Multidrug Resistance-associated Protein Gene Overexpression and Reduced Drug Sensitivity of Topoisomerase II in a Human Breast Carcinoma MCF7 Cell Line Selected for Etoposide Resistance

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ABSTRACT

A human breast cancer cell line (MCF7/WT) was selected for resistance to etoposide (VP-16) by stepwise exposure to 2-fold increasing concentrations of this agent. The resulting cell line (MCF7/VP) was 28-, 21-, and 9-fold resistant to VP-16, VM-26, and doxorubicin, respectively. MCF7/VP cells also exhibited low-level cross-resistance to 4'-[9-acyridinylamino]-methanesulfon-m-anisidide, mitoxantrone, and vincristine and no cross-resistance to genistein and camptothecin. Furthermore, these cells were collaboratively sensitive to the alkylating agents melphanal and chlorambucil. DNA topoisomerase II levels were similar in both wild-type MCF7/WT and drug-resistant MCF7/VP cells. In contrast, topoisomerase II from MCF7/VP cells appeared to be 7-fold less sensitive to drug-induced cleavable complex formation in whole cells and 3-fold less sensitive in nuclear extracts than topoisomerase II from MCF7/WT cells. Although this suggested that the resistant cells may contain a qualitatively altered topoisomerase II, no mutations were detected in either the ATP-binding nor the putative breakage/resealing regions of either DNA topoisomerase IIα or IIβ. In addition, the steady-state intracellular VP-16 concentration was reduced by 2-fold in the resistant cells, in the absence of detectable mdr1/P-gp expression and without any change in drug efflux. In contrast, expression of the gene encoding the MRPL was increased at least 10-fold in resistant MCF7/VP cells as compared to sensitive MCF7/WT cells. These results suggest that resistance to epipodophyllotoxins in MCF7/VP cells is multifactorial, involving a reduction in intracellular drug concentration, possibly as a consequence of MRP overexpression, and an altered DNA topoisomerase II drug sensitivity.

INTRODUCTION

MDR4 remains a serious problem in the chemotherapy of solid tumors. Laboratory studies over the last decade have identified at least some of the mechanisms involved in MDR. The best characterized mechanism of MDR involves the overexpression of the mdr1 gene, which encodes a Mr~180,000 membrane P-gp thought to function as a drug efflux pump. The presence of P-gp in the membrane of cells has been linked to what has been referred to as “classical” MDR, characterized by resistance to a number of diverse natural product drugs including the Vinca alkaloids vinblastine and vincristine, as well as a wide range of topoisomerase II poisons including doxorubicin and its derivatives, amrsarcine, mitoxantrone, etoposide, and teniposide (reviewed in Refs. 1–3).

Besides mdr1/P-gp a wide variety of other membrane, cytosolic, and nuclear proteins have been identified in cells selected for resistance to multiple drugs in vitro (reviewed in Ref. 4). For most of these proteins the evidence for their involvement in MDR remains circumstantial, and their biochemical functions for the most part have not been identified. Some of these proteins share sequence homology with ABC-type transport proteins, suggesting a possible role in drug transport. Recently, Cole and co-workers (5) isolated and cloned a cDNA for a putative transport protein (MRP) with a deduced molecular weight of approximately 170,000 and with some sequence homology to mdr1 and other ABC-type genes. This cDNA was isolated from MRP-overexpressing H69AR cells that were derived from a small cell lung cancer cell line (H69) by selection for doxorubicin resistance. H69AR cells did not contain increased levels of P-glycoprotein or mdr1 mRNA, and drug uptake studies have revealed that there were few differences in drug accumulation between parental sensitive H69 and resistant H69AR variant cells. Thus, these cells differ phenotypically from P-glycoprotein-overexpressing “classical” MDR cells (6–8).

