To measure drug efflux, H69 and H69AR cells were incubated with radiolabeled drug as for the accumulation studies for 60 min at 37°C. Cells were harvested by centrifugation at 4°C, washed twice in ice-cold 1% BSA/PBS, and resuspended in the same volume of drug-free 3346
medium at 37°C. Aliquots of cell suspension were removed at selected times over the next 60 min, and the cell-associated radioactivity was determined as described above.

Within each experiment, determinations were performed in duplicate or triplicate, and each experiment was repeated 2 to 4 times.

Measurement of DNA Single-Strand Breaks. H69 and H69AR cells were labeled for 18 to 24 h with [2-14C]thymidine (0.02 µCi/ml) followed by a cold chase of 18 to 24 h; L1210 cells were labeled for 18 to 24 h with [methy]-[3H]thymidine (0.05 µCi/ml). After irradiation or exposure to drug, labeled H69 or H69AR cells (1 x 10^5/ml) were mixed with an equal number of labeled L1210 cells that had been irradiated at 300 rads for the high-sensitivity assay or 1000 rads for the low-sensitivity assay and served as an internal standard. Cells were irradiated using a 137Cs irradiator (Atomic energy of Canada, Ltd.) at a dose rate of 80 rads/min. Alkaline filter elution was performed according to the method of Kohn et al. (26). Cells were deposited on chilled polycarbonate filters (Nucleopore, Toronto, Ontario, Canada) under vacuum, lysed with 2% SDS, 25 mM EDTA, 0.1 M glycine, and treated with proteinase K (0.5 mg/ml) in lysis buffer. DNA was eluted with 0.2% SDS, 20 mM EDTA in 1% tetrapropylammonium hydroxide (pH 12.1) at a flow rate of 0.035 ml/min, 3-h fractions were collected for 15 h (high-sensitivity assay) or 0.12 ml/min and 5-min fractions were collected for 30 min (low-sensitivity assay). The percentage of 14C versus the percentage of 3H remaining on the filters over time was plotted on a double log plot; the elution curves were linear for the first three fractions, and the elution rate was calculated as the slope of the linear portion of the curve.

The elution rate at various doses of radiation was determined in H69 and H69AR cells with the intent of expressing drug-induced damage as rad-equivalents, as is commonly done. However, the slope of the elution rate versus radiation dose curve was significantly less for H69 than for H69AR cells; that is, at a given dose of radiation, more SSB are produced in H69AR cells than in H69 cells (results not shown). Therefore, expressing drug-induced damage as rad-equivalents would underestimate the damage in H69AR cells and thereby inflate the estimate of its resistance to drug-induced SSB. For this reason, the elution rate (initial slope of the elution curve) was used as a direct measure of SSB.

To measure the effect of a 1-h exposure of VP-16 and a 24-h exposure of ADM on DNA SSB induction, cells were treated with drug at 37°C and washed, and high-sensitivity alkaline elution was performed as above. A 24-h exposure to ADM was needed because a 1-h exposure did not produce detectable SSB. Untreated cells were included as a negative control in each experiment.

To measure the effect of VP-16 on DNA SSB induction in isolated nuclei, cells were washed twice with cold Buffer A [1 mM KH2PO4 (pH 6.4), 5 mM MgCl2, 150 mM NaCl, 10 mM EGTA, 4 mM dithiothreitol] and then resuspended in 2 ml of Buffer A containing 0.3% Triton X-100 at 4°C for 10 min (27). Eight ml of Buffer A were added, and the nuclei were pelleted by centrifugation. The nuclei were exposed to drug for 1 h at 37°C in Buffer A and pelleted by centrifugation, and low-sensitivity alkaline elution was carried out. The optimum ATP concentration for both cell lines was found to be approximately 3 mM and was added to the nuclei with the drug. A negative control without drug was included in each experiment and indicated that little DNA damage was produced by the nuclear preparation itself. The integrity of the nuclei was confirmed by electron microscopy.

