Mechanisms of Resistance to Etoposide and Teniposide in Acquired Resistant Human Colon and Lung Carcinoma Cell Lines

Byron H. Long, Lotte Wang, Aurelio Lorico, Richard C. C. Wang, Michael G. Brattain, and Anna Maria Casazza

ABSTRACT

Stable acquired resistance to etoposide (VP-16) or teniposide (VM-26) in HCT116 human colon carcinoma cells and A549 human lung adenocarcinoma cells, was previously obtained by weekly 1-h exposures to either drug (B. H. Long, Natl. Cancer Inst. Monogr., 4: 123–127, 1987). The purpose of this study was to identify possible mechanisms of resistance present in these cells by using human mdr1 and topoisomerase II DNA probes, antibodies to these gene products, and P4 phage unknotted assay for topoisomerase II activities. HCT116(VP)35 cells were 9-, 7-, and 6-fold resistant to VP-16, VM-26, and Adriamycin, respectively, and showed no cross-resistance to colchicine and actinomycin D. These cells had no differences in mdr1 gene, mdr1 mRNA, or P-glycoprotein levels but displayed decreased levels of topoisomerase II mRNA and enzyme activity without any alteration of drug sensitivity displayed by the enzyme. HCT116(VM)34 cells were 5-, 7-, and 21-fold resistant to VP-16, VM-26, and Adriamycin; were cross-resistant to colchicine (7-fold) and actinomycin D (18-fold); and possessed a 9-fold increase in mdr1 mRNA and increased P-glycoprotein without evidence of mdr1 gene amplification. No alterations in topoisomerase II gene or mRNA levels, enzyme activity, or drug sensitivity were observed. A549(VP)28 and A549(VM)28 cells were 8-fold resistant to VP-16 and VM-26 and 3-fold resistant to Adriamycin. Both lines were not cross-resistant to colchicine or actinomycin D but were hypersensitive to cisplatin. No alterations in mdr1 gene, mdr1 mRNA, or P-glycoprotein levels, but lower topoisomerase II mRNA levels and decreased enzyme activities, were observed. Of the four acquired resistant cell lines, resistance is likely related to elevated mdr1 expression in one line and to decreased topoisomerase II expression in the other three lines.

INTRODUCTION

The active anticancer drugs VP-166 and VM-26 have been identified as potent inhibitors of eukaryote topoisomerase II (1–5). This inhibition results in the stabilization of the transient covalent intermediate formed between the enzyme and its DNA substrate (6–9). The formation of this covalent DNA-protein complex is readily observable in intact cells by using standard alkaline DNA elution techniques (10, 11). In fact, alkaline DNA elution methodology played a major role in identifying topoisomerase II as the target for VP-16 and VM-26 (1–3, 5, 12, 13) and DNA intercalating drugs (14–19). It is generally recognized that inhibition of topoisomerase II resulting in stabilization of a normally transient, covalent, DNA-enzyme inter-
free medium. Each cycle was repeated for 29, 34, or 35 weeks, as previously described (22). The generation times for HCT116, HCT116(VP)35, HCT116(VM)34, A549, A549(VP)28, and A549(VM)28 cells were 24, 23, 28, 37, and 30 h, respectively. Cells intended for alkaline elution experiments were labeled separately for 3 days with 0.1 μCi/ml [3H]thymidine in 60-cm2 plastic culture dishes to be used for experimental purposes or with 0.1 μCi/ml [3H]thymidine in 75-cm2 plastic culture flasks to be used for reference purposes (33).

Cytotoxicity Assay. The cytotoxic effects of VP-16, VM-26, and six other anticancer drugs or cytotoxic agents on the parental and acquired A549(VM)28 cells were 24, 34, 23, 28, 37, and 30 h, respectively. Cells sedimented by centrifugation, and the pellets were dissolved in 0.1 M saline, then incubated with drug for 1 h followed by incubation in drug-free medium until the flask containing untreated cells were 50 to 80% confluent, as previously described (21). Following the cell proliferation period, the surviving cells were released from flasks with trypsin-EDTA, fixed with formaldehyde, and counted by using a Model ZC Coulter Counter (Hialeah, FL). Average counts obtained from duplicate flasks of cells exposed to a given drug concentration were expressed as a percentage of the average number of untreated control cells from four flasks for each experiment. These percentages were plotted as probit values versus log of drug concentration to obtain the concentration of drug necessary to inhibit cell proliferation by 50%.