In contrast to “classical” or P-gp-mediated MDR, a different phenotype of multidrug resistance has been described that involves selective resistance to drugs that interact with the enzyme DNA topoisomerase II. Evidence from several laboratories has suggested that this form of MDR might involve an altered form of topoisomerase II (9, 10). Indeed, several cell lines exhibiting this form of MDR have been described (9–15), and studies of these cell lines have indicated that both qualitative and quantitative alterations of topoisomerase II contribute to this phenotype. In some cell lines the reduced sensitivity to topoisomerase II drugs correlated with a reduction in topoisomerase II levels and/or activity (12, 16–20). In at least one example down-regulation of topoisomerase II was shown to be due to the loss of one allele through mutation (21). Alternatively, single point mutations in the topoisomerase IIα gene have been found in several drug-resistant cell lines, suggesting that mutational alteration in the topoisomerase IIα protein can lead to a reduced sensitivity of the enzyme to topoisomerase II inhibitors (22–27). Furthermore, posttranslational modifications of topoisomerase II including alterations in phosphorylation have also been implicated in drug resistance, both in vitro and in vivo (28, 29).

Changes in drug metabolism and non-P-gp-mediated alterations in drug uptake/efflux have also been implicated in the development of MDR. For instance, several cell lines have recently been described that exhibited altered drug uptake in the absence of P-gp overexpression (16, 30–32). In this report we describe a new variant of the human breast carcinoma cell line MCF7 that was selected for resistance to the topoisomerase II inhibitor VP-16. This cell line (MCF7/VP) exhibits resistance to the epipodophyllotoxins and doxorubicin with only little cross-resistance to other drugs. Resistance in MCF7/VP cells appears to be associated with a reduction in drug accumulation and MRP overexpression and alterations in topoisomerase II drug sensitivity.

MATERIALS AND METHODS

Materials. IMEM and fetal bovine serum were obtained from Gibco (Grand Island, NY), [α-32P]dCTP and [α-32P]dATP were obtained from New England Nuclear (Wilmington, DE) and [3H]VP-16 was from Moravek (Brea, California).
CA. VP-16 and VM-26 were obtained from Bristol Myers (Syracuse, NY); genistein from Calbiochem (La Jolla, CA); vincristine, melphalan (L-PAM), of MCF7/WT cells were isolated by stepwise selection in increasing concentrations of VP-16 starting with 2× IC₅₀ (0.2 µM). When cells became confluent in medium containing VP-16, the drug concentration was increased to 3× (0.3 µM), 5× (0.5 µM), 10× (1 µM), 20× (2 µM), 30× (3 µM), 50× (5 µM), and 100× IC₅₀ (10 µM), the maximal concentration used. Cells were not exposed to mutagens prior to selection and were not clonally isolated after selection. Following this selection procedure, the MCF7/VP variant cell line was passed in drug-free medium and remained stably resistant to VP-16 for several months. However, in order to prevent the outgrowth of revertants, the cells were periodically reslected in the presence of 4 µM VP-16. Under these conditions no change in resistance was observed over 2 years. The MCF7/ADR conditions no change in resistance was observed over 2 years. The MCF7/VP cell line has been described previously (31). Human KB 3-1 cells and their colchicine-resistant derivative KB 8-5 cells (34) were obtained from Dr. I. Pasetti (Laboratory of Molecular Biology, National Cancer Institute, Bethesda, MD). GC3 cells were from a clonal subline of the human colon adenocarcinoma xenograft cell line HxGC3 (35).

Cytotoxicity Assays. MCF7/WT or MCF7/VP cells (200–500 cells/well) were plated in a 96-well microtiter plate in the presence of varying concentrations of drug. After incubation at 37°C for 7 days, cell growth was assessed by the sulforhodamin B assay (36). IC₅₀ values were determined graphically from relative survival curves.

Drug Uptake Studies. MCF7/WT or MCF7/VP cells (10⁴ cells/well) were plated into 12-well culture plates in triplicate. After incubation for 2–3 days at 37°C the cells were washed with serum-free IMEM. [³²P]JHPV-16 (0.5 ml of 100 µM, 1 µCi/ml) in serum-free IMEM was added to the cells, and incubation continued at 37°C. After 0 to 120 min, the drug was removed, and the cells were immediately washed in ice-cold PBS and then lysed with 0.2 M NaOH at room temperature overnight. The lysate was neutralized by the addition of an equal volume of 0.2 M HCl in 0.2 M Tris-HCl (pH 8.0). Aliquots were then assayed for radioactivity by liquid scintillation counting and for protein concentration using the micro BCA (bicinchoninic acid) assay (Pierce, Rockford, IL) (37).