To measure repair of VP-16-induced SSB, H69 or H69AR cells were exposed to 5 µM or 100 µM VP-16, respectively, for 1 h at 37°C, washed, resuspended in drug-free medium at 37°C, and aliquoted into glass tubes. At selected times, repair was stopped by the addition of cold medium and placing the tubes on ice. High-sensitivity alkaline elution was carried out as described above.

Immunodetection of Topoisomerase II. H69 and H69AR cells were collected by centrifugation, washed in PBS, and resuspended in solutionizing buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol] with phenylmethylsulfonyl fluoride (300 µg/ml) and aprotinin (0.5 units/ml). The cell suspensions were passed through a 27-gauge needle and boiled for 5 min. These whole-cell homogenates (50 µg of protein/lane) were subjected to electrophoresis on a 7% SDS-polyacrylamide gel. Proteins were transferred to Immobilon (Millipore, Mississauga, Ontario, Canada) at 150 mA for 18 h by the method of Towbin et al. (28). The blot was incubated with a rabbit anti-topoisomerase II antibody (diluted 1:1000 in blocking buffer consisting of 5% FBS/5% NGS in PBS/0.05% Tween 20). This antibody was raised against the recombinant M, 70,000 C-terminus of HeLa topoisomerase II (29) and was kindly provided by Dr. D. Sullivan and Dr. W. Ross (James Brown Cancer Center, Louisville, KY). Binding of the antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit IgG (diluted 1:1000 in blocking buffer) with nitroblue tetrazolium and bromochloroindolyl phosphate as substrates (14). Equal loading of protein was verified by amido-black staining of transferred proteins (not shown) and by equal nonspecific reaction of the rabbit antibody with a M, 60,000 protein.

RESULTS

Amplification of MDR1. cDNA was synthesized from mRNA isolated from the H69 and H69AR cell lines. Coamplification of a 697-base pair fragment defined by amplimers MDR1-U and MDR1-D and a 453-base pair fragment defined by amplimers ETRD-U and ETRD-D was carried out by PCR. For comparison, RNA samples from two additional human cell lines were included in these experiments as standards of drug sensitivity (K562) and resistance (8226/R40). The latter cell line is known to overexpress P-glycoprotein and thus also served as a positive control.

The amplified sequences were separated by electrophoresis and blotted. The blot was probed with an oligonucleotide specific for MDR1 (MDR1-1), stripped, and reprobed with an oligonucleotide specific for ETRD (ETRD-1). The results of these blots are shown in Fig. 1. Both H69 and H69AR express very low levels of MDR1 mRNA as evidenced by the 697-base pair MDR1-specific fragment. MDR1 expression in H69 cells was between 0.1 and 0.01% that of the 8226/R40 cell line. Interestingly, the H69AR cell line appears to express less MDR1 than the H69 cell line (only 0.01% of 8226/R40 cell expression). The drug-sensitive cell line K562 expresses levels of MDR1 greater than both H69 and H69AR cells and between 0.1 and 0.01% that seen with 8226/R40. The absence of a band in the negative control lane (ddH2O as template) indicates that none of the reaction components was contaminated with amplifiable template. A non-reverse-transcribed control was included for each sample to ensure that they were also not contaminated.

The lower autoradiogram in Fig. 1 demonstrates that H69, H69AR, and K562 cells have similar amounts of the 453-base pair ETRD DNA fragment, indicating that equivalent amounts of mRNA went into each reaction and were amplified with similar efficiency. The 8226/R40 cell line showed somewhat less ETRD fragment than the other cell lines. This may be due to inherently less esterase D expression in this cell line compared to the others. It is also possible that less mRNA went into cDNA synthesis and PCR from this cell line than the others. This is unlikely based on gel quantitation of total RNA yields. The third and most plausible explanation is that the ETRD fragment was less efficiently amplified because the high levels of MDR1 cDNA in 8226/R40 cells compete for dNTPs and Taq polymerase, as has been observed by others (23).