Alkaline Elution Assays for DNA Breakage in Intact Cells. DNA break production was quantified by standard alkaline elution techniques (10, 11). Logarithmically growing cells, containing [3H]DNA were exposed to various concentrations of VP-16 and VM-26 for 1 h. Cells were harvested and layered onto polycarbonate filters with reference cells containing [3H]DNA and exposed to 300 rads of γ-radiation as an internal elution standard. Cells were lysed with 2% sodium dodecyl sulfate, 25 mM EDTA (pH 9.6) containing proteinase K, and the DNA remaining on the filters was slowly eluted by pumping at 40 μl/min, using a buffer composed of tetrapropylammonium hydroxide and EDTA, which was adjusted to pH 12.1 to reveal both single and double strand DNA breaks. Quantification of DNA breaks was achieved by relating slopes of DNA elution curves obtained following drug treatment to those obtained following exposure to different doses of γ-radiation and calculation of true double/single strand break ratio was achieved, as previously described (3, 13, 21, 34).

VP-16 and Porfioromycin Influx and Efflux. Cells plated at a density of 5 × 10^4 cells in each 75-cm2 flask on the previous day were incubated at 37°C with 5 μCi [14C]VP-16 or [14C]Porfioromycin (Moravek Biochemicals, Brea, CA) for 30 min. The cells were immediately rinsed 4 times with ice-cold phosphate-buffered saline at the end of the incubation period, then lysed with 0.1 M NaOH. For efflux studies, cells were incubated with radiolabeled drug for 30 min; washed once with warm, drug-free medium; and incubated at 37°C for 30 to 60 min, then rinsed and processed as above. Intracellular contents of drug expressed in pmol/10^6 cells were calculated from the specific radioactivities of each drug. Cells volumes were determined by suspending 1 × 10^7 cells in 25-cm2 flasks 5 to 7 days following drug treatment. Cells were incubated with drug for 1 h followed by incubation in drug-free medium until the flask containing untreated cells were 50 to 80% confluent, as previously described (21). Following the cell proliferation period, the surviving cells were released from flasks with trypsin-EDTA, fixed with formaldehyde, and counted by using a Model ZC Coulter Counter (Hialeah, FL). Average counts obtained from duplicate flasks of cells exposed to a given drug concentration were expressed as a percentage of the average number of untreated control cells from four flasks for each experiment. These percentages were plotted as probit values versus log of drug concentration to obtain the concentration of drug necessary to inhibit cell proliferation by 50%.

Assay for Topoisomerase II Activities. To assay topoisomerase II activities in the different cell lines, 2 × 10^7 log phase cells in 75-cm2 tissue culture flasks were washed twice with ice-cold phosphate-buffered saline, then incubated with reticulocyte swelling buffer composed of 5 mM KH2PO4 (pH 7.5), 2 mM MgCl2, 0.5 mM dithiothreitol, 0.1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride, then pelleted by centrifugation at 600 × g for 20 min, then washed twice in ice-cold phosphate-buffered saline containing 5% FBS and 0.1% NaN3, and resuspended in 0.5 M NaCl (final concentration). The salt extraction was performed for 30 min at 4°C. Extracted nuclei and insoluble material were removed by centrifugation at 600 × g for 20 min, then at 12,000 × g for 10 min at 4°C. Protein quantification was assessed by the method of Bradford (37).

The strand-passing activities of topoisomerase II in serially diluted aliquots of 0.35 M NaCl extracts were monitored by using the P4 DNA unwinding assay. Reactions were initiated upon addition of 5 μl of each diluted sample to 20 μl of reaction mixture containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl2, 0.5 mM EDTA, 30 μg/ml bovine serum albumin, 0.5 mM dithiothreitol, 1 mM ATP, and 2 μg/ml P4 knotted DNA. After incubation at 37°C for 30 min, the reactions were stopped by addition of 3 μl of stopping buffer consisting of 50% glycerol, 5% sodium dodecyl sulfate, 30 mM EDTA, and 0.25% bromphenol blue. The DNA products were then separated by electrophoresis in 0.9% agarose gels in buffer consisting of 80 mM Tris-HCl (pH 8.0), 40 mM boric acid, and 2 mM EDTA. Photograph negatives from ethidium bromide-stained gels were scanned with an LKB Ultrascan XL densitometer. The linear P4 band provided an arbitrary separation point between the knotted and the unknotted DNA topoisomerers. The Topoisomerase II activities were calculated as the ratio between the unwound and the knotted forms after control background subtraction, and 1 unit of enzyme activity was defined as the concentration capable of unwinding 50% of the P4 knotted DNA in 30 min under the conditions described above.