Drug Efflux Studies. MCF7/WT and MCF7/VP cells were preincubated with 100 µM [³²P]JHPV-16 (1 µCi/ml) for 2 h in serum-free IMEM at 37°C. After washing with ice-cold PBS the cells were again incubated with 0.5 ml prewarmed (37°C) serum-free IMEM without VP-16 for 0–120 min at 37°C. After different incubation times the medium was removed, and the cells were treated as described above for drug uptake to measure the radioactivity that remained in the cells.

DNA Topoisomerase II Assays. Nuclear extracts were prepared as previously described (31) in the presence of the protease inhibitors aprotonin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride. All extracts were diluted to equal protein concentrations of approximately 1 mg/ml and stored at −20°C. Cleaveable complex formation in whole cells or nuclear extracts was measured by K/SDS assay as previously described (38). Cells were prelabeled with tritiated thymidine for 2–3 days, washed, and trypsinized, and 10⁶ cells/well were plated into a 24-well microtiter plate. After the cells had reattached to the plastic, the medium was removed and the cells were incubated for 1 h at 37°C in the presence of increasing amounts of VP-16 in serum-free medium. Nuclear extracts were incubated with increasing concentrations of VP-16 for 30 min at 37°C.

Western Blotting. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. The membrane was blocked in 5% skim milk in Tris-buffered saline, containing 0.15% Tween for 1 h at room temperature before washing three times in the same solution containing 0.01% Tween. The membrane was incubated with antibody in 0.5% skim milk for 2 h at room temperature before washing as described and incubating in a 1:500 dilution of goat anti-mouse IgG alkaline phosphatase conjugate in 0.5% skim milk–Tris-buffered saline for 1 h at room temperature. The membrane was then washed again three times before development.

RNA Protection. The RNA protection assay for mdr1 was performed as described previously (31).

Quantitative PCR. One µg of total RNA, isolated by the method of Chomczynski and Sacchi (39), from MCF7/WT or MCF7/VP cells was reverse transcribed in 20 µl of RT buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 5 mM MgCl₂; 1 mM each of dATP, dGTP, dCTP, dTTP), containing 2 units/µl RNAse inhibitor, 0.003 A₂₆₀ units random hexanucleotides (both from Boehringer Mannheim, Indianapolis, IN), and 0.4 units/µl AMV reverse transcriptase (Promega, Madison, WI). Following incubation at 25°C for 10 min and at 42°C for 15 min the reaction was terminated by incubation at 99°C for 5 min. The resulting cDNA mixture was serially diluted in RT buffer. In each dilution target sequences for DNA topoisomerase I, Ia, IIa, MRP, and G3PDH were separately amplified for 30 cycles by the polymerase chain reaction with specific primers for each gene and 10 µCi [³²P]dCTP, using the hot start modification (40). The primers used were: topo I, 5'-primer nt 1366 to 1390, 3'-primer nt 1995 to 1999 (2021); topo IIa, 5'-primer nt 1247 to 1270, 3'-primer nt 1693 to 1718 (42); topo IIβ, 5'-primer nt 894 to 918, 3'-primer nt 1206 to 1230 (42); G3PDH, 5'-primer nt 75 to 100, 3'-primer nt 670 to 696 (43); MRP, 5'-primer nt 793 to 818, 3'-primer nt 1063 to 1088 (5). Ten µl of the reaction products were separated on a 2% agarose gel in Tris-Borate-EDTA buffer. The gels were dried and exposed to X-ray film, and the radioactivity in each band was quantitated on a Molecular Dynamics Phosphor Imager (Sunnyvale, CA). The amount of each topoisomerase or MRP PCR product was normalized with G3PDH, in a manner similar to the method described by Murphy et al. (44).

Northern Blotting. The PCR product for MRP was subcloned into the Bluescript vector using Stratagene's (La Jolla, CA) pCR-Script cloning kit. The identity of the cloned fragment was verified by sequencing. This construct was then used for labeling by random priming with [³²P]dCTP and probing of Northern blots. Ten µg of total RNA were separated on a 1% agarose gel in formaldehyde/4-morpholinopropanesulfonic acid buffer. After transfer onto nylon membrane, the membrane was prehybridized and hybridized at 42°C overnight with the labeled MRP probe in 50% formamide. After washing the membrane was exposed to X-ray film for 14 days at −70°C with intensifying screens.

