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 C219 (S. E. L. Mirski et al., Cancer Res., 47: 2594, 1987). In the present study, we have used the polymerase chain reaction to verify that H69AR cells do 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 is typical 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 either by 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), [2-14C]Thymidine (specific activity, 58.3

* S. P. C. Cole, unpublished observations.
mCi/mmol), [γ-32P]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 hexa-
ucleotide primers, T4 poly nucleotide kinase, and RNAguard ribonu-
clease inhibitor were obtained from Pharmacia (Baie d'Urfé, Quebec,
Canada). Proteinase K was from BDH Chemicals (Toronto,
Canada). AMV reverse transcriptase was obtained from Life Sciences
Institute, Bethesda, MD), and its ADM-selected multidrug-resistant
variant, H69AR. was derived in this laboratory and has been described
(Madison, WI). Proteinase K was from BDH Chemicals (Toronto,
Canada), and tetrapropylammonium hydroxide was from Aldi-
Rich 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
glucose 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 drug was removed 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. D. 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 95% air/5% CO2
atmosphere without antibiotics and were negative for Mycoplasma
contamination.

Oligonucleotides. The oligonucleotides (Table 1) used as amplimers
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 structures that have been known to result
in the formation of an artifactual “primer dimer” and to avoid any
internal secondary structure. The MDR1 primers were screened for
similarity with all known P-glycoprotein cDNA sequences and several
common vectors. ETRD was used as an endogenous internal control
for cDNA synthesis and subsequent PCR as this enzyme is expressed
at relatively low but constant levels in most tissues (21). Expression
of ETRD is less dependent on the cell cycle than other genes, for example,
β- and γ-actins. Other investigators have used δ2-microglobulin as
an internal control (22, 23), but this was not included in the present study
because expression of this protein is often undetectable in SCLC cell
lines (24).

RNA Isolation, cDNA Synthesis, and Amplification by PCR. Total
cyttoplasmic RNA was isolated from K562 and 8226/R40 cells accord-
ing to the method of Gough (25) using 5 × 10^6 cells per sample. mRNA
was prepared from H69 and H69AR cells using a FastTrack mRNA
isolation kit (Invitrogen, San Diego, CA). All cDNA synthesis and
PCR procedures were set up in a laminar flow hood, using positive-
placement 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 mM 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 micro centrifuge 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 mM of each dNTP, 20 pmo l of each primer (U
and ETRD amplimer pairs 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 amplifi-
cation were performed on a PTC-2 Dri-Bloc thermocycler (Techne,
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
neutralized by immersion in 1.5 M NaCl, 0.5 mM 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 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 × 10^10 cpm/μg of DNA were obtained.
The membrane was washed for 1 h at 42°C in 0.1× SSPE and prehy-
bridized at 55°C for 5 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 hy-
bridization buffer containing 1 × 10^9 cpm/ml of the appropriate oligo-
nucleotide probe for 4 h at 55°C without agitation. The blot was
washed twice for 5 min at room temperature in 20 μl/μg/ml of 5× SSPE
60°C, 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,
95°C washes 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 × 10^6/ml) were incubated with 9 μM [3H]VP-16 (1 μCi/ml) or 5 μM
[3H]DNBR (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. After 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 solubi-
лизed with 1% SDS. Cell-associated radioactivity was determined using
liquid scintillation cocktail (Ready Gel; Beckman, Missisauga, On-
tario, Canada) and a Beckman LS1701 liquid scintillation counter equi-
pared 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

Table 1 Oligonucleotides used in amplification and detection of cDNA

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplimers</td>
<td></td>
</tr>
<tr>
<td>MDR1-U (upstream)</td>
<td>5’-ACACCGCCTTACATAGATGGCTC3’</td>
</tr>
<tr>
<td>MDR1-D (downstream)</td>
<td>5’-CAGAAGTGGTAAAGCTAAGACAT3’</td>
</tr>
<tr>
<td>ETRD-U (upstream)</td>
<td>5’-GGAGCTTCCCCCAAACTCTAAGG3’</td>
</tr>
<tr>
<td>ETRD-D (downstream)</td>
<td>5’-GCATGATGTCTGATGTGGTCAGTAA3’</td>
</tr>
<tr>
<td>Probes</td>
<td></td>
</tr>
<tr>
<td>MDR1</td>
<td>5’-CCCGAGTAAAGAATCTGGCATTGAC3’</td>
</tr>
<tr>
<td>ETRD</td>
<td>5’-GGACGAAATGCGTGAACAGATTTG3’</td>
</tr>
</tbody>
</table>
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 [\textsuperscript{3}H]thymidine (0.05 \textmu Ci/ml). After irradiation or exposure to drug, labeled H69 or H69AR cells (1 \times 10\textsuperscript{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 on ice using a \textsuperscript{137}Cs 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 glycerine, 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 \textsuperscript{3}H versus the percentage of \textsuperscript{14}C 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. 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 KH\textsubscript{2}PO\textsubscript{4} (pH 6.4), 5 mM MgCl\textsubscript{2}, 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 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 \textmu M or 100 \textmu M 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 solubilizing buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol] with phenylmethylsulfonyl fluoride (300 \mu g/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 \mu g 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% NHS 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 \textit{MDR1}. cDNA was synthesized from mRNA isolated from the H69 and H69AR cell lines. Coamplification of a 697-base pair fragment defined by amplimers \textit{MDR1}-U and \textit{MDR1-D} and a 453-base pair fragment defined by amplifiers \textit{ETRD}-U and \textit{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 \textit{MDR1} (\textit{MDR1}-1), stripped, and reprobed with an oligonucleotide specific for \textit{ETRD} (\textit{ETRD}-1). The results of these blots are shown in Fig. 1. Both H69 and H69AR express very low levels of \textit{MDR1} mRNA as evidenced by the 697-base pair \textit{MDR1}-specific fragment. \textit{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 \textit{MDR1} than the H69 cell line (only 0.01% of 8226/R40 cell expression). The drug-sensitive cell line K562 expresses levels of \textit{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 (ddH\textsubscript{2}O 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 \textit{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 \textit{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 \textit{ETRD} fragment was less efficiently amplified because the high levels of \textit{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 lines.

3347
and resistant (8226/R40 and H69AR) human tumor cell lines. The hlot was
concentrations of ADM in H69 and H69AR cells after 60-min
ences between H69 and H69AR cells in accumulation of VBL,
state concentrations of VBL were also obtained in both cell
lower autoradiogram represents an exposure of 3.5 h at room temperature without
intensifying screens, while the
hybridi/cd with the MDR1-I oligonucleolide (top}, stripped, and reprobed with

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 (v'2P|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 differ-
ences 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 ranging from 0.05 to 3 mMfor 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
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 develop-
ment of rational strategies aimed at reversing resistance in the
clinic (32). Nevertheless, it has become clear that overexpres-
sion of P-glycoprotein is likely to be only one of several resist-
ance 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-glycopro-
tein 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 an-
thracyclines, 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 resist-
ance 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

5 Unpublished observation.
Daunomycin

DRUG RESISTANCE IN SMALL CELL LUNG CANCER

VP-16

Vinblastine

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.

Cell lines which differ in their sensitivity to DNA-damaging drugs may also differ in their ability to repair DNA damage. Such differences have been reported in other ADM-selected cell lines (46), including a SCLC cell line (12). However, using the alkaline filter elution assay, we were unable to detect any differences in the rate or extent of repair of VP-16-induced 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 (O) 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 (O) 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 (O) 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, pathological, 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.

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