Slot-Blot Analysis of mdrl and Topoisomerase II mRNAs. Both RNA and DNA were extracted from 5 × 10^6 cells by using the guanidinium isothiocyanate method (38). After ultracentrifugation, RNA pellets were resuspended in 0.3 μM sodium acetate, pH 6.0, and precipitated with 70% ethanol. DNA samples in CsCl solution were ethanol precipitated then treated with proteinase K, and then phenol/chloroform extractions. DNA was recovered by ethanol precipitation. Polyadenylate-containing mRNA was isolated from total RNA extracted from A549 cells by using a QuickPrep mRNA purification kit (Pharmacia) and was stored as an ethanol precipitate at −70°C. The 1.2-kilobase clone pCa12-2 cDNA for human multidrug resistant gene mdrl was kindly provided by Dr. T. Tsuruo. The cDNA was labeled by random priming with [γ-32P]dATP (3000 Ci/mmole; NEN).
A 21-mer oligonucleotide probe for the human topoisomerase II gene with the sequence 5'-TCG TGG ACT AGC AGA ATC CTT-3' beginning at nucleotide 2389 of the cDNA sequence described by Tsai-Pflugfelder et al. (39) was synthesized and end labeled with [γ-32P]-dATP (6000 Ci/mmol; NEN), using terminal deoxynucleotidyl transferase (BRL, Bethesda, MD). A 1.8-kilobase cDNA fragment of the 3'-terminal one-third of human topoisomerase II mRNA (ZII-1.8) cloned into the EcoRI site of Bluescript SK(+) plasmid, generously supplied by Dr. Leroy Liu, was isolated from the purified plasmid by EcoRI digestion and purification in 1% SeaKem GTG (FMC Bioproducts). A 21-mer oligonucleotide probe for the human topoisomerase II gene was radiolabeled by random priming by using the Klenow fragment of DNA polymerase I in the presence of [γ-32P]-dCTP (3000 Ci/mmol; NEN).

The oligonucleotide probe was evaluated for specificity for human topoisomerase II by comparing its ability with that of the cDNA fragment to detect the 6.2-kilobase human topoisomerase II mRNA after electrophoresis in a 1.2% agarose gel containing 1.28% formaldehyde, as described by Henderson et al. (40). Following electrophoresis, the RNA was transferred to Duralose-UV nylon-reinforced nitrocellulose membranes (Stratagene, La Jolla, CA) by capillary blotting without prior alkaline treatment. RNA was bound to the membranes by UV cross-linking by using a Stratagene apparatus (Stratagene). The blot to be evaluated by using the Klenow fragment of DNA polymerase I was incubated in 6 ml of prehybridization solution at 37°C, then in 6 ml of hybridization solution containing radiolabeled probe at a final concentration of 5 x 10⁶ cpm/ml for 2 h at 37°C, as described by Henderson et al. (40). After several washes in 1X SSPE (0.15 M sodium chloride, 0.01 M sodium phosphate, and 0.002 M EDTA) containing 0.5% sodium dodecyl sulfate and 0.1% nonfat dry milk, the blot was washed first at 37°C then at 42°C for 30 min in 0.1X SSPE and 0.5% sodium dodecyl sulfate. Autoradiography was conducted by placing the nitrocellulose sheet between two DuPont Cronex Lightning Plus intensifying screens obtained from Sigma (St. Louis, MO) and Xomat-AR film sheets (Kodak) and exposed at −70°C overnight.