DNA Sequencing. Cyttoplasmic RNA was isolated from wild-type and resistant cells as described by Sambrook et al. (45). Separate primer pairs (24 mer to 27 mer) that bracketed either the putative ATP binding B region or the putative breakage/rescaling region of topoisomerase Ila or IIβ were prepared and used for reverse transcription followed by PCR amplification, using either a high-temperature or a regular DNA/PCR kit from Perkin Elmer (Norwalk, CT). Following purification by two isopropanol precipitations the amplified products were then sequenced using the CircumVent sequencing kit from New England Biolab (Beverly, MA) and analyzed on a denaturing polyacrylamide sequencing gel. The primer pairs used were: ATP binding region: topo Ila, 5'-primer nt 1246 to 1270, 3'-primer nt 1515–1539; topo IIβ, 5'-primer nt 897 to 920, 3'-primer nt 1181 to 1206 (42); breakage/rescaling region: topo Ila, 5'-primer nt 2049–2074, 3'-primer nt 2622 to 2648; topo IIβ, 5'-primer nt 1641 to 1666, 3'-primer nt 2238 to 2264 (42).

Protein Determination. Protein concentrations in nuclear extracts were measured by the method of Bradford (46).

RESULTS

Drug Sensitivity. As shown in Table 1, the MCF7/VP cells were 28- and 21-fold resistant to the epipodophyllotoxins VP-16 and VM-26, respectively, when compared to the sensitivity of wild-type MCF7/WT cells. Although the MCF7/VP cell line was 9-fold and 5-fold cross-resistant to doxorubicin and vincristine, respectively, these cells remained relatively sensitive (less than 4-fold resistant) to all of the other drugs examined. Interestingly, MCF7/VP cells were 2-fold more sensitive than the wild-type cells to the alkylating drugs.
melphalan and chlorambucil, an observation that had also been noted in other cell lines selected for resistance to topoisomerase II poisons (16).

**DNA Topoisomerase II.** The predominant resistance of MCF7/VP cells to VP-16 and VM-26 suggested an alteration in DNA topoisomerase II activity in the resistant cells. We therefore examined topoisomerase II levels and activities in MCF7/WT and MCF7/VP cells. As shown in Fig. 1A there was little, if any, difference in topoisomerase II protein levels between the two cell lines. The upper two bands presumably represent the Mr 180,000 IIβ and the Mr 170,000 IIα forms of topoisomerase II. A Mr 160,000 species was also observed in both cell lines, similar to a Mr 160,000 form of topoisomerase II recently described by Harker et al. in a mitoxantrone-resistant HL-60 cell line (47). However, in contrast to the results described by these authors we observed that the Mr 160,000 species was equally expressed in both wild-type and resistant cells. This result suggested that the corresponding protein was not involved in the resistant phenotype, and this band may possibly represent a proteolytic breakdown product of either topoisomerase IIα or IIβ, despite the presence of a cocktail of protease inhibitors during nuclear extract preparation. Thus, these results indicated that topoisomerase II levels and species distribution were similar in the MCF7/WT and MCF7/VP cell lines. Also, no difference in the levels of topoisomerase I protein was detected between MCF7/WT and MCF7/VP cells (Fig. 1B).

When we examined the mRNA levels of topoisomerase I, IIα, and IIβ by quantitative PCR using primers specific for each transcript we found that expression of topoisomerase I, IIα, and IIβ was essentially equal between MCF7/WT and MCF7/VP cells (data not shown). Thus, there appeared to be no quantitative difference in topoisomerase I and II expression between MCF7/WT and MCF7/VP cells.

We next examined the formation of drug-induced cleavable complexes by K/SDS precipitation assay. Using a whole cell assay (Fig. 2) we found a marked (approximately 8-fold) dose-dependent increase in protein-DNA complex formation after a 1-h exposure to 100 µM VP-16 in MCF7/WT cells as opposed to only an approximately 2-fold stimulation in MCF7/VP cells. Similarly, a dose-dependent stimulation of cleavable complex formation up to approximately 6- and 3-fold after 30 min of incubation was also seen with nuclear extracts from MCF7/WT and MCF7/VP cells, respectively (Fig. 3). Analysis of the initial slopes of the graphs for drug-induced complex formation revealed that 7-fold higher VP-16 concentrations were needed to produce the same stimulation of drug-induced protein-DNA complexes in MCF7/VP cells than in MCF7/WT cells and 3-fold more VP-16 was required in nuclear extracts from MCF7/VP cells than in extracts from MCF7/WT cells. These results suggested that topoisomerase II from MCF7/VP cells was at least 3-fold less sensitive to drug-induced cleavable complex stimulation and that the interaction between topoisomerase II, DNA, and drug was qualitatively altered in the resistant cells. Thus, the resistance of MCF7/VP cells to epipodophyllotoxins apparently involved a change(s) in the sensitivity of topoisomerase II to drug-induced cleavable complex formation. A similar difference in drug-induced cleavable complex formation was also observed between nuclear extracts from MCF7/WT and MCF7/VP cells with mAMSA as the stimulating agent (data not shown). These data suggested that the underlying defect of topoisomerase II in MCF7/VP cells was not specific for etoposide.