Drug Uptake and Efflux. The time courses of accumulation and efflux for DNR, VP-16, and VBL are presented in Fig. 2. Steady-state concentrations of DNR and VP-16 were achieved by 30 min in both H69 and H69AR cells; there were no significant differences between the sensitive and resistant cell
and resistant (8226/R40 and H69AR) human tumor cell lines. The blot was hybridized with the MDR1-I oligonucleotide (top), stripped, and reprobed with the ETRD-I oligonucleotide (bottom). In both cases, the blot was probed with 1 x 10^6 cpm of [γ-32P]ATP end-labeled oligonucleotide. The upper autoradiogram represents an exposure of 29.5 h at -70°C with intensifying screens, while the lower autoradiogram represents an exposure of 3.5 h at room temperature without intensifying screens.

Fig. 1. Southern blot analysis of PCR products from sensitive (H69, K562) and resistant (8226/R40 and H69AR) human tumor cell lines. The blot was hybridized with the MDR1-I oligonucleotide (top), stripped, and reprobed with the ETRD-I oligonucleotide (bottom). In both cases, the blot was probed with 1 x 10^6 cpm of [γ-32P]ATP end-labeled oligonucleotide. The upper autoradiogram represents an exposure of 29.5 h at -70°C with intensifying screens, while the lower autoradiogram represents an exposure of 3.5 h at room temperature without intensifying screens.

lines in levels of accumulation of VBL. Equivalent steady-state concentrations of VBL were also obtained in both cell lines but were achieved by 5 min. Thus, there were no differences between H69 and H69AR cells in accumulation of VBL, DNR, or VP-16. These results are in agreement with those of Dr. W. Dalton (Arizona Cancer Center) who found equal concentrations of ADM in H69 and H69AR cells after 60-min exposure to this drug using high-performance liquid chromatography.5

Efflux of DNR, VP-16, and VBL was measured after "loading" the cells with drug to achieve steady-state drug levels. No significant differences in rates of efflux were observed between the sensitive and resistant cells with any of the three drugs (Fig. 2). After 60-min incubation in drug-free medium, 50 to 60% of DNR and VBL and 25% of VP-16 remained associated with the cells.

DNA Damage and Repair. The induction of SSB by exposure of H69 or H69AR cells to VP-16 and ADM was investigated. Readily detectable SSB were produced in H69 cells by 5 μM VP-16 (Fig. 3, top). In contrast, 100 μM VP-16 was required to induce similar amounts of damage in H69AR cells. Thus, H69AR cells are approximately 20-fold resistant to the induction of SSB by VP-16. When H69 cells were exposed to concentrations of ADM ranging from 0.05 to 3 μM for 1 h, no SSB were detected (data not shown). However, when the ADM exposure time was extended to 24 h, SSB were detected in H69 cells but not H69AR cells (Fig. 3, bottom). Maximal damage in H69 cells was produced by 0.3 μM ADM. There was less damage at higher ADM concentrations as has been reported in other studies with DNA-intercalating agents (30, 31).

Elution experiments were also performed on isolated nuclei. No SSB were detected in the absence of added ATP. H69AR nuclei were approximately 10-fold resistant to the induction of SSB by VP-16 compared to H69 nuclei (Fig. 4). In addition, the maximal induction of SSB was less in H69AR nuclei.

The ability of H69 and H69AR cells to repair DNA damage was examined after exposure of the cells to VP-16 (Fig. 5). In both cell lines, there was a plateau in the repair of drug-induced SSB by 30 min after removal of the drug. Moreover, there was no significant difference in the initial rate of repair (up to 10 min).

Immunodetection of Topoisomerase II. Immunoblots of cell lysates were performed using a rabbit anti-topoisomerase II antibody. A single Mr 170,000 to 180,000 band was much more intense in H69 cells compared to H69AR cells (Fig. 6).

**DISCUSSION**

The elucidation of the function of P-glycoprotein has had a major impact on the understanding of multidrug resistance in human tumors. More importantly, it has enabled the development of rational strategies aimed at reversing resistance in the clinic (32). Nevertheless, it has become clear that overexpression of P-glycoprotein is likely to be only one of several resistance mechanisms that will be clinically significant (33).