Slot blots of DNA and RNA in quantities ranging from 5 to 0.5 μg per slot on nitrocellulose filter membranes were prepared by using a Manifold II Slot Blotter (Schleicher & Schnell, Keene, NH), which were processed according to the manufacturer's protocol. Hybridization conditions were essentially the same for both RNA and DNA blots, depending on the probe used in each case. Filters were prehybridized, hybridized, and washed as described previously (41). This oligo probe identified two EcoRI restriction fragments in digested cellular DNA, following electrophoresis in agarose gels and transfer to nitrocellulose filters. The filters were hybridized overnight at 37°C and washed at 40°C for 20 min in 0.1 X SSC (75 mM sodium chloride and 7.5 mM sodium citrate). The DNA blots were normalized with a chicken muscle glyceraldehyde-3-phosphate dehydrogenase DNA probe obtained from Dr. K. Mulder (Bristol-Baylor Laboratory, Baylor College of Medicine, Houston, TX). End-labeled oligodeoxythymidylate₁₀ (Pharmacia, Piscataway, NJ) was used to normalize RNA blots (42). Quantification of slot hybridization results was accomplished by densitometric analyses of the autoradiographs by using a Bio-Rad (Rockville, NY) Model 620 video densitometer.

RESULTS

Cross-Resistance Properties of Acquired Resistant Human Carcinoma Cell Lines. Table 1 reveals that the four acquired resistant cell lines previously described (22) are 5- to 9-fold less sensitive to VP-16 than the parental lines. This resistance is stable and has not diminished over months of continuous culture. Data reported in Table 1 show that an extensive multidrug resistance profile was observed for only one line, HCT116(VM)₃⁴, which was resistant to all of the drugs shown in Table 1 except csPt and MMC. This cell line is 20-fold resistant to ADM, while displaying only a 9-fold resistance to VM-26, the pressuring agent. One interesting observation regarding this cross-resistance profile was the increased sensitivity observed for csPt that accompanied resistance to VP-16 and VM-26. A similar observation was made for the inherently VP-16 resistant human small cell lung carcinoma cell line SW900, which is hypersensitive to MMC and csPt due to less efficient repair of the monoadducts before DNA cross-links were formed (23). The other three acquired resistant cell lines are cross-resistant only to the other demethyllepidopodophyllotoxin and to ADM.

Alkaline Elution Analysis of Topoisomerase II Inhibition. It is well documented that VP-16 and VM-26 are potent inhibitors of eukaryote topoisomerase II (1-5), resulting in the stabiliza-

Table 1 Comparison of median cytotoxic concentrations of common anticancer drugs in parental and acquired resistant cell lines

Cells were exposed to different concentrations of drug for 1 h in tissue culture flasks before allowing growth to continue for 5 to 9 days in the absence of drug. Median cytotoxic drug concentrations were derived from at least 3 separate experiments as described in "Materials and Methods." Each value has a standard deviation of less than 20%.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>VP-16 (μM)</th>
<th>VM-26 (μM)</th>
<th>ADM (μM)</th>
<th>ActD (μg/ml)</th>
<th>Colchicine (μg/ml)</th>
<th>MMC (μM)</th>
<th>csPt (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116</td>
<td>10</td>
<td>0.8</td>
<td>0.4</td>
<td>0.03</td>
<td>0.2</td>
<td>1.3</td>
<td>10</td>
</tr>
<tr>
<td>HCT116(VP)₃⁵</td>
<td>86</td>
<td>5.6</td>
<td>2.5</td>
<td>0.01</td>
<td>0.3</td>
<td>0.9</td>
<td>6</td>
</tr>
<tr>
<td>HCT116(VM)₃⁴</td>
<td>53</td>
<td>5.3</td>
<td>8.6</td>
<td>0.55</td>
<td>1.3</td>
<td>1.2</td>
<td>6</td>
</tr>
<tr>
<td>A549</td>
<td>8</td>
<td>0.5</td>
<td>0.7</td>
<td>0.06</td>
<td>0.3</td>
<td>1.3</td>
<td>26</td>
</tr>
<tr>
<td>A549(VP)₃⁵</td>
<td>65</td>
<td>4.1</td>
<td>2.3</td>
<td>0.09</td>
<td>0.4</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>A549(VM)₃⁴</td>
<td>67</td>
<td>3.4</td>
<td>2.3</td>
<td>0.03</td>
<td>0.3</td>
<td>2.6</td>
<td>9</td>
</tr>
</tbody>
</table>