Recent studies have identified mutations in the topoisomerase IIα gene in mammalian cells selected for resistance to various topoisomerase II poisons (22-27). We therefore examined the nucleotide sequences of the conserved regions of the topoisomerase IIα and IIβ genes in which mutations had been previously identified in drug-resistant cells. Following reverse transcription and amplification by the polymerase chain reaction, the putative ATP-binding B domain (comprising aa 416 to 513 in topoisomerase IIα and aa 299 to 306 in topoisomerase IIβ) and the putative breakage/resealing domain (the area around the active tyrosine based on homology with gyrA of E. coli, comprising aa 686 to 882 in topoisomerase IIα and aa 547 to 754 in topoisomerase IIβ) were sequenced. No evidence for mutations was found in either of these regions of topoisomerase IIα or IIβ (data not shown). Thus, the reduced sensitivity of the topoisomerase II from MCF7/VP cells to stimulation of cleavable complex formation by VP-16 or mAMSA was apparently not associated with a mutation within one of these two conserved regions of the topoisomerase IIα or IIβ genes. However, we cannot exclude the possibility of mutations in other regions of the topoisomerase IIα or IIβ genes that might play a role in drug resistance.

**Drug Uptake and Efflux.** As described above, the difference in drug-induced cleavable complex formation between wild-type and resistant cells was more than 2-fold greater when assayed in whole cells instead of nuclear extracts. This suggested that alterations in intracellular drug accumulation may also be involved in the resistant

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**Table 1  Sensitivity of MCF7/WT and MCF7/VP cells to various anti-cancer agents**

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 (µM) ± SE</th>
<th>IC50 (µM) ± SE</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP-16</td>
<td>124 ± 15</td>
<td>3520 ± 220</td>
<td>28.4</td>
</tr>
<tr>
<td>VM-26</td>
<td>17 ± 4.8</td>
<td>368 ± 93</td>
<td>21.6</td>
</tr>
<tr>
<td>Genistein</td>
<td>1260 ± 2500</td>
<td>33700 ± 100</td>
<td>1.5</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>3.3 ± 0.4</td>
<td>30 ± 3</td>
<td>9.1</td>
</tr>
<tr>
<td>mAMSA</td>
<td>24 ± 3</td>
<td>91 ± 17</td>
<td>3.8</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>0.5 ± 0.02</td>
<td>1.4 ± 0.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>4.3 ± 1.0</td>
<td>5.5 ± 1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>10,11-Methylenedioxyamethothecin</td>
<td>0.7 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.6</td>
</tr>
<tr>
<td>9-Aminomethothecin</td>
<td>1.5 ± 0.1</td>
<td>3.8 ± 0.01</td>
<td>2.5</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.3 ± 0.05</td>
<td>1.5 ± 0.07</td>
<td>5.0</td>
</tr>
<tr>
<td>Melphalan</td>
<td>1.1 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>6.5 ± 0.4</td>
<td>3.7 ± 0.3</td>
<td>0.6</td>
</tr>
</tbody>
</table>

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Fig. 1. Western blot for DNA topoisomerases I and II with nuclear extracts from MCF7/WT and MCF7/VP cells. Nuclear extracts containing 50 µg protein from MCF7/WT or MCF7/VP cells were separated on a 7.5% SDS/polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was incubated with a polyclonal rabbit antibody against DNA topoisomerase I or II followed by goat anti-rabbit IgG coupled to alkaline phosphatase, as described in “Materials and Methods.” The results presented are from one representative of several experiments.

