Cellular Adaptation to Drug Exposure: Evolution of the Drug-resistant Phenotype

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ABSTRACT

The efficacy of all chemotherapeutic agents is limited by the occurrence of drug resistance. For etoposide (VP-16), increased expression of MDR-1 or MRP and alterations in topoisomerase IIα have been shown to confer tolerance. To further understand resistance to VP-16, three sublines, designated MCF-7-VP17, ZR-75B-VP13, and MDA-MB-231-VP7, were initially isolated as single clones from parental cells by exposure to VP-16. Subsequently, a population of cells from each subline was exposed to 3-fold higher drug concentrations, allowing stable sublines to be established at higher extracellular drug concentrations. Characterization of the resistant sublines demonstrates the adaptation that occurs with advancing drug concentrations during in vitro selections. Reduced topoisomerase IIα mRNA and protein levels were observed in the initial isolates. This reduction was accompanied by a decrease in topoisomerase II activity and cellular growth rate and was associated with 6–314-fold resistance to topoisomerase II poisons. With advancing resistance, MRP expression increased and VP-16 accumulation decreased. This adaptation allowed for partial restoration of topoisomerase II activity as a result of increased expression (MCF-7-VP17 and ZR-75B-VP13) or hyperphosphorylation (MDA-MB-231-VP7), with a resultant increase in growth rate. In MDA-MB-231-VP7 cells, hyperphosphorylation coincided with increased casein kinase IIα mRNA and protein levels, suggesting a role for this kinase in the acquired hyperphosphorylation. In this cell line, hyperphosphorylation mediated the increased activity despite a fall in topoisomerase IIα protein levels secondary to an acquired 600-bp deletion in one topoisomerase IIα allele, which resulted in reduced protein levels. In all three sublines, high levels of resistance were attained as a result of synergism between the reduced topoisomerase IIα levels and MRP overexpression. These studies demonstrate how cellular adaptation to increasing drug pressure occurs and how more than one mechanism can contribute to the resistant phenotype when increasing selecting pressure is applied. Reduced expression of topoisomerase II is sufficient to confer substantial resistance early in the selection process, with synergy from MRP overexpression helping to confer high levels of resistance.

INTRODUCTION

The epipodophyllotoxins VP-16 and VM-26 are useful antineoplastic agents with activity in both hematological malignancies and solid tumors (1). The anticancer activity of these agents is thought to result at least in part from stabilization of the cleavable complex, an intermediate in which topoisomerase II is covalently bound to DNA in a step that precedes DNA religation (2–5). This mechanism of action is shared by other antitumor agents, including the anthracyclines, the mitomycins, and the plastic agents with activity in both hematological malignancies and solid tumors (1). The order of the first two authors is to be considered arbitrary.

The efficacy of the epipodophyllotoxins is limited by the occurrence of drug resistance in the tumor cell population. Cellular insensitivity to drugs that stabilize the cleavable complex is frequently expressed as MDR (7). In some cell lines, overexpression of MDR-1/P-glycoprotein or the MDR-associated protein, MRPs, has been demonstrated and implicated as the mechanism of resistance (8–12). Typically, these cells have reduced drug accumulation, secondary to increased drug efflux. In other cell lines, an atypical MDR phenotype has been identified, and the predominant mechanism of resistance has been shown to be qualitative and/or quantitative changes in the levels and activity of topoisomerase II (13–18).

Phosphorylation of topoisomerase II occurs primarily on serine residues in the COOH-terminal domain of the protein (19). Studies using both synchronized cells and purified topoisomerase II have demonstrated a correlation between topoisomerase II activity and phosphorylation, suggesting that this posttranslational modification may regulate catalytic activity (20, 21). Both increased and decreased activity have been reported following phosphorylation, consistent with differential effects of phosphorylation at divergent sites (20, 22–24).