Fig. 1. Total DNA breaks in cells exposed to different concentrations of VP-16 (A) or VM-26 (B). Cells were incubated in cell culture dishes with different concentrations of drug for 1 h and then processed for alkaline elution at pH 12.1 to reveal total cellular DNA breaks, as described in "Materials and Methods."
to cellular exportation by the transport protein P-glycoprotein (for reviews see Refs. 31, 32, 43, 44) is unknown. Experiments performed on the HCT116 parental cell line and its two sublines are illustrated in Fig. 2. When cells were incubated with 5 μM VP-16 (Fig. 2A) or PFM (Fig. 2B), the drugs rapidly accumulated in parental cells, reaching plateaus in 10 to 20 min. When the medium-containing drug was exchanged for drug-free medium, the intracellular concentrations of both VP-16 and PFM rapidly decreased to nearly undetectable levels for VP-16 and to 10% for PFM within 30 min. However, little drug entered the cells upon addition of drug to the medium or exited from the cells at 4°C when the cells were loaded with drug at 37°C. Similar results were seen by Yallowich and Ross (45, 46).

It can be seen from Fig. 3 that both HCT116(VM)34 and HCT116(VP)35 had significantly decreased intracellular accumulations of VP-16, which was especially true of HCT116(VM)34 cells, relative to their parental line. However, only the HCT116(VM)34 line displayed a significantly lower accumulation of PFM, a finding consistent with the multidrug resistance properties of this line and the lack of multidrug resistance properties for the HCT116(VP)35 cell line (Table 1). Both A549 acquired resistant lines displayed no abnormal accumulations of PFM but A549(VP)29 accumulated significantly less VP-16 than the parental line (Fig. 3).

Intracellular Accumulation and Efflux of VP-16 and Porfiromycin. Decreased drug accumulation may account for the much lower levels of topoisomerase II inhibition observed in acquired resistant HCT116 cells. Therefore, influx and efflux of radioactive VP-16 and PFM in the acquired resistant cells were studied. The MMC analogue PFM, which is readily prepared in a radioactive form, was used in these studies as an alternative anticancer drug with a mechanism of action unrelated to topoisomerase II inhibition. The susceptibility of MMC and PFM

5 B. Long, unpublished results.

Fig. 2. Intracellular accumulation and efflux of radiolabeled VP-16 (A) and porfiromycin (B). Cells in cell culture flasks were incubated with 5 μM [1H]VP-16 or porfiromycin for different times up to 30 min at 37°C to assess intracellular accumulation, or for 30 min then for different lengths of time in drug-free medium at 37°C to assess efflux of drug from cells. Points, means of 3 separate experiments. ■—■, HCT116; ○—○, HCT116(VM)34; ■—■, HCT116(VP)35 at 37°C. ■—■, HCT116; ○—○, HCT116(VM)34; ■—■, HCT116(VP)35 with either influx or efflux conducted at 0 instead of 37°C.
Flow Cytometric Analysis for P-glycoprotein. The expression of P-glycoprotein on the surface of cells was evaluated by using flow cytometry of indirect immunofluorescent stained cells. In these studies, mouse monoclonal antibody MRK16, which recognizes an external epitope of human P-glycoprotein in living cells, was reacted first with the cells, then stained with a fluorescein-conjugated F(ab')2 fragment of goat anti-mouse antibody. Both parental lines HCT116 and A549 contain little, if any, P-glycoprotein on the cell surface (Fig. 4). Of the three acquired resistant cell lines evaluated in this manner, only HCT116(VM)34 clearly displayed increased amounts of P-glycoprotein on the cell surface, whereas both HCT116(VP)35 and A549(VP)29 had essentially the same levels of P-glycoprotein as their parental lines (Fig. 4).