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**EPIPODOPHYLLOTOXIN-RESISTANT BREAST CANCER CELLS**

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30-min incubation with VP-16 at 37°C as described in “Materials and Methods.” The formation of DNA topoisomerase II-DNA cleavable complexes was examined of the 3'-end-labeled substrate used.

The results shown are the means ± SE of three independent experiments. The maximal amount of radioactivity precipitated corresponded to approximately 5% to 10% of the total activity used in the assay.

The reduced intracellular drug concentration could be the result of decreased drug uptake as well as increased drug efflux. Therefore, MCF7/WT and MCF7/VP cells were preloaded with radiolabeled VP-16 to steady-state levels, followed by measuring the loss of intracellular VP-16 over time. The efflux of intracellular drug was rapid and similar in the two cell lines, with more than half of the initial amount of drug lost from both cell lines within 5 min. This initial rapid efflux was followed by a slower phase to essentially identical levels of less than 20% of the initial concentrations in both cell lines within 1–2 h (Fig. 4). Thus, the mechanism of decreased drug accumulation in MCF7/VP cells does not appear to involve enhanced drug efflux.

**mdrl/P-Glycoprotein Expression.** Since the expression of P-gp had previously been associated with a decrease in the accumulation of epipodophyllotoxins and multidrug resistance, we examined the levels of mdrl/P-gp expression in both cell lines using RNase protection and Western blot assays. As shown in Fig. 5, we were unable to detect mdrl RNA in either MCF7/WT or MCF7/VP cells. Similarly, Western blot analysis failed to detect P-glycoprotein in either cell line (Fig. 6).

Thus, the reduction in drug accumulation and the development of drug resistance in the MCF7/VP cells was not associated with mdrl/P-gp overexpression.

**MRP Expression.** Recently Cole and co-workers (5) described a new putative (drug)-transport protein associated with multidrug resistance, called MRP. Since our results suggested the involvement of a defect in drug transport in the multidrug resistance of MCF7/VP cells, we also examined the expression of the MRP gene in MCF7/VP cells. MRP mRNA was readily detectable in both MCF7/WT and MCF7/VP cells by PCR analysis. However, quantitative PCR analysis demonstrated that the level of MRP mRNA in MCF7/VP cells was at least 10-fold higher than in MCF7/WT cells (Fig. 7). Using Northern blot analysis we found that in MCF7/WT cells MRP mRNA was undetectable and in MCF7/VP cells was detectable only after prolonged exposure (Fig. 8). Thus, although these results suggested that MRP is involved in the resistance mechanism of MCF7/VP cells, MRP mRNA appeared to be expressed only at low levels in our cells. It remains to be seen whether this is also reflected in low protein levels. However, such studies await the availability of antibodies against the MRP protein.

Drug uptake studies with radiolabeled VP-16 revealed that MCF7/VP cells accumulated 2- to 3-fold less VP-16 than MCF7/WT cells (Fig. 4). The reduced intracellular drug concentration between MCF7/WT and MCF7/VP cells under steady-state conditions was independent of the extracellular drug concentration used. Furthermore, the time course of drug uptake in MCF7/WT cells was relatively constant at all drug concentrations examined (data not shown). Thus, the resistance of MCF7/VP cells is associated, at least in part, with a defect in intracellular drug accumulation.

The uptake of drug was therefore analyzed in both cell lines. Drug uptake studies with radiolabeled VP-16 revealed that MCF7/VP cells accumulated 2- to 3-fold less VP-16 than MCF7/WT cells (Fig. 4). While the absolute amount of intracellular VP-16 was dependent on the drug concentration used for the experiment (uptake studies were performed using drug concentrations ranging from 0.1 μM to 100 μM VP-16), the relative difference in intracellular drug concentration between MCF7/WT and MCF7/VP cells under steady-state conditions was independent of the extracellular drug concentration used. Furthermore, the time course of drug uptake was similar at all drug concentrations examined (data not shown). Thus, the resistance of MCF7/VP cells is associated, at least in part, with a defect in intracellular drug accumulation.