Using a single clone selection process, we have isolated and characterized six breast cancer cell lines isolated for resistance to VP-16. The data show an initial decrease in topoisomerase IIα mRNA, protein, and activity, followed by restoration of activity coincident with increased levels or phosphorylation of topoisomerase II. In the latter, increased casein kinase IIα mRNA and protein are demonstrated. With further selection at higher drug concentrations, synergyism from increased expression of MRP with reduced VP-16 accumulation occurred. The evolution of the drug-resistant phenotype is described as cells adapt to increasing drug exposure.

MATERIALS AND METHODS

Cell Lines and Cell Cultures. The cell lines described in the present report were isolated as single clones, and the clones were then exposed as a population to increasing concentrations of drug. Three parental breast carcinoma cell lines were used: MCF-7 and ZR-75B (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative). The initial selections were performed at a different drug concentration for each cell line because clones were picked from the highest drug concentration at which single colonies could be isolated, and this varied among the three cell lines. The initial isolates were designated MCF-7-VP17(500), ZR-75B-VP13(300), and MDA-MB-231-VP7(1000) because they were isolated from 500, 300, and 1000 nm VP-16, respectively. Subsequently, a population of cells from each initial isolate was exposed to a 3-fold higher concentration of drug, and three additional sublines were established: MCF-7-VP17(1500), ZR-75B-VP13(900), and MDA-MB-231-VP7(3000). These were maintained at 1500, 900, and 3000 nm VP-16, respectively, and are referred to as the populations advanced to higher drug concentrations or the sublines maintained at higher drug concentrations.

All cells were grown in monolayer in Eagle’s MEM containing 10% fetal bovine serum, 2 mm L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO2 at 37°C. The resistant sublines have been maintained continuously in VP-16 at the concentrations indicated in parentheses.

Cytotoxicity Assays. Cytotoxicity assays were performed as described previously (25). Three hundred to 1000 cells/well plated in 96-well plates in 96-well dishes were incubated overnight, drug was added, and the cells were incubated an additional 5 days.

Cloning of Topoisomerase IIα, MRP, and CKIIα cDNA by RT-PCR. Synthetic oligonucleotides corresponding to the published cDNA sequence of human topoisomerase IIα, human MRP, and human CKIIα were used to isolate by RT-PCR-specific products for direct cloning into pGEM-3z vectors (Promega). The identity of the cDNA clones was confirmed by direct sequence analysis prior to their use as probes for Northern analysis. The sequences of the oligonucleotides used in the RT-PCR were as follows: topoisomerase IIα: 5'TGGTTGAGAAGCCGCTTGTGC3' and 465'TAGTTACTAGAGTTAGGCGCTG3'; MRP, 251'sCATGTCAGCAGTAAGCAGC3' and 390'sAACGACGACGACCTTCC3'; and CKIIα, 275'sAGCCTTGTTGGAAAAATAGGCC3' and 1079'sCTCATCACGCGACGTTGCTC3'.
Table 1 Cross-resistance