Topoisomerase II Activities. The activities of topoisomerase II in 0.35 M NaCl extracts of nuclei isolated from parental and acquired resistant cell lines were compared by using the topoisomerase II specific unknotting reaction. In this assay, only topoisomerase II activity is capable of converting highly knotted P4 phage DNA into the circular, covalently closed DNA form (47). Fig. 5 shows results obtained with nuclear extracts from HCT116 parental and acquired resistant cell lines. Undiluted extract caused complete conversion of the knotted P4 phage DNA used as substrate to the covalently closed, circular, unknotted DNA product. Progressive serial dilutions of each extract resulted in the dilution of enzyme activities until less than 50% of the substrate was converted into the unknotted DNA product. It is clear from Fig. 5 that the level of enzyme present in salt extracts of HCT116(VM)34 nuclei was essentially the same as that obtained from HCT116 nuclei, whereas substantially less enzyme activity was extracted from the nuclei obtained from HCT116(VP)35 cells (Fig. 5). Densitometric scans of the negatives of photographs taken of the gel shown in Fig. 5 and of a gel obtained from an experiment comparing the enzyme activities in A549 parental and acquired resistant cell lines (not shown) provides a means of quantifying and comparing enzyme activities in the different cell lines (Table 2). As suggested from Fig. 5, salt extracts of nuclei obtained
from HCT116(VP)35 cells had almost one-half of the topoisomerase II activity found in extracts from HCT116 nuclei. Topoisomerase II activities in similar salt extracts of nuclei from HCT116 and HCT116(VP)35 cells normalized for activity were equally sensitive to different concentrations of VP-16 up to 200 μM, indicating that resistance is not due to altered drug sensitivity.

Although salt extracts from A549 nuclei had 3.5 times more activity than HCT116 parental cells, the salt extracts obtained from nuclei of both A549 acquired resistant cell lines contained only one-fourth as much enzyme activity (Table 2). It is paradoxical that HCT116 cells have low topoisomerase II activity relative to A549 cells and not be resistant to VP-16 and VM-26. This difference is presently under investigation.

Slot-Blot Analysis of mdr1 and Topoisomerase II mRNA Levels. The observation that 0.35 M NaCl extracts of nuclei from acquired resistant cells contain substantially less enzyme activity than similar extracts from parental cells could be accounted for by differences in extractability or differences in catalytic activities between parental and their respective acquired resistant cells. Slot-blot analysis of topoisomerase II and mdr1 mRNA levels in parental and acquired resistant cells were conducted to address this question. Although the mdr1 cDNA probe used in this study has been well characterized (48), the oligodeoxyribonucleotide probe for human topoisomerase II was heretofore undescribed. The specificity of this probe was verified by Northern transfer analysis of mRNA extracted from A549 cells and purified by passage through an oligodeoxymidine column. A single mRNA species was detected that migrated in an agarose gel to the same extent (Fig. 6A) as a band identified by a cDNA probe for human topoisomerase II (Fig. 6B).

Fig. 7 shows that topoisomerase II mRNA levels in HCT116(VP)35, A549(VM)29, and A549(VP)29 cells were lower than in the respective parental cells. When the same mRNAs on the filter were evaluated with a DNA probe recognizing mdr1 gene expression, only HCT116(VM)34 revealed increased levels of mdr1 mRNA (Fig. 7). For comparison purposes, RNA extracted from human KB cells displaying different degrees of acquired resistance to colchicine and the parental line KB-3-1 were evaluated for topoisomerase II and mdr1 mRNA levels. The KB-8-5 cell line was reported to display enhanced expression without gene amplification and the KB-8-5-11 line display enhanced expression of the mdr1 gene with gene amplification (48). As previously reported, KB-8-5-11 cells contained very high levels, KB-8-5 cells contained intermediate levels, and the parental cell line contained barely detectable levels of mdr1 mRNA (48). Although HCT116(VM)34 cells clearly overexpressed mdr1 mRNA, no amplification of the mdr1 gene was detected from DNA slot-blot analysis (results not shown). In fact, only KB-8-5-11 cells of all of the cell lines tested contained amplification of the mdr1 gene, in agreement with Shen et al. (48).