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An increasing number of mammalian cell lines have recently been isolated that display relatively specific resistance to one or several topoisomerase II inhibitors. The phenotype of these cell lines (termed \textit{atMDR} for altered topoisomerase multidrug resistance by Beck and co-workers) (9, 10), differs considerably from that of "classical" MDR cell lines, the multidrug resistance of which is associated with the overexpression of \textit{mdrl/P-glycoprotein}. In contrast to "classical" MDR, \textit{atMDR} cells do not express \textit{mdrl}/P-gp and in general show little if any cross-resistance to the \textit{Vinca} alkaloids vincristine and vinblastine (9, 10, 12, 13, 16, 20, 32, 48-50). Studies in cell lines displaying \textit{atMDR} indicated that resistance is associated with quantitative and/or qualitative alterations of topoisomerase II. However, the precise mechanisms responsible for \textit{atMDR} are still unclear. In some drug-resistant cell lines a point mutation in the putative ATP-binding region and near the active tyrosine of topoisomerase II has been detected, and it has been suggested that this change may alter the enzyme and render it resistant to topoisomerase II inhibitors (22-27). In other drug-resistant cells, a decrease in topoisomerase II levels was noted (12, 16, 18, 19, 32), suggesting that overall topoisomerase II levels are correlated with drug sensitivity. Additional mechanisms affecting drug sensitivity have also been proposed, including changes in drug metabolism (51, 52), alterations in the phosphorylation of topoisomerase II (29), and non-P-glycoprotein-mediated changes in drug uptake and/or efflux (10, 24).

In the present study we have characterized a drug-resistant variant of the human breast carcinoma cell line MCF7 that displays relatively selective resistance to the epipodophyllotoxins VP-16 and VM-26. Analysis of topoisomerase II levels and activity as well as drug uptake studies indicated that resistance in this cell line apparently involves multiple mechanisms, although we cannot exclude the formal possibility that we have a mixed population of several cell lines, each with its own distinct mechanism of resistance. However, we consider this unlikely since we did not observe any changes in the phenotype over the last 2 years. While there were no detectable differences in topoisomerase II levels between wild-type and drug-resistant cells, topoisomerase II from MCF7/VP cells appeared 3-fold less sensitive to drug-induced cleavable complex formation in nuclear extracts from MCF7/VP cells and 7-fold less sensitive in whole cells relative to topoisomerase II from MCF7/WT nuclear extract or cells, respectively. These studies suggested that resistance was related, at least in part, to a reduced sensitivity of topoisomerase II to drug-induced cleavable complex formation. Although previous reports had identified point mutations in drug-resistant cells, we found no evidence for mutations in the sequence of the ATP B-consensus and the area around the active tyrosine of the topoisomerase II\alpha and II\beta genes. These findings were further supported by our observations that the strand passing reaction with nuclear extracts from wild-type and resistant cells had similar ATP requirements and that there was no difference in the stability of the drug-induced cleavable complexes to reversal at 65°C between nuclear extracts from MCF7/WT and MCF7/VP cells (E. Schneider, unpublished results).

So far we have been unable to identify any direct or indirect alterations in the topoisomerase II enzyme from the resistant cells that could explain the apparent resistance to drug-stimulated cleavable complex formation. Other studies have suggested that hyperphosphorylation of topoisomerase II may be associated with drug resistance in human KB/VP cells (29). Additional evidence implicating a role for phosphorylation of topoisomerase II in drug sensitivity has recently been obtained from studies using purified enzyme (28). These studies demonstrated that phosphorylation of topoisomerase II resulted in a reduction of the level of VP-16- or mAMSA-induced DNA cleavage. Thus, it is possible that the reduction of cleavable complex formation in nuclear extracts from MCF7/VP cells is associated with an alteration in the state of phosphorylation of topoisomerase II, and studies to address this question are currently under way.

In addition to being unable to directly identify any changes in topoisomerase II, we found that MCF7/WT and MCF7/VP cells also had approximately equal levels of topoisomerase I protein. Similar to what was seen in VM-26 resistant human KB cells (16), we observed some collateral sensitivity to alkylating agents. At present, however, it is unclear what causes this sensitivity, and further studies are in progress to address this question.

**DISCUSSION**

**Fig. 5.** RNase protection assay for \textit{mdrl} RNA in MCF7/WT and MCF7/VP cells. Total cellular RNA (20 \mu g) from MCF7/WT, MCF7/VP, KB 3-1 (negative control), KB 8-5 (positive control), or GC3 (positive control) cells was hybridized with a radiolabeled \textit{mdrl} RNA probe as described (31).