<table>
<thead>
<tr>
<th></th>
<th>VC50 (^{a})</th>
<th>VC50 (^{a})</th>
<th>RR (^{b})</th>
<th>VC50 (^{a})</th>
<th>VC50 (^{a})</th>
<th>RR (^{b})</th>
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<tr>
<td></td>
<td>MCF7</td>
<td>VP17(500)</td>
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<tr>
<td>VP-16</td>
<td>76 ± 36</td>
<td>1289 ± 355</td>
<td>17</td>
<td>3689 ± 1628</td>
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<tr>
<td>mAMSA</td>
<td>0.47 ± 0.15</td>
<td>46 ± 73</td>
<td>98</td>
<td>29 ± 0.66</td>
<td>62</td>
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<tr>
<td>Mitoxantrone</td>
<td>0.10 ± 0.02</td>
<td>15 ± 5.4</td>
<td>150</td>
<td>12 ± 7.1</td>
<td>120</td>
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<tr>
<td>Adriamycin</td>
<td>0.63 ± 0.09</td>
<td>5.3 ± 0.2</td>
<td>8.4</td>
<td>16 ± 4.7</td>
<td>25</td>
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<tr>
<td>Vincristine</td>
<td>3.3 ± 1.6</td>
<td>9.9 ± 4.1</td>
<td>3.0</td>
<td>11 ± 5.6</td>
<td>3.3</td>
<td>1.1</td>
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<td>ZR-75B</td>
<td>1.5 ± 0.36</td>
<td>479 ± 324</td>
<td>319</td>
<td>3396 ± 1615</td>
<td>2264</td>
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<tr>
<td>mAMSA</td>
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<td>14 ± 7.2</td>
<td>156</td>
<td>11 ± 3.2</td>
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<td>MDA-231</td>
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<td>19671 ± 3351</td>
<td>635</td>
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<td>VP7(1000)</td>
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<tr>
<td>VP-16</td>
<td>31 ± 3.3</td>
<td>1456 ± 200</td>
<td>47</td>
<td>19671 ± 3351</td>
<td>635</td>
<td>13.5</td>
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<td>27 ± 2.5</td>
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<td>0.24 ± 0.04</td>
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\(^{a}\) dose in ng/ml.
\(^{b}\) RR, relative resistance: ratio of IC50 of the individual cell lines.

**Northern Blotting.** Total RNA (20 μg/lane) was separated on a 6% formaldehyde gel and transferred to Hybond N \(^{+}\) and hybridized at 42°C overnight.

**Immunoblotting.** Nuclear extracts for topoisomerase II immunoblots were resolved on a 6% SDS-polyacrylamide gel. Transfer to an Immobilon-P membrane was followed by incubation with a 1:1000 dilution of a polyclonal antibody against human topoisomerase II (TopoGEN, Columbus, OH) for 1 h at room temperature. The membranes were washed with TBS-T (50 mm Tris-HCl, pH 7.5, 1% NP-40, 0.25% sodium deoxycholate, 150 mm NaCl, 1 mm EGTA) and then incubated with biotinylated secondary antibody and developed using a chemiluminescence detection system (ECL, Amersham Corp.). Immunoblotting for MRP was performed using a 1:1000 dilution of a polyclonal antibody against human topoisomerase II (Upstate Biotechnology, Lake Placid, NY). After three washes in TBS-T, the membranes were incubated with horseradish peroxidase-conjugated antimouse secondary antibody at a dilution of 1:1000 for 1 h. The membrane was followed by incubation with a 1:1000 dilution of a polyclonal antibody at 4°C overnight, and the autoradiographs were analyzed using the method of the Melton (26).

**DNA Topoisomerase II Activity Assay.** The decatenation reaction of catenated DNA was carried out with serial dilutions of nuclear extract and 0.1 μg of kinetoplast DNA (TopoGEN, Columbus, OH). Samples were separated by electrophoresis through a 1% agarose gel. After staining with ethidium bromide, films were photographed under UV illumination (14). Activity was calculated by determining the amount of nuclear extract required to convert 25 and 50% of kinetoplast DNA to minicircles. Activities are expressed as percentage of activity relative to parent. These percentages were derived by dividing the amount of nuclear extract of parental cells by that of the resistant cells. In all cases, the 25 and 50% values were comparable.

**DNA Topoisomerase II Phosphorylation.** Phosphorylation and immunoprecipitation were performed using a modification of a technique described previously (14). Cells (5 X 10\(^{5}\) in 100-mm dishes were incubated with \(^{32}\)P-iP, for 1 h prior to preparation of nuclear extracts. The nuclear extracts were incubated with antibody against human topoisomerase IIa at 4°C overnight, after which time protein A-Sepharose was added for 2 h. The mixture was centrifuged, the supernatant was removed, and the pellet was washed three times.
times with buffers containing NP-40. The pellets were then resuspended in loading buffer and boiled. The supernatants were subjected to electrophoresis on 6% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane. Following autoradiography, immunoblotting for topoisomerase IIα protein was performed on the same membrane.