Analysis of MDR1 gene expression in SCLC cell lines and tumor samples from SCLC patients using a PCR-based assay and RNA analysis have led to the conclusion that P-glycoprotein does not play a major role in the drug resistance observed in this disease (8, 22). The PCR data in the present study (Fig. 1) provide convincing evidence that the multidrug-resistant H69AR SCLC cell line expresses very low levels of MDR1 and, more importantly, that this gene is not overexpressed in H69AR cells relative to H69 cells. Therefore the H69AR cell line is a model of drug resistance that is potentially clinically relevant. H69AR is one of several ADM-selected human tumor cell lines that displays multidrug resistance (i.e., resistance to the vinca alkaloids, vinblastine, VBL, and the epipodophyllotoxins, as well as Vinca alkaloids) but does not overexpress P-glycoprotein. Others include the SCLC cell line GLC4/ADR (12), the fibrosarcoma cell line HT1080/DR4 (34), two variants of the HL60 myeloid leukemia cell line (35, 36), and variants of two NSCLC cell lines, SW-1573 (37) and COR-L23 (38). Baas et al. (37) have suggested that, because several of these cell lines have similar features, they may share a common mechanism of resistance. However, the H69AR cell line is clearly different from these other cell lines because it does not display decreased drug accumulation. The H69 and H69AR cell lines have an equal capacity to accumulation anthracyclines (DNR and ADM) as well as the Vinca alkaloid, VBL, and the epipodophyllotoxin, VP-16 (Fig. 2). These observations, together with the PCR data (Fig. 1), indicate that a mechanism(s) other than P-glycoprotein or a similar drug transporter is responsible for the multidrug resistance phenotype in H69AR cells.

Although decreased accumulation is not a factor in H69AR cell drug resistance, it is still possible that the sequestering of the drugs within the cell has changed such that the drugs are...
Daunomycin

DRG RESISTANCE IN SMALL CELL LUNG CANCER

VP-16

Vinblastine

20 30 40 SU 60

time (min) time (min) time (min)

Fig. 2. Drug accumulation and efflux in sensitive H69 (○) and resistant H69AR (●) cells. For the drug accumulation studies, cells were incubated in the presence of drug at 37°C for the times indicated. Points, mean of values obtained in two or more independent experiments; bars, SD where three or more independent experiments were performed. For drug efflux studies, cells were incubated in the presence of drug for 60 min at 37°C, washed, and exposed to drug-free medium. Cell-associated radioactivity was determined at the times indicated. Points, mean of values obtained in three or more independent experiments; bars, SD.

less able to exert their cytotoxic actions. Some investigators have taken advantage of the fluorescent properties of the anthracyclines and used fluorescence microscopy to determine the intracellular distribution of these drugs. Alterations in the subcellular localization of ADM and DNR have been found in both cell lines that do (39, 40) and those that do not overexpress P-glycoprotein (41). Unfortunately, this technique cannot be used with the nonfluorescent Vinca alkaloids or epipodophyllotoxins and, thus, the question of whether multidrug resistance ensues from altered subcellular distribution cannot be addressed in this manner. Nevertheless, it will be of interest to determine if differences in distribution play a role in the anthracycline resistance of H69AR cells.

It is widely held that the cytotoxic effects of ADM and VP-16 occur through direct or indirect damage of DNA. The finding that a number of ADM-selected multidrug-resistant cell lines are less vulnerable to drug-induced DNA damage is consistent with this view (42–44). Reduced numbers of drug-induced SSB were found in intact H69AR cells compared to H69 cells after exposure to either VP-16 or ADM (Fig. 3). Since drug accumulation is not reduced in H69AR cells, the lower incidence of DNA SSB in these cells cannot be attributed to a lower intracellular drug concentration (45). Resistance to the induction of SSB by VP-16 in isolated H69AR nuclei was similar to that found in intact H69AR cells (Fig. 4). Furthermore, the detection of SSB in isolated nuclei required the addition of ATP. These data indicate that a nuclear mechanism such as altered topoisomerase II is likely to be involved in the resistance phenotype of H69AR cells.

DNA damage (Fig. 5), suggesting that enhanced DNA repair does not contribute to resistance in H69AR cells.