Quantification of the differences seen in Fig. 6 by scanning these autoradiograms with a densitometer revealed a direct correlation between elevated mdr1 mRNA levels and P-glycoprotein on the cell surface, decreased intracellular drug accumulation, and the presence of a multidrug resistance phenotype (Table 3). These properties were observed only of HCT116(VM)34 cells. Likewise, a direct correlation was observed between decreased topoisomerase II mRNA levels and enzyme activity in salt extracts of nuclei and cross-resistance only to topoisomerase II inhibitors (Table 3). The other three acquired resistant cell lines possessed these properties, in that they were cross-resistant to VP-16, VM-26, and Adriamycin but were sensitive to actinomycin D and colchicine and displayed varying degrees of collateral hypersensitivities to cisplatinum.

Table 2 Topoisomerase II activities obtained in 0.35 M NaCl extracts of parental and acquired resistant cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Activity (units/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116</td>
<td>14</td>
</tr>
<tr>
<td>HCT116(VP)35</td>
<td>8</td>
</tr>
<tr>
<td>HCT116(VM)34</td>
<td>15</td>
</tr>
<tr>
<td>A549</td>
<td>50</td>
</tr>
<tr>
<td>A549(VP)28</td>
<td>11</td>
</tr>
<tr>
<td>A549(VM)28</td>
<td>12</td>
</tr>
</tbody>
</table>

* R. Woessner, unpublished results.
VP-16 AND VM-26 RESISTANT HUMAN CELL LINES

Fig. 6. Northern blot analysis of mRNA isolated from A549 cells examined with a radiolabeled oligonucleotide probe (A) or a cDNA probe (B). Lane 1, 10 μg of total cellular RNA; Lane 2, 2 μg; Lane 3, 4 μg; and Lane 4, 8 μg of polyadenylated RNA. RNA aliquots for A and B were subjected to electrophoresis in the same agarose gel. After transfer of RNA to nitrocellulose membranes by capillary blotting, the membrane was cut in half and probed separately, as described in “Materials and Methods.” Kb, kilobase.

DISCUSSION

Two primary and well-documented mechanisms have been described by which cancer cells resist the cytotoxic effects of the anticancer drug VP-16. The initial work from the laboratories of Biedler and Ling revealed that cells resistant to high concentrations of one cytotoxic agent were generally highly resistant to many structurally different natural product cytotoxins due to the presence of a specific membrane protein (49, 50). This extensively studied drug resistance mechanism is characterized by decreased intracellular accumulation of drug facilitated by overexpression of the human mdr1 gene, causing overproduction of P-glycoprotein. This cell membrane protein acts as an export pump for a wide variety of unrelated foreign natural products (for reviews see Refs. 31, 32, 43, 44). By maintaining lower intracellular levels of drug, less drug would be available to the target, which has been shown to be topoisomerase II (1–5). A second mechanism has come to light recently, which relates directly to the target enzyme, namely that either low enzyme levels (24–26) or altered sensitivity of the enzyme for the drug (27–29) confer resistance to that drug. This mechanism also confers a form of multidrug resistance, in that resistance to one topoisomerase II inhibitor through decreased or altered topoisomerase activity generally translates into resistance to most other topoisomerase II inhibitors (51).

Of the four acquired resistant cell lines described here, three are resistant to VP-16 and VM-26 by a decreased topoisomerase II expression mechanism without any involvement of an mdr1-related mechanism of resistance and one line is resistant to VP-16 and VM-26 in part, if not exclusively, through an mdr1,

Table 3 Relative levels of genes, gene expression, protein levels, and enzyme activities in parental and acquired resistant cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>mdr1 DNA</th>
<th>mdr1 RNA</th>
<th>mdr1 Protein</th>
<th>topoll DNA</th>
<th>topoll RNA</th>
<th>topoll Protein</th>
<th>Activity</th>
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</thead>
<tbody>
<tr>
<td>HCT116</td>
<td>9</td>
<td>&gt;</td>
<td>1.4</td>
<td>0.7</td>
<td>&lt;</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>HCT116(VM)34</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>0.4</td>
<td>ND</td>
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<tr>
<td>HCT116(VP)35</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>0.5</td>
<td>ND</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

Therefore, three of the four acquired resistant cell lines we have studied are resistant to VP-16 and VM-26 in part, if not exclusively, by a decreased topoisomerase II expression mechanism without any involvement of an mdr1-related mechanism of resistance. Conversely, the HCT116(VM)34 line is resistant to VP-16 and VM-26 in part, if not exclusively through an mdr1 mechanism reflecting decreased intracellular drug accumulation, rather than a topoisomerase II related mechanism.