**Cellular Accumulation and Efflux Assay for [3H]VP-16.** In each well of a six-well plate, 3.5 × 10⁵ cells were plated, and cells were incubated for 24 h at 37°C. Growth medium was then replaced with serum-free Eagle’s MEM, and the cells were incubated with 2.6 μM [3H]VP16 for various times up to 90 min at 37°C. At the completion of the incubation period, cells were quickly washed three times with ice-cold PBS, lysed with 2 ml of 0.05% SDS, and mixed with 3 ml of Scintisol. Similarly treated wells were used for determination of cell counts. Relative cell volumes were determined on a Becton Dickinson FACScan by measuring forward and side scatter. The data were analyzed using CellQuest (Becton Dickinson, San Jose, CA).

**RESULTS**

Table 1 depicts the cross-resistance profile of the six sublines to various chemotherapeutic agents. Cross-resistance to four topoisomerase II poisons was observed in the clones isolated in the first step of each selection; there was little or no cross-resistance to the microtubule active agent vincristine. The populations maintained at higher drug concentrations were significantly more resistant to VP-16 and Adriamycin but were similarly or less resistant to mAMSA, mitoxantrone, and vincristine.

When expression of topoisomerase IIα was examined, the results shown in Fig. 1 were obtained. Northern analysis using a cDNA probe encoding residues −91 to 449 showed decreased mRNA expression in the three initial isolates compared to parental cells [quantitation of five different experiments using 28S as an internal control: MCF-VP17(500), 60.9 ± 9.6% of parental MDA-MB-231; ZR-VP13(900), 13.4 ± 7.05% of parental ZR-75B; and MD-VP7(3000), 60.9 ± 9.67% of parental MDA-MB-231]. With the exception of the MDA subline maintained at higher drug concentrations [VP7(3000)], protein levels, as shown in the lower panels, paralleled the mRNA levels. The discordance seen in MDA cells is discussed below.

Previous investigations have implicated mutations at several sites as responsible for the resistant phenotype (30–35). To identify acquired mutations, RNase protection assays were performed using 10 probes encompassing the entire topoisomerase IIα coding sequence and 91 residues of the 5′ untranslated region (not shown). Using this approach, no mismatches were detected in the MCF-7 or the ZR-75B sublines. However, mismatches were identified in MDA VP7(3000) cells using as probes fragments corresponding to residues 2814–3433 and 3767–4388. Sequence analysis revealed the mismatches were the result of a 615-bp deletion comprising residues 3194–3808. In the RNase protection experiments with 2814–3433 and 3767–4388, fragments corresponding to both the full length and a shorter size consistent with the deletion were observed (not shown). This indicated that in addition to the gene with the 615-bp deletion, a gene with the wild-type sequence was expressed at comparable levels and that the cells were in effect heterozygous.

To further characterize the 615-bp deletion, genomic DNA from parental cells was amplified by PCR using a series of primers in an attempt to better define the genomic organization of the topoisomerase IIα gene in this region. These results are summarized in schematic form in Fig. 2. Using the primers summarized in Fig. 2 and conditions that have been used successfully to clone PCR products in excess of 6 kb, two small introns and a putative third large intron were identified in this region with parental DNA. The two smaller introns were subcloned and sequenced, allowing for identification of consensus residues at the 5′ and 3′ ends. The putative third intron is located closest to the 3′ end and was not further characterized. The results are