Resistance to drugs that generate DNA strand breaks by interacting with topoisomerase II has been attributed to a decrease in the quantity and activities of this enzyme in a number of different cell lines (47). It is noteworthy that H69AR cells contain less immunoreactive enzyme than do the sensitive parent cells (Fig. 6), a finding consistent with the cross-resistance of these cells to topoisomerase II-targeting drugs (9). The role of topoisomerase II in SCLC resistance is potentially complex. A variety of biochemical and molecular alterations in this enzyme have been described in drug-resistant cultured cell lines. For instance, some cell lines contain two forms of the enzyme (topoisomerase II-α and topoisomerase II-β) coded for by similar-sized mRNAs (48, 49), while others display a reduction in the catalytic activity of a single enzyme (10, 28, 50); two differently sized topoisomerase II mRNAs have been reported in yet another cell line (51). At least three different allelic mutations have been found (51–53), and differences in gene methylation have also been described (53). Thus, an array of genetic changes in topoisomerase II have been observed in resistant cells, although there is no evidence to indicate if any one alteration is related to the drug used in selection or to a given tumor type or species. Experiments are under way to examine H69AR cells for further quantitative or qualitative alterations of both topoisomerase II-α and topoisomerase II-β proteins and their mRNAs. The possibility of topoisomerase II gene rearrangements and altered methylation status is also under investigation.

In conclusion, the results presented in this paper indicate a role for topoisomerase II in the resistance phenotype of H69AR cells. However, changes in this enzyme cannot explain the resistance of these cells to such drugs as the Vinca alkaloids and gramicidin D. Like other ADM-selected cell lines, the H69AR cell line possesses biochemical and pharmacological...
Fig. 3. Effect of VP-16 (top) and ADM (bottom) on SSB induction in H69 (○) and H69AR (●) cells. Points, mean of 2 to 8 independent determinations; bars, SD where 3 or more experiments were done.

Fig. 4. Effect of VP-16 on DNA SSB induction in isolated nuclei from H69 (○) and H69AR (●) cells. Points, mean of one to 4 independent determinations; bars, SD where 3 or more experiments were done.

Fig. 5. Time course of DNA repair of VP-16-induced SSB in H69 (○) and H69AR (●) cells after a 1-h exposure to 5 μM and 100 μM VP-16, respectively. Points, mean of 2 to 4 experiments; bars, SD where 3 or more experiments were done.

Fig. 6. Immunoblots of topoisomerase II in H69 and H69AR total cell lysates. The position of the protein detected by the rabbit anti-topoisomerase II antibody is indicated by the arrow to the left of the blot. Numbers to the right of the blot indicate position and size of the molecular weight markers (kDa). Equal loading of protein was confirmed by amido-black staining of transferred proteins (not shown) and by equal nonspecific reaction of the rabbit antibody with a M, 60,000 protein.
properties consistent with multiple resistance mechanisms (12, 41, 44, 46, 54).

Innovative approaches to new therapies in SCLC are required to overcome the therapeutic plateau that has been reached in this disease (3). Rational development of these therapies will be best achieved when the biology of drug resistance in SCLC is understood. Such understanding should be facilitated by investigations of the mechanisms of resistance in the H69AR cell line and other SCLC model systems (10–13, 55, 56) which reflect the clinical, pathologic, and biological complexity of this disease. Ultimately, information derived from these experimental model systems must be integrated and correlated with clinical studies so that relevant resistance mechanisms can be identified that may be amenable to therapeutic intervention.

ACKNOWLEDGMENTS

The technical assistance of D. Clements and E. Vreekom is gratefully acknowledged. The authors wish to thank Dr. K. Deugau, Dr. R. Deelew, Dr. L. Erickson, Dr. R. Ramharack, Dr. W. Ross, Dr. D. Sullivan, and Dr. L. Zwiller for helpful discussions. We thank B. Bryant Harris for assistance with the manuscript.

REFERENCES


Non-P-glycoprotein-mediated Multidrug Resistance in a Small Cell Lung Cancer Cell Line: Evidence for Decreased Susceptibility to Drug-induced DNA Damage and Reduced Levels of Topoisomerase II


Updated version  Access the most recent version of this article at:  http://cancerres.aacrjournals.org/content/51/13/3345