Fig. 7. Composite presentation of slot-blot analysis of topoisomerase II and mdr1 gene expression. Total RNA was extracted from cells and 0.5 to 5.0 μg of RNA were placed in each slot over a nitrocellulose filter membrane. After blotting and drying, slots on the filter were analyzed for topoisomerase II, mdr1, and total polyadenylate-containing mRNA levels using 32P-labeled oligonucleotide, cDNA, and oligodeoxynucleotide probes, respectively. X-ray films were exposed to the washed and dried filter to obtain autoradiograms. See “Materials and Methods” for further details.
rather than a topoisomerase II-related mechanism. It should be noted that efforts were taken to develop low levels of resistance so as to limit the number of mechanisms of resistance functioning in a given cell line. Furthermore, the resistance described for these acquired resistant sublines is stable and has not diminished substantially over 1 year in culture. Therefore, it is possible to make some generalizations about cross-resistance properties displayed by the two different mechanisms.

First, the mdr1-resistant cell subline HCT116(VM)34, which is 5- to 6-fold resistant to VP-16 and VM-26, is cross-resistant to ADM, actD, and colchicine but not to MMC or csPt (Table 1). It is well recognized that elevated P-glycoprotein does not confer resistance to csPt, but the lack of resistance to MMC cannot be explained in light of the observation of substantially decreased accumulation of PFM by these cells (Figs. 2 and 3). The higher resistance to ADM, actD, and colchicine (21-, 18-, and 7-fold, respectively) relative to resistance to VP-16 and VM-26 (5- and 6-fold, respectively) is a property commonly seen for cell lines with elevated P-glycoprotein. Therefore, it is possible that P-glycoprotein is less effective at transporting VP-16 and VM-26 than ADM, actD, and Vinca alkaloids out of the cell. In this regard, we find no difference in sensitivity of HCT116 and HCT116(VM)34 cells for podophyllotoxin.2

Second, the two cell sublines with lower topoisomerase II activity levels are cross-resistant to only those agents that are potent topoisomerase II inhibitors, such as ADM (52, 53). It is important to notice that even though actD is reported to be an inhibitor of eukaryote topoisomerase II (52–54), it is obvious that the cytotoxicity produced by actD in HCT116 and A549 cells is not related to topoisomerase II inhibition because of the lack of cross-resistance to this drug in those cell lines possessing lower topoisomerase II levels (Table 1). This conclusion is supported by the observation of Trask and Muller that actD preferentially inhibits topoisomerase I (55).

Third, a pronounced hypersensitivity (collateral sensitivity) to csPt was observed in both of the acquired resistant A549 cell sublines. Varying hypersensitivity to MMC was observed, as well. Since our initial report describing collateral sensitivity between the alkylating agents csPt and MMC and the topoisomerase II inhibitor VP-16 (56), others have made similar observations (57–59). Tan et al. have found that human Burkitt lymphoma cells with an unstable acquired resistance to nitrogen mustard possess elevated levels of topoisomerase II and thus, are hypersensitive to VP-16, relative to the parental cell line (57–59). This nitrogen mustard-resistant line is also hypersensitive to the topoisomerase II inhibitor novobiocin (60).

This collateral sensitivity existing between topoisomerase II inhibitors and alkylating agents in vitro may provide an explanation for the advantage for combination therapies of cisplatin and etoposide in the clinic. In fact, the suggestion that these two drugs may have synergistic effects in mice (61) has led to a number of clinical trials exploiting cisplatin and etoposide combinations.

It was not entirely surprising that those cell lines displaying decreased topoisomerase II expression levels also displayed decreased intracellular accumulation of VP-16 even though no evidence for elevated expression of P-glycoprotein was found in these cell lines. Other laboratories have observed similar results with cells displaying acquired resistance to VP-16 (62–64). The reason for these decreased accumulations of VP-16 remains to be defined. However, it is possible that a yet unidentified regulator of VP-16 transport is increased in these resistant cell lines, although other explanations cannot be excluded.

We are presently utilizing these resistant cell lines as solid tumor model systems for the evaluation of VP-16 analogues in vitro and in vivo, both as subrenal capsule and as s.c. implants (65).

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