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![Fig. 2. Map of the confirmed and putative genomic organization surrounding the area of deletion in MDA-VP7(3000) cells. Exons are shown in uppercase and introns in lowercase. The exon sequences contained within the deletion are shown in boldface, and consensus residues are underlined. Below the map are shown the primers used to clone the intervening introns and their respective products, as well as the primer combinations that failed to generate a product, suggesting the presence of a large intervening intron.](image-url)
was calculated as described in "Materials and Methods" (gel not shown). Topoisomerase activity in the initial MCF-7 and ZR isolates was decreased [VP17(500), 14.6%; VP13(300), 14.4%], consistent with the decrease in mRNA and protein levels shown in Fig. 1. With further selection, activity was partially restored [VP17(1500), 64.5%; VP13(900), 44.7%], in agreement with the partial normalization of mRNA and protein levels observed in these sublines. In contrast in the MDA sublines, a decrease was seen in the initial step [VP7(1000), 12.8% of control], consistent with the fall in topoisomerase II mRNA and protein levels; however, restoration of activity to nearly normal levels was observed with further selection [VP7(3000), 91% of parental], a finding that was unexpected, because protein levels remained low. This discrepancy could not be explained by the levels of topoisomerase IIα because these were similar in the resistant sublines and parental cells (not shown).

Consequently, as shown in Fig. 3, metabolic labeling studies were performed to examine phosphorylation of topoisomerase IIα from parental cells and the resistant sublines. The extent of phosphorylation of the topoisomerase recovered by immunoprecipitation was quantitated by autoradiography following electrophoresis and transfer to an

Fig. 4. Growth curves. Growth of parental cells and their respective drug-resistant sublines over a 1-week period of incubation is shown.

Fig. 5. Expression of casein kinase II. Top panel, Northern analysis using a cDNA encompassing residues 275–1079 of CKIIα; middle panel, ethidium bromide-stained RNA prior to transfer; bottom panel, immunoblotting using a polyclonal CKIIα antibody. The Northern membrane used here was that used in Fig. 1.

Fig. 6. Expression of MRP. Upper panel: Northern analysis using MRP cDNA (residues 251 to 965). Middle panel: Ethidium bromide stained RNA prior to transfer. Lower panel: Immunoblotting using a monoclonal antibody against MRP. The Northern membrane used here was that used in Figs. 1 and 5.
Immobilon membrane. Once adequate exposures had been obtained, the blot was probed with an antitopoisomerase IIα antibody, and the amount of protein was quantitated by chemiluminescence. This latter approach allows for more precise quantitation because the actual amount of protein successfully recovered following the immunoprecipitation can be determined. As shown in Fig. 3, phosphorylation of topoisomerase IIα was increased 451% in VP7(3000) cells, providing a potential explanation for the discrepancy between protein levels and activity. Whereas the levels of protein kinase α, β, γ, δ, and μ were similar in all three cell lines (not shown), CKIIα mRNA and protein levels were elevated in VP7(3000) cells, as shown in Fig. 5, providing a possible explanation for the increased phosphorylation. Phosphorylation of topoisomerase IIα in the other resistant sublines was not altered (not shown).

Because previous studies have shown correlations between topoisomerase II levels and growth rate, we next examined the doubling times of the parental cells and the resistant sublines. As shown in Fig. 4, the growth rate of the initial isolates was reduced compared to parental cells, consistent with the reduced levels and activity of topoisomerase II in these cells. With continued selection, growth rate increased as topoisomerase II activity increased. The increase in growth rate was greatest for MDA-VP7(3000) cells, which demonstrated the largest increase in topoisomerase II activity.

The reduced topoisomerase II mRNA and protein levels provided a potential explanation for the resistance observed in the initial isolates. However, with advancing selection and increasing resistance, topoisomerase II levels increased [MCF-VP17(1500) and ZR-VP13(900)] or remained low [MDA-VP7(3000)]. Because a similar cross-resistance profile has been reported in cells expressing the multidrug resistance-associated protein, MRP, expression of MRP and accumulation of VP-16 were examined. As shown in Fig. 6, mRNA levels in parental MCF-7 and ZR-75B cells is low; somewhat higher levels were observed in MDA-MB-231 cells. Protein was not detectable by immunoblotting. In the first step of each selection, significant changes in mRNA were not detected, and protein levels remained below the threshold of detection. In contrast, the three sublines maintained at higher drug concentrations all had marked increases in mRNA levels and readily detectable protein by immunoblotting. These latter results are supported by the experiments shown in Fig. 7 examining the accumulation of VP-16, which showed reduced accumulation in the cells lines maintained in higher drug concentrations. These observations were thought to provide an explanation for the increased resistance in the cells maintained at higher drug concentrations.