**Fig. 6.** Western blot analysis for P-glycoprotein in MCF7/WT and MCF7/VP cells. Plasma membranes (100 \mu g protein) from MCF7/WT, MCF7/VP, or MCF7/ADR (positive control) cells were separated by SDS/polyacrylamide electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with monoclonal anti-P-glycoprotein antibody C219 (3 \mu g/ml), followed by goat anti-mouse IgG coupled to alkaline phosphatase.
One of the best studied mechanisms of multidrug resistance involves reduced drug accumulation and overexpression of P-glycoprotein, a membrane protein that functions as a drug efflux pump. Although the accumulation of VP-16 in MCF7/VP cells was 2-fold lower than in wild-type MCF7/WT cells, there was no detectable expression of mdrl mRNA or P-glycoprotein in either cell line. Thus, P-glycoprotein is not involved in the reduction of drug accumulation or, consequently, in the development of drug resistance in the MCF7/VP cells. Several cell lines have been described that exhibit an apparent defect in drug accumulation in the absence of mdrl/P-gp expression, including a mitoxantrone-resistant MCF7 cell line that was recently described (31). Other studies have also investigated the relation between drug uptake and sensitivity to VP-16, both in mdrl1-expressing and non-mdrl-expressing cells (30, 53-56). However, no single mechanism relating drug accumulation with the development of drug resistance has been identified, and it remains to be determined whether a nonspecific reduction in drug accumulation is a general phenomenon in the development of resistance to VP-16.

Recent studies with a doxorubicin-resistant human lung cancer cell line revealed the presence of a new protein, termed MRP, that is overexpressed in the resistant cells as compared to the sensitive parent cells. This protein shares homology with several members of an ATP-binding cassette superfamily of proteins that also includes P-glycoprotein and was therefore thought to be involved in the multidrug resistance of these lung cancer cells (5). When we compared the expression of MRP in MCF7/VP and MCF7/WT cells, we found that MRP was at least 10-fold overexpressed in the resistant cells. Therefore it is possible that MRP overexpression is responsible for the multidrug resistance phenotype of MCF7/VP cells. However, while we found a clear reduction in drug accumulation in the MCF7/VP cells that overexpress MRP, no change in drug accumulation was observed in the H69AR cells in which MRP was originally identified (7). Therefore, although it appears that MRP overexpression is involved in epipodophyllotoxin resistance in MCF7/VP cells, its role in reduced drug accumulation remains to be clarified. So far all cell lines reported to overexpress MRP were selected for doxorubicin resistance (5, 57, 58). Therefore we believe the MCF7/VP cell line to be the first example of an MRP-overexpressing cell line that was not selected for doxorubicin, further substantiating the possibility that MRP overexpression might be a more general mechanism for acquisition of non-P-glycoprotein-mediated multidrug resistance.

In conclusion, we have isolated a new MCF7 variant cell line that is relatively selectively resistant to epipodophyllotoxins. While DNA topoisomerase II levels were unchanged, there was a reduction in drug-stimulated cleavable complex formation, suggesting a qualitative modification of topoisomerase II. In addition, resistance in these cells was associated with decreased drug accumulation, possibly due to the overexpression of MRP.

REFERENCES


Fig. 7. Quantitative PCR assay for MRP in MCF7/WT and MCF7/VP cells. Total RNA was isolated from MCF7/WT and MCF7/VP cells and reverse transcribed as described in “Materials and Methods.” The resulting cDNA was serially diluted, and each dilution was separately amplified with primer pairs for MRP or G3PDH, respectively. Ten μl of each amplified reaction mixture were separated on a 2% agarose gel in Tris-Borate-EDTA. The gels were dried and exposed on X-ray film overnight. The cDNA dilutions used for each amplification reaction are indicated above each lane. The results of a representative experiment are shown.

Fig. 8. Northern blot analysis for MRP expression in MCF7/WT and MCF7/VP cells. Total RNA was isolated from MCF7/WT and MCF7/VP cells and separated on a 1% agarose gel and probed for MRP as described in “Materials and Methods.” Exposure was for 14 days at -70°C with intensifying screens.
Multidrug Resistance-associated Protein Gene Overexpression and Reduced Drug Sensitivity of Topoisomerase II in a Human Breast Carcinoma MCF7 Cell Line Selected for Etoposide Resistance

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