**DISCUSSION**

The present study describes the characterization of multidrug-resistant sublines derived from three parental breast carcinoma cell lines by exposure to VP-16. Characterization of three clones is described, including the initial isolates as well as sublines derived by exposure of the initial isolates to higher drug concentrations. The results demonstrate the adaptation that occurs during the course of *in vitro* selections. The first adaptation observed in the initial isolates was a reduction in topoisomerase II levels and activity. With advancing resistance, topoisomerase II activity was partially restored and the growth rate increased as a result of increased expression or hyperphosphorylation. However, because of increased expression of the drug efflux pump, MRP, and a resultant decrease in VP-16 accumulation, the increase in topoisomerase II activity did not result in greater drug sensitivity.

The results described in the present study support and extend previous observations. The initial change observed was a decrease in the expression and activity of topoisomerase II. Previous studies have documented reduced expression of topoisomerase II with drug selection, and this has been proposed as a mechanism of drug resistance (13–18). In our own experience, we have found decreased expression of topoisomerase in 50 of 53 single-step isolates, including the three described in the present report, suggesting that reduced expression is a common adaptation. Although reduction of topoisomerase levels effectively diminishes the main intracellular target of VP-16 and other topoisomerase poisons and can confer broad cross-resistance to these agents, such a reduction also leads to a decrease in growth rate. Such a decrease was observed in the initial isolates described in the present study. As the drug concentration was advanced and sublines established at higher extracellular drug concentrations, the growth rate increased. This coincided in two of the isolates with a partial restoration of topoisomerase levels and activity and in the third with restoration of activity. In the latter case, the MDA-VP7(3000) subline, increased activity occurred without an increase in the protein level. Acquisition of a 615-bp deletion in one topoisomerase II allele resulted in a persistence of reduced topoisomerase II protein levels. The increase in activity was associated with an increase in phosphorylation. Previous reports have established that topoisomerase II exists as a phosphoprotein (14, 19–21, 36). This posttranslational modification occurs

*Unpublished observations.*

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Fig. 7. VP-16 accumulation in parental cells and the drug-resistant sublines. Accumulation over a 90-min period is displayed as pmol of VP-16/10^7 cells. Cell volumes for the resistant sublines relative to parental cells were VP17(500), 106%; VP17(1500), 102%; VP13(300), 97%; VP13(900), 105%; VP7(1000), 91%; and VP7(3000), 93%.
primarily on the serine residues in the COOH-terminal domain of the enzyme (3, 19, 23, 37). Studies using synchronized cells and in vitro phosphorylation of purified topoisomerase II have demonstrated a correlation between phosphorylation and topoisomerase II catalytic activity, suggesting that phosphorylation may be an important mechanism for regulating topoisomerase II function (21). For example, the extent of phosphorylation of topoisomerase II increases as cells enter the G2-M phase of the cell cycle, when activity of the enzyme is the greatest (37). More recently, peptide mapping of topoisomerase II at various times after synchronization of Chinese hamster ovary cells revealed that the distribution of phosphorylated serine residues changes as cells progress through the cell cycle, suggesting that CKIIα is a major kinase responsible for phosphorylation of topoisomerase IIα (23, 37). However, several reports suggest that protein kinase C may also play a role in phosphorylation and/or regulation of topoisomerase II (3, 20, 22). In MDA-VP7(3000) cells, the increase in phosphorylation was associated with an increase in CKIIα expression without an increase in protein kinase C levels. These results are in agreement with the studies demonstrating a role for casein kinase II phosphorylation and represent an example of an acquired increase in CKIIα expression with phosphorylation of topoisomerase II in a drug-resistant cell line (14, 22).

Previous reports describing characterization of MRP-overexpressing cell lines have reported modest relative resistance ratios of 3.5—28.4 for VP-16 and 6—22.5 for Adriamycin in the majority of cell lines (10—12, 38—40); higher and more variable ratios were reported in a cell line that previous studies had identified as having altered topoisomerase II levels (41, 42). The high levels of cross-resistance achieved in the resistant sublines, especially the ZR-75B and MDA-MB-231 sublines maintained at higher drug concentrations, exceeds these relative resistance values. However, if the contribution of MRP overexpression is estimated by comparing the relative resistance of the sublines maintained at higher drug concentrations to the initial isolates, as summarized in the right column of Table I, more modest values are obtained, in agreement with those in the literature (MCF-VPI(1500)/MCF-VPI(1700): Adriamycin, 3.0 and VP-16, 2.9; ZRVPI/13(900)/ZR-VPI(300): Adriamycin, 6.1 and VP-16, 7.1; MDA-VPI(3000)/MDA-VPI(1000): Adriamycin, 8.6 and VP-16, 13.5).

High levels of resistance were achieved because more than one mechanism contributed to the resistant phenotype. This demonstrates how two mechanisms can synergize and confer very high levels of drug tolerance. It is also interesting to note that advancing the initial isolates to higher levels of extracellular drug resulted in reduced tolerance to the topoisomerase poisons mAMSA and Mitoxantrone in the MCF-VPI(1500) and ZR-VPI(900), but not in MDA-VPI(3000), whereas resistance to the selecting agent, VP-16, increased in all of the sublines. This difference in behavior is explained by the fact that in the MCF-7 and ZR-75B sublines, topoisomerase II levels increased, whereas in MDA-VPI(3000) cells, protein levels remained stable or fell secondary to the acquired mutation. In MDA-VPI(3000) cells, increased activity and growth rate were achieved instead by enhanced topoisomerase II phosphorylation. The latter maintained the levels of protein (drug target) low.

It was somewhat surprising to find that alterations in topoisomerase II levels conferred such a high degree of cross-resistance compared to that due to MRP overexpression. High levels of resistance were obtained in the initial single-step isolates in which topoisomerase IIα levels were 13.4—22.5% of parental levels. Synergy with MRP resulted in high levels of cross-resistance, with only modest reductions in topoisomerase IIα levels (48.9—67.5% of parental levels) in the sublines maintained at higher drug concentrations. To be sure, advancing the cells further would likely lead to higher levels of MRP and greater cross-resistance. However, the results suggest that reduced topoisomerase IIα levels can confer a high degree of resistance and cross-resistance. Furthermore, the observation that MRP overexpression occurred with advancing drug concentration suggests that at higher levels of drug, protection is best achieved by reducing intracellular drug levels, whereas changes in target level were sufficient at lower extracellular drug concentrations. The increase in MRP levels, by reducing intracellular drug concentrations, allowed for partial restoration of topoisomerase II levels and for a concomitant increase in growth rate. This was an advantageous adaptation. Indeed, it is likely that over several months of selection, a cell in the population with acquired overexpression of MRP and higher topoisomerase II levels was able to grow faster, so that its offspring were the predominant cells in the population that now grew more rapidly.

In summary, the present study demonstrates how cellular adaptation to drug exposure occurs and how more than one mechanism can contribute to the resistant phenotype. Reduced expression of topoisomerase IIα was the first change observed in the selection process. Further adaptations with advancing drug selection (increased MRP) demonstrate that additional mechanisms can contribute to the initial phenotype and that synergism can confer high levels of resistance. These cellular adaptations allowed more resistant, faster growing cells to overtake the population in a manner reminiscent of some acquired drug-resistant tumors in clinical oncology.

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